Statement of Originality

The accompanying thesis submitted for the degree of Doctor of Medicine is entitled 'Blood Monocytes in Cystic Fibrosis.' This thesis is based on the work conducted by the author in the Department of Infection, Immunity and Inflammation, University of Leicester during the period between September 2004 and January 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.

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Acknowledgements

Abstract

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Title: Blood Monocytes in Cystic Fibrosis

Background: Neutrophilic inflammation causes lung damage in cystic fibrosis (CF). Recent data from animal models and other chronic pulmonary inflammatory conditions suggest that the monocytes/macrophages may be an important driver of airway inflammation. CF may be associated with increased airway levels of chemoattractants for monocytes and resulting expansion of CD14++ small macrophages. I sought to assess the levels of monocyte chemoattractants CCL2 and CX3CL1 in the blood and airways of CF patients, and expression of their respective receptors CCR2 and CX3CR1 on monocytes. In a pilot study, I sought evidence for expansion of airway CD14++ small macrophages.

Methods: Blood was obtained from 32 CF patients and 25 healthy controls; and induced sputum (IS) from 24 CF patients and 17 healthy controls. Flow cytometry was used to determine expression of CCR2 and CX3CR1 on CD14++ and CD14+CD16+ blood monocytes and to characterise IS airway macrophages. CCL2 and CX3CL1 levels in blood and IS were determined by ELISA.

Results: Absolute count of total monocytes and monocytes subpopulations was not different between CF and controls. Serum CCL2, but not CX3CL1, was increased in CF patients (p=0.006). Similarly, CF was associated with increased IS CCL2, but not CX3CL1 (190.6 *vs*. 77.3 pg/mL; p=0.029). CCR2 was expressed on CD14++ monocytes but not on CD14+CD16+ monocytes. Both CD14+CD16+ and CD14++ cells expressed CX3CR1 but the expression was higher in CD14+CD16+ cells compared to the CD14++ cells. There was no difference in expression of both chemokine receptors by either monocyte subpopulation between CF and controls. Small macrophages were significantly increased in CF airways (p=0.018).

Conclusion: CCL2, but not CX3CL1 is increased in the airway and blood of CF patients. Blood monocytes from CF patients are phenotypically competent to respond to CCL2, since they express normal levels of CCR2. Preliminary results suggest an expansion of small macrophages in CF airways.

Abbreviations

α- Alpha

AF- Autoflouroscence

APC- Allophycoerthryin cyanin

ARDS- Acute respiratory distress syndrome

ASL- Airway surface fluid

β- Beta

BAL- Bronchoalveolar lavage

BD- Becton Dickinson

DNA- Deoxyribonucleic acid

CF- Cystic fibrosis

CFTR- Cystic fibrosis transmembrane regulator

CL^- - Chloride

CO_2- Carbondioxide

COPD- Chronic obstructive pulmonary disease

DC- Dendritic cell

DEPC- Diethylpyrocarbonate

dF508- Deletion of phenylalanine (F) at position 508

dH_2O- De-ionised water

DTT- Dithiothreitol

EC- Epithelial cells

E. coli- Escherichia coli

EDTA- Ethylenediaminetetraacetic acid

ENaC- Epithelial sodium channel

Fc- Conserved fragment

FCS- Fetal calf serum

FEV1- Forced expiratory flow in 1 second

FITC- Fluorescin isothyocyanate

FL- Fluorescence

FRC- Functional residual capacity

FSC- Forward scatter

FTK- Fractalkine (now known as CX3CL1)

γ- Gamma

GM- Geometric mean

GM- CSF- Granulocyte/monocytes colony stimulating factor

HCO_3^- - Hydrogen bicarbonate

H. Influenzae- Hemophilus influenzae

HLA- Human leucocyte antigen

HMC-1- Human mast cell 1

H_2O- Water

IFN- Interferon

IgG- Immunoglobulin

IL- Interleukin

IPF- Idiopathic pulmonary fibrosis

K^+- Potassium ion

KCl- Potassium chloride

$KHCO_3$- Potassium hydrogen carbonate

KH_2PO_4- Potassium dihydrogen phosphate

LDL- Low density lipoprotein

LPB- Lipopolysaccharide binding protein

LPS- Lipopolysaccharide

LRI- Leicester Royal Infirmary

LTB4- Leukotriene B4

mAB- Monoclonal antibody

MACS- Magnetic activated cell sorting

MARCO- Macrophage receptor with collagenase structure

MBL- Mannan binding lecithin

M-CSF- Monocyte colony stimulating factor

MDM- Monocyte derived macrophages

MHC- Major histocompatibility complex

min- Minute

MCP-1 - Monocyte chemoattractant protein 1 (now known as CCL2)

MIP- Macrophage inflammatory protein

ml- 10^{-3} litre

MM6- Matrix metalloproteinase

MPO- Myeloperoxidase

mRNA- Messenger ribonucleic acid

n- Number

N/A- not applicable

Na^+- Sodium

NaCl- Sodium chloride

Na2HPO4- Disodium hydrogen phosphate

ng- 10^{-9} grammes

NH4CL- Ammonium chloride

NSG- No significant growth

P. aeruginosa- *Pseudomonas aeruginosa*

PBMC- Peripheral blood mononuclear cells

PBS- Phosphate buffered saline

PC-5- Phycoerythrin cyanin 5

PCR- Polymerase chain reaction

PC- Phycoerythrin

PerCP- Perdinin chlorophyll protein

pg- 10^{-12} grammes

PK- Protein kinase

PMT- Photomultiplier tube

RNA- Ribonucleic acid

rpm- Revolutions per minute

RT- reverse transcriptase

s- Seconds

S. aureus- *Staphylococcus aureus*

SD- Standard deviation

sp.- Species

SR- Scavenger receptor

SSC- Side scatter

TGF- Transforming growth factor

TLR- Toll like receptor

TNF- Tumour necrosis factor

μg- 10^{-6} grammes

μl- 10^{-6} litres

μm- 10^{-6} metres

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1 Introduction

1.1 Cystic Fibrosis

Cystic fibrosis (CF) is the most common inherited disease in the western population with an incidence of 1 in 2500 live-births. It is an autosomal recessive condition characterised by a defective cystic fibrosis transmembrane regulator (CFTR) gene that results in a multi-system disorder affecting mainly the lungs, pancreas and liver. Chronic progressive lung damage is the most common cause of mortality and morbidity in cystic fibrosis.

1.1.1 History of cystic fibrosis

Some of the characteristic symptoms of cystic fibrosis and associated high mortality were described back in the medieval times (Quinton 1999). In medieval folklore from Eastern Europe, there are references to early mortality in infants who tasted "salty" when kissed and that these infants were "hexed". In fact, salty sweat forms the basis of the diagnostic sweat test for cystic fibrosis. From then and until the early 1900s, it appears that CF was thought to be similar to the celiac disease affecting mainly the digestive tract. It was in fact known as 'cystic fibrosis of pancreas' and this was based on the pathological appearances of inspissated and cystic ductal changes of pancreas and intestines. It was probably in 1936 when Fanconi et al described "cystic fibromatosis with bronchiectasis" that CF was recognised as a multi-system disease affecting lungs as well as pancreas for the first time. In 1938, Anderson compiled the first comprehensive list of CF symptoms and a few years later in 1946, (Di Sant'Agnese and Andersen 1948)

described the autosomal recessive nature of inheritance (Anderson and Hodges 1946). At this stage the disease process was attributed to the "generalised state of thickened mucus". In 1948 during a heat wave in New York a number of these patients presented with excessive dehydration. Puzzled by this phenomenon, Di Sant'Agnese from Columbia (Di Sant'Agnese, Darling et al. 1953; Di Sant'Agnese, Darling et al. 1953) subsequently discovered that these patients sweat excessively, forming the basis of the modern day sweat test (Kessler and Andersen 1951). In the next 5 years or so, the sweat test developed to become a reliable diagnostic test for cystic fibrosis. The next key milestone in the CF history was in early 1980s when the electrolyte transport defect as the cause of organ dysfunction and damage was recognised. A major breakthrough however was in 1989. Tsui and Riordan from Canada discovered the gene responsible for CF on chromosome 7q and called the product of the gene as cystic fibrosis transmembrane regulator (CFTR) (Kerem, Rommens et al. 1989).

1.1.2   CFTR

Cystic fibrosis is caused by a mutation in a large gene (230kb) on chromosome 7 which codes for CFTR (Ratjen and Döring 2003). Over 1000 mutations have been described so far. The most common mutation is caused by deletion of codon coding for phenlyalanine at position 508 of the CFTR and accounts for over 80% of cases in the western Caucasian population. The early enthusiasm of this discovery was somewhat subdued following observations that there are wide phenotypic variations between patients with same mutations suggesting that other factors such as environment and epigenetic factors modify the disease severity and progression.

The CFTR mutations could be grouped into six classes:

1.Class 1 (example G542X): CFTR is not synthesised due to presence of premature stop codon leading to mRNA degradation

2.Class 2 (example F508del): defective processing; most common mutation leading to retention of mis-folded protein in the endoplasmic reticulum and subsequent degradation

3.Class 3 (example G551D): defective regulation resulting in impaired channel opening

4.Class 4 (example R334W): defective conductance through the channel

5. Class 5 (example aberrant splicing 3272-26A>G): deficient production of CFTR albeit with normal function

6.Class 6: defective regulation of other channels

The CFTR (1480 aminoacid polypeptide) is a chloride channel located on the apical surface of the epithelial cells in the target organs such as airways, pancreatic ducts, biliary ducts and sweat glands. It is regulated by c-AMP dependent protein kinase and adenosine tri-phosphate (ATP). In the lungs, CFTR defect leads to reduced chloride transport and therefore reduced water secretion resulting in poor hydration of the airway surface liquid (ASL). Optimum depth of airway surface liquid is necessary for effective ciliary function and reduced ASL leads to impaired ciliary mucus clearance which in turn pre-disposes to infections. In addition, CFTR is a conductance regulator which also seems to regulate the function of other channels in the epithelium for e.g. epithelial sodium channel (ENaC). ENaC is expressed in the airway and alveolar epithelium and acts by

sodium (with water) resorption to maintain a relatively constant depth of the airway surface liquid. Defective CFTR results in increased activity of ENaC and therefore in increased sodium and water resorption contributing further to depletion of ASL (Chmiel and Davis 2003). CFTR is also believed to impact on the immune response in the airways and defective CFTR could lead to poor eradication of infection. For e.g. there is evidence to suggest that CFTR acts as a specific receptor for *P.aeruginosa* and lack of CFTR could result in poor internalisation and clearance of this organism (Chmiel and Davis 2003). A number of other factors have been implicated as potential causes too; poor non-specific airway defences (e.g. reduced airway nitric oxide), modified cell surface molecules with altered binding of micro-organisms and excessive inflammation contributing to tissue damage providing favourable microbial environment. Certain characteristics of infecting organisms also contribute to abnormal retention and persistent inflammatory response. For instance by strategies such as production of mucoid exopolysachharide, formation of biofilm and quorum sensing, *P.aeruginosa* is protected from host defense response and antibiotic therapy (Chmiel and Davis 2003).

Chronic irreversible lung damage secondary to recurrent and persistent lower airway infection is the main cause of morbidity and mortality in CF. In infants and toddlers, the recurrent lower airway infection is due to mainly *Staphylococcus aureus* and *Haemophilus influenzae*. However, majority of the children acquire *P.aeruginosa*, which in non-mucoid form infects the airways intermittently. However, the non-mucoid strain could subsequently transform in to mucoid alginate producing strain resulting in chronic airway infection and progressive

decline of the lung function. Therefore early recognition and aggressive anti-microbial treatment of pseudomonal infection is of paramount importance (Valerius, Koch et al. 1991; Frederiksen, Koch et al. 1997; Döring, Conway et al. 2000; Høiby, Frederiksen et al. 2005).

Until recently, the most common clinical presentations of cystic fibrosis were recurrent chest infections, failure to thrive (secondary to pancreatic exocrine dysfunction). And today, with the recent introduction of neonatal CF screening in the United Kingdom majority of infants are diagnosed even before they become symptomatic. However, certain centres such as Sheffield, Nottingham and Leicester have had the screening programme for over a decade. Sweat test however is the diagnostic test for CF. In the sweat gland, defective CFTR leads to impaired absorption of chloride resulting in high chloride concentration in sweat and forms the basis for sweat test. A concentration of sweat chloride greater than 60mmol/L on repeated measurements is diagnostic of cystic fibrosis (Ratjen and Döring 2003).

The prognosis for cystic fibrosis has improved significantly over the last 2 decades. It is now expected that majority of the CF patients will survive until the fourth or fifth decade of life (Dodge, Lewis et al. 2007). This has been largely due to the implementation of specialised multi-disciplinary care with emphasis on regular chest physiotherapy, prompt treatment of chest infections and aggressive nutritional intervention. This trend in improved survival is likely to continue due to further advances in CF care. For example newborn screening has enabled recognition and treatment of CF even before the development of any symptoms.

There is already emerging evidence of better nutritional outcome with this programme (Grosse, Rosenfeld et al. 2006; Sims, Clark et al. 2007). Gene therapy aimed at rectifying the basic genetic defect has shown some early promise although considerable progress still need to be made for it to be effective and widely available (Moss, Rodman et al. 2004; Moss, Milla et al. 2007). Several pharmacological agents such as gentamicin, PTC124 have been used to improve the function of mutated CFTR with variable results (Clancy, Rowe et al. 2007). Gentamicin acts by suppressing premature stop codon on the CFTR gene, which then enables translational readthrough and production of full-length functional CFTR. Premature stop codons are found in about 10% of CF population but can be much higher in specific population. For example approximately, 85% of CF patients with Ashkenazi Jewish descent have premature stop codon mutation (Clancy, Rowe et al. 2007). Similarly activating alternative chloride channels for example activating P2Y2 channel with denufosol is currently being studied in a phase 3 trial (Deterding, Lavange et al. 2007). However, airway inflammation still remains a key feature of CF lung disease (Konstan and Berger 1997 Aug). This has generated interest in developing novel anti-inflammatory therapy targeting specific pathways thereby modulating inflammation and minimising the side-effects associated with non-specific anti-inflammatory therapy such as systemic steroids. Better understanding of the onset and drivers of persistent inflammation in CF airways is crucial if we are to progress in this area. In the following sections, I present some of the recent advances and also highlight gaps in our understanding of CF airway inflammation. But first, I will start with the description of inflammation.

1.1.3 Description of inflammation

The lungs in cystic fibrosis appear to be normal at birth (Konstan and Berger 1997 Aug; Chmiel and Davis 2003). Histopathological examination of the lungs of uninfected neonates who died of meconium ileus shows little evidence of inflammation apart from mild dilation of the acini of tracheal submucosal glands. Plugging of small airways by abnormal mucus is the prominent early finding followed by endobronchial inflammation. The most characteristic feature later on is the persistent excessive neutrophilic inflammation that does not convert to a chronic pattern. When present in excess, neutrophils and their products impair host's ability to clear bacterial infection. Neutrophils are a major source of DNA that makes the CF sputum more tenacious. Studies have shown that the exacerbations of lung infection is associated with increase in amounts of DNA that are of human origin in the sputum (Smith, Redding et al. 1988 Apr). Further, neutrophils are also a source of an arsenal of oxidants and proteases including neutrophil elastase (Konstan and Berger 1997 Aug; Chmiel and Davis 2003). Neutrophil elastase directly damages the airways by its direct action on elastin and other structural proteins leading to bronchiectasis. It interferes with host defense responses by cleaving opsonins and receptors necessary for phagocytosis, thereby contributing further to persistence of infection. Finally, elastase promotes transcription of potent chemoattractants such as IL8 from epithelial cells. These together with other chemoattractants from epithelial cells, macrophages and neutrophils recruit more neutrophils in to the airways, thereby maintaining a vicious cycle of inflammation that eventually leads to lung damage. Persistence of neutrophilic inflammation may also in part be due to longer survival of neutrophils in the CF airways. Recent studies have shown that neutrophil apoptosis is

defective due to presence of excess GM-CSF and lack of IL-10, which inhibit neutrophil apoptosis (Saba, Soong et al. 2002 Nov). The time of onset and drivers of persistent neutrophilic inflammation has been the subject of recent research in cystic fibrosis.

1.1.4 Airway inflammation in cystic fibrosis

The traditional view has been that persistent inflammation is secondary to chronic bacterial infection of the lower airways (Figure 1.1-1). However, recently I have contributed to revisiting the debate on relationship between infection and inflammation, specifically whether inflammation in fact predates infection (Rao and Grigg 2006). One clinical study that raises this possibility more strongly than any other is by Khan et al (Khan, Wagener et al. 1995 Apr). In their study of 16 CF infants (mean age 6 months), 7 infants had no evidence of bacterial, viral or fungal infection at the time of their broncho-alveolar lavage. But the degree of inflammation in this group was significantly higher than the normal controls although they were lower than that seen in CF infants positive for microorganisms. Similar results were found in other longitudinal studies of clinically asymptomatic young cystic fibrosis patients (Balough, McCubbin et al. 1995 Aug; Grosse, Rosenfeld et al. 2006). These studies have demonstrated that in the majority of children, significant airway inflammation is present without any demonstrable airway infection or symptoms and despite normal lung function. However all these studies have limitations. The studies are often small with various confounding variables. It is difficult to exclude preceding or co-existing infection completely. BAL was performed on one of the lobes of the lungs and it is quite possible that the sampled area was free of infection and the inflammation

was in response to infection present elsewhere in the lungs. Equally concomitant antibiotic therapy may have cleared the infection and what is detected is in fact the resolving inflammation. Furthermore, some of the studies have older patients with chronic symptoms and inflammation could also be explained by other co-morbidities such as gastro-oesophageal reflux or asthma. The only clinical study that has looked at infection-naive neonates so far is by Armstrong et al (Armstrong, Grimwood et al. 1997 Oct). BAL was performed on neonates at 3 months age, diagnosed by neonatal screening and without any present or past chest symptoms. In this infection-naïve group the neutrophil count was similar to that in the non-CF control group. This study seemed to suggest that inflammation is secondary to infection. However the neutrophil count in the control group was higher than that reported in previous studies. So even this study fails to convince us one way or the other. Nevertheless they all provide us with sufficient proof that the inflammation begins very early in life, in absence of any symptoms and associated infection makes inflammation worse.

Irrespective of whether inflammation precedes infection or not, there seems to be convincing evidence that the inflammatory response is exaggerated to a given degree of infection. Studies such as Dakin et al (Dakin, Numa et al. 2002) indicate that the neutrophil count and pro-inflammatory cytokine (example IL8) levels are remarkably out of proportion to the degree of airway infection. In other words, presence of even low levels of commensals and/or pathogens is associated with significantly higher inflammatory response compared to controls. One explanation for this finding could be that although the bacterial count is similar in CF and non-CF controls, the bacterial antigen load in cystic fibrosis airway might be higher

due to impaired clearance. Muchlebach et al (Muhlebach, Stewart et al. 1999 Muhlebach and Noah 2002) have addressed this very question and shown that the endotoxin load correlated with the bacterial antigen load in both CF and non-CF patients. These findings would suggest that there is a shift in the inflammatory response in cystic fibrosis to a pro-inflammatory state leading to an excessive and exaggerated response to an otherwise transient/trivial airway infection. And the inflammatory response continues to reverberate even after the infection is controlled.

Whilst clinical studies have been inadequate to investigate the primary abnormality in inflammatory response in CF, more convincing evidence is available from post-mortem studies of infection-naïve CF foetuses and neonates. The histopathological examination of lungs in infection-naïve CF foetus and neonates who died of meconium ileus was normal but the airway tissue grafted in to the backs of immuno-compromised mice show that the uninfected CF airways were in a pro-inflammatory state. Long-term CF grafts, but not non-CF grafts, underwent intraluminal neutrophilic infiltration and tissue destruction independent of infection (Tirouvanziam, de Bentzmann et al. 2000). The amount of pro-inflammatory cytokine and chemoattractant IL8 was 8-fold higher in the airway fluid of CF grafts compared to non-CF grafts. Furthermore, there was an exacerbated response to *P.aeruginosa* primary infection in CF airway grafts in the form of extensive exfoliation and shedding of epithelial cells, which was not seen in the non-CF grafts. These studies fundamentally raise the possibility of an intrinsic defect in the immune system due to the CFTR mutation that renders it pro-inflammatory and that infection merely amplifies this process **(Figure 1.1-1)**.

In which case, treating infection alone is not sufficient to limit the inflammatory response and ensuing lung damage. There is a clear need for developing other anti-inflammatory strategies to complement the current treatment. Hence understanding the key drivers of inflammation has been the subject of recent research as modulating inflammation offers a prospect of minimising irreversible lung damage.

A

Defective CF gene
↓
Defective or deficient CFTR
↓
Thick viscous secretions
↓
Bronchial obstruction
↓
Infection
↓
Inflammation
↓
Bronchiectasis

B

Defective CF gene
⇓
Defective or deficient CFTR
⇓
Increased activation of resident airway inflammatory cells

Infection →amplification→

Increased neutrophil and monocyte recruitment
⇓
Increased neutrophil products e.g. elastase
and increased release of neutrophil chemoattractants from monocytes
⇓
Bronchiectasis

Figure 1.1-1: Traditional and proposed model of the pathogenesis of lung disease in cystic fibrosis. Traditionally, infection was believed to be the main trigger for airway inflammation and damage (A) whereas it is now believed that CF intrinsically results in a pro-inflammatory state in CF airways and infection amplifies these changes (B). (Rao and Grigg 2006)

1.1.4.1 Does inflammation lead to lung damage?

The relationship between infection, inflammation and lung damage has been studied to a certain extent in established cystic fibrosis. Several studies have provided an indirect link between inflammation and lung damage (Dean, Dai et al. 1993 Aug; Watkin, Elborn et al. 1994 Jan; Balough, McCubbin et al. 1995 Aug; Salva, Doyle et al. 1996 Jan; Konstan and Berger 1997 Aug; Nixon, Yung et al. 1998 Jun; Hendry, Elborn et al. 1999 Aug). Numerous inflammatory indices in serum, broncho-alveolar lavage and sputum have been studied; IL-8, TNF-α and neutrophil density being the commonly measured ones. There appears to be an increase in the basal level of inflammatory mediators in BAL, sputum samples (most studies) and in serum (in some studies) in CF patients compared to the non-CF controls. Some of these mediators (example IL-8) correlate with the clinical status, lung function and severity of disease. For instance, higher levels of IL-8 are seen in patients with exacerbations of chest symptoms, poor lung function and more severe lung disease. Furthermore the improvement in clinical status and lung function is associated with decrease in the level of inflammatory mediators. Similar response has been documented with other inflammatory indices such as sputum neutrophil density and TNF-α. These findings would suggest that inflammation probably plays an important role in lung damage. Furthermore recent studies have shown that structural and functional damage is seen early in the disease and in absence of significant symptoms.

Structural abnormalities such as dilated and thickened airways on HRCT (Long, Williams et al. 2004 Feb) and lung function abnormalities(Godfrey, Mearns et al.

1978 Jan; Tepper, Hiatt et al. 1988) have been shown in infants and pre-school children. However there are only a few studies that describe a direct relationship between airway inflammation and lung damage. This has been due to the lack of a sensitive and specific test that would detect early structural and functional lung damage as well as lack of a reliable non-invasive method of monitoring lung inflammation in this age group. There is lack of consensus among the experts about specific lung function measurements that would detect early changes reliably. In addition, the complexity and duration of testing makes it difficult to repeat the tests as frequently as desired. It may further be difficult to justify repeated measurements, as there is no benefit for individual patients especially at a time when the family are coming to terms with the diagnosis and treatment of an incurable illness. Nevertheless it is important to establish the relationship between inflammation and lung damage as early institution of anti-inflammatory therapy even before patients become symptomatic could potentially limit the lung damage and enhance survival.

At present, the findings from the few studies that have attempted to document the relationship between inflammation and lung function in early disease are not conclusive. Studies involving older children with more chronic symptoms such as Dakin et al (Dakin, Numa et al. 2002 Apr 1), seems to suggest a negative correlation between the degree of inflammation *per se* and lung function. This was further compounded by the presence of any infection. In this study of 22 children (age range 6.7-44 months, mean 23.2 months), specific compliance and FRC_{N2}/TLC_{N2} correlated negatively with degree of neutrophilia and IL8 concentration in the BAL fluid. The inflammatory response was compounded

even by presence of otherwise trivial infection. In a similar study, the FRC was found to be significantly lower in the CF group where no pathogen was isolated compared to the groups where $<10^5$cfu/ml or $>10^5$cfu/ml of pathogens were isolated from BAL (Balough, McCubbin et al. 1995). Similar correlation between the lung function and inflammation has been demonstrated by Brennan et al (Brennan, Hall et al. 2005) by the use of low frequency forced oscillation technique. Whereas in another study of younger stable CF children predominantly diagnosed by neonatal screening, poor lung function was associated with airway infection, respiratory symptoms and unrelated to inflammation(Nixon, Armstrong et al. 2002 Oct). These differences between the studies could be explained by different patient groups, lung function techniques, measurements and timing of BAL sampling in relation to infant lung function testing. Further each individual study is small and given the lack of uniformity in techniques and measurements comparisons between different studies is difficult.

1.1.4.2 Drivers of airway inflammation

Airway infection is not the only major driver of inflammation and it has been recognised that there are other drivers too. Much of work on inflammation in cystic fibrosis has concentrated on the role of airway epithelial cell as a driver of inflammation. The defect in CFTR function renders the CF airway epithelial cells more pro-inflammatory compared to normal epithelial cells by different mechanisms. They have high expression of pro-inflammatory cytokine IL-8, ICAM-1 (important ligand for major neutrophil adhesion molecule) and low expression of anti-inflammatory cytokine IL10. Further alterations in cell surface glycoconjugates and in the ionic milieu contribute to increased adherence of

bacteria such as *S.aureus, H.influenzae and P.aeruginosa* and defective phagocytic function of epithelial cells. The result is a persistent ineffective inflammation that not only fails to clear infection effectively but also contributes to the lung damage.

It is now becoming apparent that effects of a defective CFTR is not limited to epithelial cells but instead could have a broader impact on the immune system. The evidence at present is limited but nevertheless seems to suggest that both the innate and adaptive immune systems could be affected. Study of fetal CF lung tissue at various stages of development has shown an increase in the macrophage population in the airways of CF fetuses at later stage of development when compared with non-CF controls (Hubeau, Puchelle et al. 2001). This might suggest that this increase in the macrophage cell population might result be responsible for an early inflammatory response but there is currently no direct evidence and hence this needs to be studied further. The macrophages derived in vitro from peripheral blood monocytes in both CF patients and mice exhibit greater pro-inflammatory properties in form of increased expression of pro-inflammatory cytokines IL8 and TNF-α in response to stimulation with lipopolysachharide (LPS) compared to healthy controls (Pfeffer, Huecksteadt et al. 1993; Thomas, Costelloe et al. 2000; Zaman, Gelrud et al. 2004). Similarly in a study of young CF patients, T-cells were shown to have an altered cytokine profile (Hubeau, Le Naour et al. 2004). These findings suggest that the CF genotype produces an altered leucocyte trafficking in to the airways, which might partly or wholly contribute to the early inflammation. If this is true then blocking of transmigration of these inflammatory cells could provide a novel therapeutic

strategy to limit inflammation. A similar strategy of blocking monocyte migration in to the joints has been successful in reducing inflammation and improving patient's symptom in rheumatoid arthritis (Haringman, Kraan et al. 2003). However, at present we do not know if the altered function of macrophages and T-cells is a clinically relevant problem or just an interesting epiphenomenon. Further work in to attempting to establish a correlation between abnormal immune function and clinical status of the patients and/or effects of therapy on various aspects of inflammation is necessary.

In CF, until recently the majority of the research concentrated on epithelial cells and infection as the main drivers of inflammation. However, we can see from the evidence above that other immune cells may also modulate inflammation. And in that respect it is important to study the role of monocytes/macrophages. Monocytes/macrophages as the mononuclear phagocytes play a key role in immune modulation and maintaining tissue homeostasis. In the following sections, I hope to present some of the relevant evidence for importance of monocyte/macrophage as a key player in maintaining chronic inflammation and discussed the implications of these findings for our study. I will start with a review of peripheral blood monocytes, its heterogeneity and chemotactic properties.

For purpose of review, I performed search for articles using comprehensive electronic databases, handsearched relevant journals, published abstracts, relevant textbooks in Immunology and flow cytometry, proceedings from various conferences and following references from the some of the key articles.

1.2   Peripheral Blood Monocytes

Monocytes originate from the bone marrow from the precursors cells- monoblasts and pro-monocytes (van Furth 1970). The monocytes and neutrophils share common progenitor cell in the bone marrow called colony forming unit, granulocyte-macrophage (CFU-GM) (van Furth, Cohn et al. 1972). The monoblast is the least mature cell of the mononuclear phagocytic system. The division of monoblast results in 2 pro-monocytes, which are direct precursors for monocytes. Newly formed monocyte remains in the marrow for less than 24 hours and then enters circulation where the half-life of circulation could be up to 70 hours in humans (Whitelaw 1966; van Furth and Sluiter 1986).

In adults, the monocyte constitute between 1 and 6 percent of circulating white cell population. More than 95% of tissue macrophages are derived from circulating monocytes (van Furth 1980).

1.2.1   Description of main surface receptors CD14 and CD16

The classical monocytes are characterised by a high expression of the surface antigen CD14 (Schütt 1999). The human CD14 is a 55 kDa glycoprotein and acts as a receptor for the endotoxin lipopolysachharide (LPS) which is a major component of the cell membrane of gram negative bacteria (Schütt 1999). The CD14 protein is a glucosyl-phosphatidylinositol (GPI)-anchored cell surface molecule. Binding of the LPS to CD14 requires LPS binding protein (LBP). When LPS is released by the bacteria, it binds to the LPB in plasma which then

facilitates the binding of LPS to CD14 (Schütt 1999). Both CD14 and LPB are believed to be pattern recognition receptors (PRR) (Pugin, Heumann et al. 1994) and hence play an important role in the innate immune system.

A variety of factors affect the expression of CD14 on monocytes. LPS is known to be a potent inducer of CD14 expression (Schütt 1999). LPS challenge in mice results in a transient increase in CD14 expression and similar results are available from the in vitro studies on human myeloid cells. Other factors that up regulate CD14 expression include TNF-α and IFN-γ (Schütt 1999).

Binding of LPS to CD14 results in cellular activation and production of inflammatory mediators such as TNF-α and IL-8 as well as anti-inflammatory cytokines like IL10 and TGF-β (Schütt 1999). CD14 can also bind the whole gram-negative bacteria and hence is not completely dependent on liberation of endotoxin from bacteria (Grunwald, Fan et al. 1996). It is believed that CD14 may also have an important role to play in phagocytosis of the apoptotic cells (Savill 1998).

Another key surface receptor CD16 which is a FcγRIII receptor is expressed on about 5-10% of the circulating monocytes (Frankenberger, Menzel et al. 2004). This receptor has low affinity for monomeric IgG but can effectively bind immune complexes (Unkeless 1989). The attachment of Fc region of IgG molecule to this receptor triggers various functions including secretion of potent inflammatory mediators and phagocytosis (Unkeless 1989). It is now recognised that the blood

monocyte population is heterogenous and exhibit different phenotypic and functional characteristics.

1.2.2   Heterogeneity of Blood Monocytes

Recently using fluorescence-activated cell sorter (FACS), different subpopulations of the blood monocytes have been defined. Majority of the circulating monocytes have a strong expression of the surface receptor CD14 with no expression of the CD16 receptor. These cells constitute about 90% of the monocyte population. A second population of cells that stain for both CD14 and CD16 can be identified when both CD14 and CD16 antibodies are used in the FACS analysis (Frankenberger, Menzel et al. 2004). This group of CD14+CD16+ monocytes can be differentiated from granulocytes, T-cells, B-cells, NK cells and dendritic cell precursors by lack of the receptors specific to the respective groups. The CD14+CD16+ monocytes are smaller than the classical monocytes and are situated in the monocyte/lymphocyte overlap on the forward scatter.

The CD14+CD16+ monocytes are also different to the classical monocytes in their surface antigen expression, functional characteristics and chemotactic properties (Frankenberger, Menzel et al. 2004). They exhibit lower levels of CD11b, CD33 and CD64 but higher levels of intercellular adhesion molecule (ICAM-1), major histocompatibility complex (MHC) class II and VLA-4. This pattern of surface molecule expression suggests that they are more-mature form of circulating monocytes with properties similar to tissue macrophages. The lower expression of CD33, a myelo-monocytic stem cell antigen, is consistent with higher maturation and also, the expression of CD16 and MHC class 2 with low expression of CD14, CD33, and CD11b is similar to the pattern seen in the alveolar macrophages. It has

been suggested that this distinct surface receptor expression might result in different functional characteristics for the CD14+CD16+ monocytes. But there is still considerable debate as to whether this indeed is the case.

Various studies have shown that there is in fact no difference in the level of various pro-inflammatory cytokines production between the two groups (Frankenberger, Menzel et al. 2004). Both CD14++ and CD14+CD16+ groups were noted to have similar level of expression of mRNA of important pro-inflammatory cytokines such as TNF- α, IL-1 and IL-6 production in a study that used the technique of reverse transcriptase PCR. But there was significantly decreased expression of mRNA for a potent anti-inflammatory cytokine, IL-10 (Frankenberger, Menzel et al. 2004; Mizuno, Toma et al. 2005). The IL-10 level was almost undetectable in the CD14+CD16+ monocytes whereas in CD14++ monocytes it was readily detected. Hence there is an argument that the decreased anti-inflammatory capability together with similar inflammatory profile renders CD14+CD16 more pro-inflammatory compared to the classical monocytes. In other words, CD14+CD16+ monocytes could be capable of an inflammatory response that is more pronounced than classical monocytes. Further with higher expression of MHC class II antigens, CD14+CD16+ monocytes are potent antigen-presenting cells (APC), which means that they have an increased ability to modulate inflammation with their interaction with the lymphocytes. Preliminary experiments, however, do not show any difference in the APC activity between CD14++ and CD14+CD16+ monocytes and more work is therefore needed to confirm these results.

Another important difference in functional significance is that the CD14+CD16+ cells could be readily recruitable in to the tissues compared to the CD14++ monocytes. They have a higher expression of adhesion molecules receptors for ICAM-1 and VCAM-1, CD11a and VLA-4 respectively (Frankenberger, Menzel et al. 2004). This up regulation is believed to increase their interaction with the endothelial cells and hence increase transmigration in to the tissues.

Further, it is well recognised that the CD14+CD16+ monocytes have distinct migratory properties. CD14+CD16+ monocytes are characterised by strong expression of CX3CR1 (receptor for fractalkine or CX3CL1) and CCR5; weak expression of CCR2 (receptor for MCP-1 or CCL2). In contrast, classical monocytes exhibit strong expression of CCR2 and weaker expression of CX3CR1 and CCR5 receptors (Weber, Belge et al. 2000). This distinct chemokine receptor expression would suggest that the mechanisms that govern transmigration are likely to be different for the different monocyte subpopulations. In some studies, CD14+CD16+ monocytes failed to respond to MCP-1 (CCL2) (Weber, Belge et al. 2000) but instead fractalkine (CX3CL1) preferentially mediated the migration (Ancuta, Rao et al. 2003 Jun 16).

Therefore, in summary, CD14+CD16+ monocytes constitute a distinct population of mature monocytes, with potentially enhanced pro-inflammatory and transmigratory capabilities.

1.2.3 CD14+CD16+ monocytes- a marker of systemic monocyte activation?

The CD14+CD16+ monocytes population is expanded in various acute and chronic inflammatory conditions. In acute severe sepsis, Fingerele et al (Fingerle, Pforte et al. 1993) have demonstrated that in some patients there is a ten-fold increase in the CD14+CD16+ numbers. In other acute inflammatory conditions such as Kawasaki's disease (Katayama, Matsubara et al. 2000) there is two-fold increase in the CD14+CD16+ population. Similar results have also been reported in patients with chronic inflammatory conditions such as rheumatoid arthritis (Kawanaka, Yamamura et al. 2002 Oct), sarcoidosis (Okamoto, Mizuno et al. 2003), tuberculosis (Vanham, Edmonds et al. 1996) and AIDS dementia (Pulliam, Sun et al. 2004 Dec; Ellery and Crowe 2005). In patients with chronic renal failure on hemodialysis who were clinically stable, the CD14+CD16+ population was significantly elevated (14% compared to 8% in controls) whereas the CD14++ population remained normal (Saionji and Ohsaka 2001). The expansion of this cell population appears to be selective and specific and not just an associated epiphenomenon due to a non-specific increase in cytokines (TGF-b1, IL-10) that induce maturation to CD14+CD16+ type. This selective expansion further increased with associated intercurrent infections and decreased with successful antibiotic treatment whereas in contrast the CD14++ population did not change significantly.

Further the CD14+CD16+ population may be a better marker of severity of disease in various acute and chronic conditions compared to CD14++ monocytes. In rheumatoid arthritis (Kawanaka, Yamamura et al. 2002 Oct) a positive

correlation has been shown between increased joint tenderness, raised CRP, erythrocyte sedimentation rate (ESR), rheumatoid factor titres and high CD14+CD16+ count. Similar findings have been reported in acute inflammatory conditions such as Kawasaki's disease (Katayama, Matsubara et al. 2000 Sep). Moreover, in some of the longitudinal studies in rheumatoid arthritis (Kawanaka, Yamamura et al. 2002), CD14+CD16+ monocyte appeared to be a reliable marker of disease activity and was useful to predict the group of patients who would respond to therapy. Kawanaka et al (Kawanaka, Yamamura et al. 2002 Oct) grouped patients to active disease and inactive disease groups based on clinical symptoms, serum CRP and RF levels following a course of anti-inflammatory therapy, and compared CD14+CD16+ counts between these groups. In both groups, the baseline CD14+CD16+ was elevated compared to healthy controls. They found that CD14+CD16+ count remained high in patients with active disease, but it returned to normal levels in patients who responded to treatment and were in remission (active RA 74 +/- 51/ µl, inactive RA 43 +/- 31/µl and healthy control 37 +/- 14/µl). Similar findings have been described in other inflammatory conditions. In a study of patients with systemic vasculitis and nephritis (Scherberich and Nockher 1999), the clinical improvement with glucocorticoid therapy was associated with a significant drop in the CD14+CD16+ monocyte count. Further, the fall in cell count correlated with improvement in other clinical parameters such as rise in glomerular filtration rate (GFR), decrease in proteinuria and fall in acute phase reactants. These data provide convincing evidence that certain local and systemic inflammatory diseases lead to systemic monocyte activation which in turn results in preferential expansion of CD14+CD16+ monocytes. In other words, CD14+CD16+ monocyte population

could be a marker of systemic monocyte activation. So what is known about CD14+CD16+ monocytes in lung inflammation?

1.2.4   CD14+CD16+ Monocytes and Lung Inflammation

The evidence for a key role for the CD14+CD16+ monocytes in lung inflammation is derived predominantly from research in sarcoidosis (Okamoto, Mizuno et al. 2003) and pulmonary tuberculosis (Vanham, Edmonds et al. 1996). In a study of patients with sarcoidosis, high CD14+CD16+ number has been recently described. This increase moreover, correlated with disease severity and improved with treatment. Again in this condition, CD14+CD16+ proportion was better indicator of disease severity and a better predictor of response to treatment compared to CD14++ monocytes. Similar results have also been shown in tuberculosis.

Further evidence is available in allergic conditions such as asthma. Rivier et al (Rivier, Pène et al. 1995) have shown that untreated active asthma is associated with an increase in expansion of CD14+CD16+ monocyte population. But the clinical significance of this finding is unclear.

The pathophysiological role of CD14+CD16+ monocytes appears to be complex and has been poorly understood. Some murine and rat models (Yrlid, Milling et al. 2006) seem to suggest that CD14+CD16+ monocytes key role is in maintaining normal transmigration in to non-inflamed tissue (Tacke and Randolph 2006) and that they are incapable of migrating in to the inflammatory sites (Geissmann, Jung et al. 2003 Jul; Sunderkötter, Nikolic et al. 2004). However, contradictory results

are available from different animal models (Maus, von Grote et al. 2002) suggesting that whether or not these cells are recruited in to the inflammatory sites could be partly explained by different experimental models used. Therefore, it is conceivable that these cells may also play an important role in maintaining inflammation because of their enhanced pro-inflammatory and transmigratory properties. Even so, there is still considerable uncertainty about their specific role. At present there is no direct evidence to suggest that the expanded tissue macrophage population in various inflammatory conditions is in fact due to an increase in recruitment of these cells. Even in conditions such as rheumatoid arthritis where increase in proportion of CD16+ synovial macrophages has been demonstrated (Iwahashi, Yamamura et al. 2004), it is still possible that they are derived from transmigrated CD14++CD16- monocytes under the influence of cytokine such as transforming growth factor- β (TGF- β) and not from CD14+CD16+ monocytes (Wahl, Allen et al. 1992). Nevertheless, it is accepted that these cells are distinct to classical monocytes with respect to their function and migratory properties and that it is important not to overlook their possible role in inflammatory conditions including CF.

In CF, studies on monocytes are limited and have so far concentrated on demonstrating differences in the pro-inflammatory properties of CF monocyte-derived macrophages. To date, there is no data on monocyte populations, drivers of monocyte recruitment and on nature of their role once recruited into the lungs. Importance of such research in CF is highlighted by the fact that recently therapeutic strategies to modulate monocyte transmigration, thereby minimising the inflammation and tissue damage have been developed in other conditions such

as rheumatoid arthritis. One of the initial steps in developing such a therapeutic strategy would include demonstration of systemic monocyte activation and characterisation of monocyte subpopulations as the mechanisms that govern transmigration of each of sub-groups are likely to be different. Therefore, in this study, I sought to measure the monocyte subpopulations in a group of patients with cystic fibrosis and compared with healthy controls. My hypothesis was that CF is associated with systemic activation of peripheral blood monocytes. The following section concentrates on various techniques of characterisation of blood monocytes and their implications in developing the methodology for my study.

1.2.5   Characterisation of blood monocyte subpopulations

The blood monocytes were originally characterised by various cell separation methods such as sedimentation, FcγR expression and fibronectin (Fn) adhesion. Zembala et al (Zembala, Uracz et al. 1984) originally showed that FcγR + cells were expanded in sepsis and had greater antigen expression properties. Graige-Griebenow (Grage-Griebenow, Lorenzen et al. 1993) attempted to define them by use of high affinity FcγR (FcγRI; CD64). In this study CD64- cells had higher antigen expression capacity and were pro-inflammatory. However, in the above methods, there are possibilities of contamination by other leucocytes and activation of the cells during cell preparation which could potentially influence the above results. In order to minimise these problems, Ziegler-Heitbrock et al (Passlick, Flieger et al. 1989; Frankenberger, Menzel et al. 2004) used fluorescence activated cell sorter (FACS) and employed two colour immunofluorescene with monoclonal antibodies against CD14 and CD16. The mononuclear cells from the peripheral blood were initially obtained by density-gradient sedimentation using Ficoll-Hypaque. The cells were then sorted using

antibodies against CD14 and CD16; the CD14+CD16+ monocytes could be separated from CD14++ classical monocytes based on their expression of CD16 and low expression of CD14. They could also be clearly distinguished from other cells types such as NK cells and dendritic precursors as these cell types expressed CD14-CD16+ and CD14+CD16- respectively. The same group have shown further that the characterisation of monocytes could be performed on whole blood sample and does not require to separation of mononuclear cells (Fingerle, Pforte et al. 1993). This technique is simple and requires minimal handling which means that possibility of artefactual cell activation affecting the results are overcome considerably. I have therefore used this methodology to characterise monocyte subpopulations and to determine their absolute counts. Various studies since have provided more information on differential expression of cell surface molecules in CD14+CD16+ monocytes compared to CD14++ monocytes (Frankenberger, Menzel et al. 2004) (Table 1.2-1).

Surface marker expression	CD14+CD16+ monocytes	CD14++ monocytes
CD14	+	++
CD15	-	+
CD16	+	-
Cd64	-	+
CCR2	-	++
CX3CR1	++	+
DR	++	+
CD38	-	++

Table 1.2-1: Differential Expression of surface receptors in CD14+CD16+ monocytes as compared to CD14++ monocytes.

1.2.6   Flow Cytometry

Flow cytometry is a technique that provides rapid measurements of multiple characteristics of single cells as they flow in a fluid stream. It measures the optical and fluorescence characteristics of each individual cell within the suspension and not just as average values for the whole population. Flow cytometry are used for immunophenotyping of variety of specimens for example blood, cerebrospinal fluid (CSF), sputum etc. Cell size, cytoplasmic constituents, wide range of intra-cellular and membrane bound proteins, DNA and RNA are some of the characteristics that can be measured. Further, flow cytometry has also been used to separate different cell populations for various applications.

1.2.6.1 Principles of Flow Cytometry

Flow cytometry uses principles of light scattering, light excitation and emission of flurochrome molecules. The cells in suspension move past an excitation source which is usually a beam of monochromatic light from a laser. The light hitting individual cells are either scattered or absorbed initially and then re-emitted (fluorescence). The scattered and re-emitted lights are then detected by photomultiplier tubes after passing through the optical filters which isolate particular wavelength bands.

The pattern of scatter of the light by the cells either by reflection or refraction is dependent on the cell size and shape and hence gives information about these characteristics of the cell. This can be displayed as a dot plot of forward and side scatter where the cells are displayed grouped based on their size and granularity (figure 1.2-1). Further the cells can be sorted in to different collection tubes based on their shapes and size by the use electrostatic deflection for other applications.

For fluorescence-based detection, the cells are labelled with specific fluorochromes, a fluorescent marker. These fluorochromes are excited by specific wavelength of light and results in emission of secondary light at a specific wavelength which is detected by photomultiplier tubes, enhanced by use of optical filters which block the original light source thereby reducing the background. Detection of the second wavelength is used as a measure of the presence of the dye on the cell and thus the component it is labelling (figure 1.2-2).
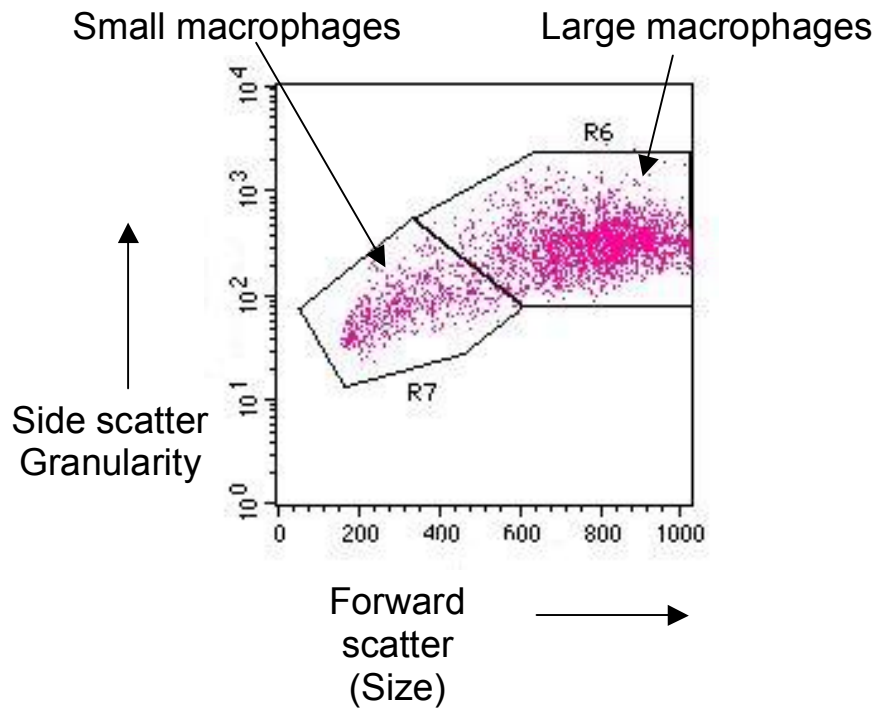
Figure 1.2-1: Dot plot showing light scatter properties of the macrophages from induced sputum sample. Based on the light scatter properties, in this example the macrophage populations can be divided in to small (R7) and large macrophage (R6) sub-populations
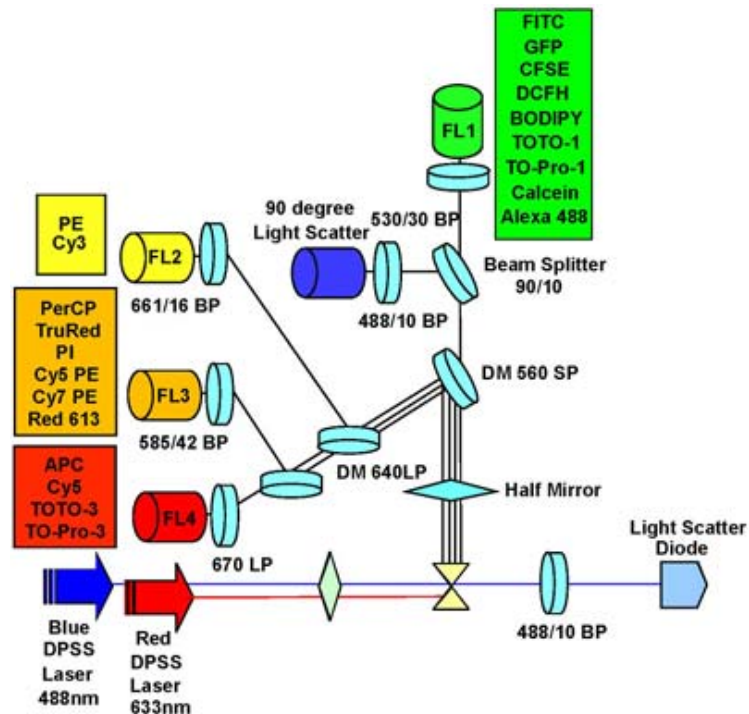
Figure 1.2-2: A schematic of optical layout of FACS Calibur (Courtesy Becton Dickinson UK)

The data from the flow cytometer is displayed either as a dot plot or a histogram. In the dot plot the X and Y axes measure different emission and each cell with that particular emission is displayed as a dot. Depending on the pattern of emissions of different cell populations, each population is represented in particular quadrants of the dot plot (figure 1.2-3).

In the histogram, the X-axis shows the intensity of the detected signal and Y axis the number of cells (events) counted. Histograms often display the output of two (or more) samples using a single fluorochrome. In an experiment determining the presence or absence of a particular cell marker or a relative increase or decrease of a marker after experimental treatment, a histogram shows the shift in the fluorescence intensity of the sampled cells is represented (figure 1.2-4).
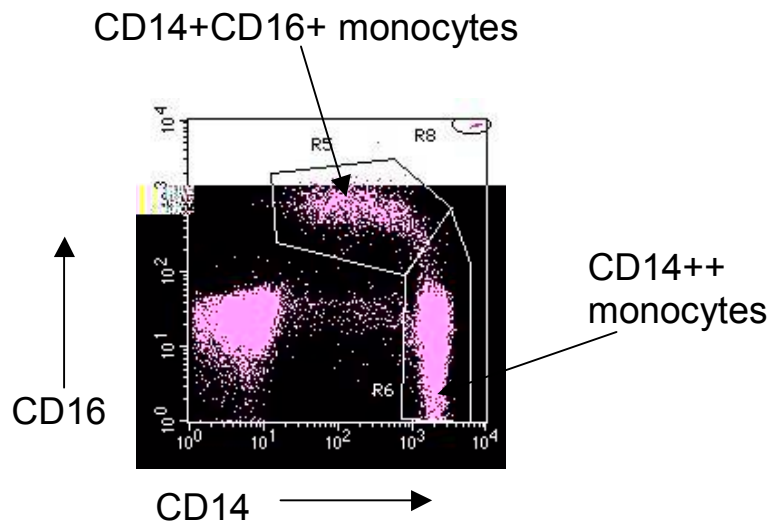
Figure 1.2-3: Dot plot showing expression of surface receptor CD14 FITC and CD16 PE along X and Y axes respectively. In this example, using flow cytometry 2 different populations of monocytes are identified as shown in the figure.
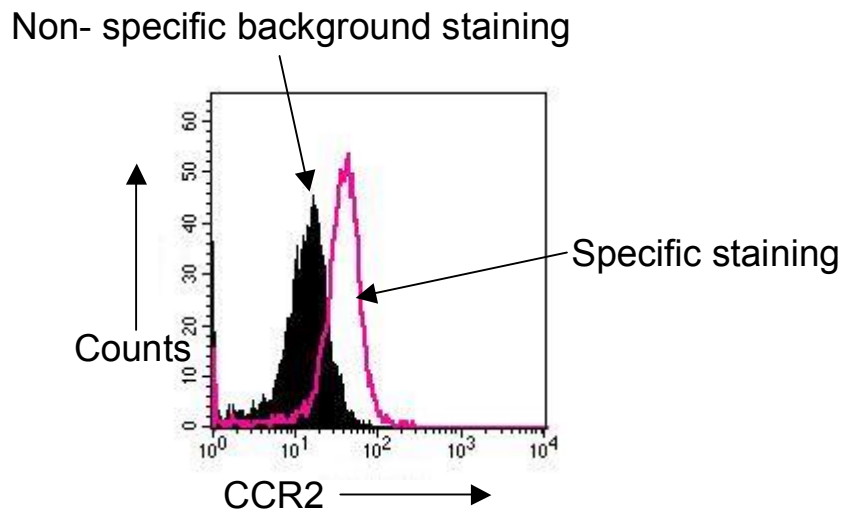
Figure 1.2-4: Histogram showing expression of chemokine receptor CCR2 in classical monocytes. The black shaded area denotes non-specific background staining whereas pink open histogram represents specific CCR2 staining. The intensity of CCR2 receptor expression is the difference between the median fluorescence intensity of specific and background staining.

1.3 Monocyte Chemoattractant Activity and Chemokine Receptor Expression

The transmigration of leucocytes in to the tissues involves a cascade of molecular events including elaboration of chemokines. Chemokines aid leucocyte/endothelial interaction and facilitates leucocyte transmigration through the blood vessel. The first step in the classical pathway of transmigration involves selectin-mediated transient and weak interaction between the leucocyte and endothelial cells. But the next step that results in definitive adhesion between leucocytes and endothelial cells is mediated by the chemokines through the up regulation of integrins cascade. Chemokines can be grouped broadly in to 2 groups based on their functions namely homeostatic chemokines and inflammatory chemokines (Moser and Loetscher 2001; Moser and Loetscher 2001). Homeostatic chemokines are responsible for basal leucocyte trafficking in to tissues and lymph nodes whereas inflammatory chemokines recruit leucocytes in response to a stress for example infection. Chemokines thus have important roles in both maintaining homeostasis and in stress example infection. However, inappropriate over-expression of inflammatory chemokines may result in exaggerated recruitment of activated leucocytes and thus lead to extensive tissue damage is seen in various chronic inflammatory conditions (Moser 1998; Gerard and Rollins 2001).

Study of chemokines offers the prospect of developing a novel strategy to modulate inflammatory response to the host's advantage in conditions where inappropriate inflammation leads to irreversible tissue damage for example in rheumatoid arthritis and other auto-immune conditions. By using specific

chemokines blockers, excessive leucocyte trafficking could be modulated and thereby minimising disease progression. Further, in certain infection such as HIV, there is also evidence to suggest that chemokines receptors (in this case CCR5) in fact could act as receptor to the virus enabling them to enter in to the immune cells which is an essential pre-requisite to their replication and dissemination.

Chemokines are a family of structurally related glycoproteins with potent leukocyte activation and/or chemotactic activity (Zlotnik and Yoshie 2000). They are 70 to 90 amino acids in length and approximately 8 to 10 kDa in molecular weight. Most of them fit into two subfamilies with four cysteine residues. These subfamilies are based on whether the two amino terminal cysteine residues are immediately adjacent or separated by one amino acid. The α chemokines, also known as CXC chemokines, contain a single amino acid between the first and second cysteine residues; ß, or CC, chemokines have adjacent cysteine residues. Most CXC chemokines are chemoattractants for neutrophils whereas CC chemokines generally attract monocytes, lymphocytes, basophils, and eosinophils. There are also 2 other small sub-groups. The C group has one member (lymphotactin). It lacks one of the cysteines in the four-cysteine motif, but shares homology at its carboxyl terminus with the C-C chemokines. The C chemokine seems to be lymphocyte specific. The fourth subgroup is the C-X3-C subgroup. The C-X3-C chemokine (fractalkine/neurotactin) has three amino acid residues between the first two cysteines. It induces both adhesion and migration of leukocytes (Umehara, Bloom et al. 2004).

1.3.1   Chemokines and Monocytes

Monocyte trafficking is influenced by a wide range of chemokines as suggested by the expression of multiple chemokines receptors and diversity of receptor interactions with more than one ligand (Patel, Charlton et al. 2001). But, monocyte chemoattractant protein-1 (MCP-1/ CCL2) is believed to be the main chemokine responsible for monocyte trafficking. It has also been realised recently that different monocyte populations have distinct migratory properties. For instance, The CCR2 expression in the CD14+CD16+ monocytes is weak compared with the classical monocytes. Which means that CCL2 is a potent chemoattractant for the classical monocyte population but has little effect on CD14+CD16+ monocytes (Geissmann, Jung et al. 2003 Jul). In contrast CD14+CD16+ monocytes have high expression of CX3CR1 receptor suggesting a key role for CX3CL1, which is CX3CR1 ligand.   The discussion in the ensuing sections will demonstrate the importance of CCL2 and CX3CL1 as key mediators of monocyte transmigration.

1.3.2   CCR2 and CCL2

MCP-1/CCL2 belongs to CC families of chemokines and is expressed by epithelial cells, endothelial cells and macrophages. The receptor for CCL2 binding on the monocytes is CCR2 (Frankenberger, Menzel et al. 2004). It is known that there are at least five other ligands for this receptor in mouse and monocytes express other chemokine receptors as well. But there is convincing evidence that CCL2 and CCR2 interaction is very important for monocyte trafficking. Inactivation of CCL2 or CCR2 receptor either by specific antibodies or in knock-out models is sufficient to arrest the recruitment of monocytes (Daly and Rollins

2003). Evidence to support this is available in various inflammatory conditions but has been best studied in atherosclerosis and multiple sclerosis.

1.3.3 CCL2/CCR2 and Inflammation

Atherosclerosis is characterised by inflammatory reaction resulting in the deposition of lipid and cholesterol in the arterial wall. The key role for CCL2 as the main chemo-attractant for the monocytes was convincingly shown in a study of genetically modified mice that were deficient in CCL2/CCL2 expression. These mice had 83% less lipid deposition in spite of being fed high cholesterol diet which was still sustained after 20 weeks of the study (Tabary, Zahm et al. 1998 Sep). Conversely, an increased atheroma formation was demonstrated in another mice model where over-expression of CCL2 was genetically induced (Aiello, Bourassa et al. 1999). Further, use of a direct anti-CCL2 therapy (N-terminally truncated CCL2 variant 7ND) substantially reduced the lesion formation in coronary arteries in a hypertensive rodent model (Egashira, Koyanagi et al. 2000). Similar to the above studies, reduced atheroma correlated with reduction in macrophage number. Further, we can find similar evidence in another inflammatory condition, multiple sclerosis, which is associated with demyelination. In this disease, in two separate models, one using antibody against CCL2 (Karpus and Kennedy 1997) and other with CCL2 deficient mice (Fife, Huffnagle et al. 2000), a marked reduction in inflammation has been demonstrated when CCL2 was blocked.

In humans, key role for CCL2/CCL2 in monocyte recruitment is clearly defined in conditions such as atherosclerosis (Szalai, Duba et al. 2001) and rheumatoid arthritis (RA) . Genetic polymorphisms that result in increased expression of CCL2 are associated with 2-fold increase in the incidence of coronary artery disease secondary to atherosclerosis (Szalai, Duba et al. 2001). In rheumatoid arthritis, plasma CCL2 levels correlate with severity of joint swelling and it is increased in patients with active disease (Yao, Kuo et al. 2006). Therapeutic benefit of modulation of CCL2 has been shown in experimental conditions in RA; reduction of CCL2 expression resulted in beneficial effects on joint inflammation (Dawson, Miltz et al. 2003). In fact there is evidence to suggest that CCL2 is much more than a chemoattractant and has the potential to induce inflammatory response. Viedt et al in their study of tubulo-interstitial renal disease found that CCL2 activates tubular epithelial cells leading to secretion of pro-inflammatory cytokine interleukin (IL)-6 and intercellular adhesion molecule-1 (ICAM-) in vitro (Viedt and Orth 2002). However, these results need to be confirmed by further in vivo studies.

Monocytes express several chemokines receptors (Patel, Charlton et al. 2001). But, there is substantial body of evidence to suggest that CCR2 receptor (with CCL2 as its ligand) is the key receptor for monocyte transmigration. Similar to CCL2 studies, most of the evidence originates from experiments in mice using CCR2- knock out models in conditions such as atherosclerosis (Boring, Gosling et al. 1998) and multiple sclerosis (Fife, Huffnagle et al. 2000) where CCR-2 deficient mice demonstrated weaker inflammatory response.

1.3.4 CCR2 and Monocyte Chemo-attractant protein (CCL2) in Lung Inflammation

Maus et al in a series of experiments in mice have provided convincing evidence for the importance of CCL2/CCR2 interaction in monocyte recruitment in to the lungs. In the initial experiments, they showed that intra-tracheal instillation of CCL2 (JE MCP-1) resulted in monocyte accumulation in lungs (Maus, Herold et al. 2001; Maus, von Grote et al. 2002 Aug 1), which was exaggerated in the presence of infection with *E coli* (Maus, Huwe et al. 2001) . However the importance of specific interaction between CCR2 and CCL2 was established in their subsequent experiments in which the mice were divided in to 4 groups; wild type mice, CCR2 deficient, CCR2 knock-out transplanted with wild type, and wild type transplanted with CCR2 (Maus, Waelsch et al. 2003). They showed that the CCL2 could not induce monocyte transmigration in the absence of CCR2 receptor. The monocyte accumulation in to the lungs in response to CCL2 and LPS was significantly reduced in the CCR2 deficient mice and this reversed when the CCR2 knock-out mice were transplanted with wild type monocytes that expressed CCR2. Another significant finding was that the monocytes were potent inducers of neutrophilic influx in to the airways. The presence of CCR2+ cells in the lungs induced a more pronounced neutrophilic influx. There was reduction in the degree of neutrophilic inflammation in CCR2 deficient mice compared to the wild type in response to LPS. And this effect was reversed when CCR2 deficient mice were transplanted with wild type alveolar macrophages that expressed CCR2.

In humans, CCL2 has been studied in various inflammatory conditions of the lung. In COPD, de Boer et al have demonstrated 1.5 fold increase in CCL2 mRNA

expression in the bronchial epithelium which correlated with increased expression of CCR2 on macrophages and number of intra-epithelial macrophages suggesting a key role for CCL2/CCR2 interaction in monocyte recruitment (de Boer, Sont et al. 2000). In another study of BAL samples, the bronchitis group and asymptomatic smoker group had significantly higher concentration of CCL2 compared with healthy non-smoking group (Capelli, Di Stefano et al. 1999). Further in the same study, cessation of smoking was associated with decrease in CCL2 levels suggesting a relationship between smoking induced airway inflammation and CCL2. Similar evidence of raised CCL2, which correlates with inflammatory indices and clinical parameters, is available in other inflammatory lung conditions such as interstitial lung disease (Suga, Iyonaga et al. 1999). Based on the above findings we can conclude that CCL2 is one of the main mediators of monocyte transmigration into the lungs.

CCL2 might also have a key role in resolution of inflammation and repair. In one experiment CCL2 promoted resolution and repair by increasing alveolar macrophages ingesting neutrophils. And instillation of anti-MCP1 antibodies reversed this effect causing more inflammation and tissue destruction (Amano, Morimoto et al. 2004).However, there are other studies with conflicting findings. In a mice model of non-infectious lung injury following allogeneic bone marrow transplantation (idiopathic pneumonia syndrome), CCR2+ monocytes contributed to more inflammation and neutralising CCL2/CCR2 either by transplantation of CCR2 deficient cells or by using CCL2 neutralising antibodies resulted in significantly reduced inflammation and tissue destruction (Hildebrandt, Duffner et al. 2004).

In CF, the role of CCL2/CCR2 has been poorly studied. To date, there is only indirect evidence of their role in sustaining airway inflammation. In two clinical studies, CCL2 was detectable in blood (Augarten, Paret et al. 2004) and induced sputum samples (McAllister, Henry et al. 2005) of CF patients. As there were no control samples in these studies, it is not possible to conclude that there is increase in airway and systemic CCL2 activity. Nevertheless, these results do suggest a role for CCL2 in CF. Further, in vitro experiments with CF and non-CF epithelial cells, have shown expression of CCL2 in the CF epithelial cells (Schwiebert, Estell et al. 1999) and *P.aeruginosa* exoenzyme results in stronger expression of CCR2 in peripheral blood mononuclear cells isolated from healthy controls (Epelman, Bruno et al. 2000). This finding could be of specific relevance to CF as majority of patients develop *P.aeruginosa* colonisation in their airways with advancing age.

1.3.5   CX3CR1 and CX3CL1

CX3CL1 is the first CX3C chemokine to be described (Umehara, Bloom et al. 2004). CX3C chemokine and thereby CX3CL1 are unique in many of their characteristics. First, there are 3 amino acids between the cysteines residues. Second, they are large with a sequence that continues for 241 residues unlike other chemokines, which terminate at 70-80 amino acids. Above all, they consist of 4 domain, characteristic of this group of chemokines; an extracellular domain with 76 amino acids, a mucin like stalk, a transmembrane domain and an intracellular domain. The CX3C chemokines are membrane bound unlike other cehmokines

that are soluble, and by this virtue, they can function both as chemokines as well as adhesion molecules (Umehara, Bloom et al. 2004).

CX3CL1 plays an important role in the trafficking of T-cells, NK cells and monocytes. It is mainly expressed by the endothelial cells and not leucocytes (Umehara, Bloom et al. 2004). On induction by various mediators such as TNF-a, IL-1 and γ-interferon, the chemokine domain is presented at the top of the mucin stalk extracellularly. CX3CR1 expressing cells bind rapidly to these receptors with more affinity compared to vascular adhesion molecules (VCAM-1) (Haskell, Cleary et al. 1999). Further soluble CX3CL1 can also be induced by various mediators and is chemotactic to T-cells, NK cells and monocytes. In addition to its intrinsic adhesive property, the CX3CR1 induces the integrin cascade as well thereby resulting in a greatly enhanced cell adhesion compared to each system alone (Goda, Imai et al. 2000). Thus CX3CL1 functions both as an effective chemokine as well as adhesion molecule.

CX3CL1 receptor (CX3CR1) appears to be preferentially expressed in Th1 cells compared to Th2 cells thereby suggesting that it plays an important role in mediating Th1 response. Th1 cells include CD4+ T cells, CD8+ T cells and NK cells. The CX3CR1 is mainly expressed in T-cells however recent evidence shows that it is also expressed in CD14+CD16+ monocytes and that CX3CL1/CX3CR1 interaction plays an important role in CD14+CD16+ monocyte trafficking (Ancuta Rao et al. 2003).

1.3.6   CX3CL1 and Inflammation

Similar to CCL2 most of the evidence for a key role for CX3CL1 originates from studies in atherosclerosis, renal disease, HIV infection, allograft rejection and other inflammatory disease such as rheumatoid arthritis (Umehara, Tanaka et al. 2006). In atherosclerosis, inducing CX3CR1 deficiency results in decreased atherosclerotic lesion in the arteries (Umehara and Imai 2001). Similarly in acute allograft rejection models, blocking of CX3CL1 by neutralising antibodies prolongs the survival of allogeneic grafts which is associated with reduction in leucocyte infiltration including macrophages (Haskell, Hancock et al. 2001). In glomerulonephritis rat model anti-CX3CR1 treatment blocked leucocyte infiltration, prevented crescent formation, improved renal function suggesting a key role for CX3CR1/fractalkine interaction in inflammatory renal diseases (Feng, Chen et al. 1999).

In patients with rheumatoid arthritis, synovial macrophages, fibroblasts, endothelial cells and dendritic cells have been shown to express CX3CL1 and CX3CR1 (Umehara and Imai 2001). In all the other studies, the impact of CX3CR1/CX3CL1 interaction is not specific to monocytes and on the contrary is broad with influences over various inflammatory cells including NK cells and T-cells. Nevertheless, it affects monocyte trafficking, specifically targeting the pro-inflammatory CD14+CD16+ monocytes. Therefore, dysregulation of CX3CR1/CX3CL1 could result in exaggerated recruitment of pro-inflammatory monocytes into the tissues.

1.3.7 CX3CL1 and Lung Inflammation

There is limited understanding of the role of CX3CR1/CX3CL1 interaction in lung disease. It appears that CX3CL1 is up regulated in the inflamed lungs as demonstrated in a septic rat model by Beck et al (Becker, Sauer et al. 2004 Mar 1). This effect in fact could lead to increased monocyte adherence and transmigration. Fujimoto et al (Fujimoto, Imaizumi et al. 2001) performed in vitro studies using human bronchial epithelial cell lines and lung biopsy specimens obtained from patients undergoing surgery for suspected lung cancer. They showed that the enhanced mononuclear cell adherence following induction of CX3CL1 in bronchial epithelial cells and this effect was neutralised by use of antibody against CX3CL1. In the same study bronchoalveolar lavage (BAL) was obtained from patients with range of acute and chronic lung inflammatory conditions and positive correlation between the CX3CL1 concentration in and mononuclear cell counts was demonstrated. CX3CL1 is also expressed by airway smooth muscles in response to TNF-α (Sukkar, Issa et al. 2004) and might have an important role in recruiting leucocytes for example mast cells in asthma (El-Shazly, Berger et al. 2006). These results show that CX3CL1 does have a role in leucocyte recruitment in to the lungs.

There is no current data on the role of CX3CL1 as inflammatory mediator in cystic fibrosis. Given its unique chemotactic and adhesive properties with an ability to influence transmigration of a wide range of inflammatory cells, one can speculate that CX3CL1 plays an important role in sustaining inflammation in CF.

The summary of the above sections is that CCL2 and CX3CL1 are the principal chemokines for CD14++ monocytes and CD14+CD16+ monocytes respectively. The chemokines act via their main respective cognate receptors CCR2 and CX3CR1 respectively. Also there is good evidence for a key role of the CCL2/CCR2 and CX3CL1/CX3CR1 interaction in other chronic inflammatory conditions. It is not clear at this stage if there is such an interaction in CF. Therefore in my study, I evaluated if there was an increase in systemic and airway monocyte chemoattractant activity (CCL2 and CX3CL1) and if there was any altered chemokines receptor expression.

1.4 Airway Macrophage heterogeneity and lung inflammation

Macrophages and monocytes are derived from the myelo/monocytic stem cell in the bone marrow. The latter progenitor cells develop into monoblasts and then monocytes. Monocytes are released into the blood and then migrate into tissue at which stage they are termed macrophages. Tissue macrophages can be very heterogeneous with respect to morphology and function as exemplified by microglia cells in the brain, peritoneal macrophages, osteoclasts or Kupffer cells in the liver.

Similar to blood monocytes, the airway macrophage population is heterogenous and it appears that each group has distinct phenotypic and functional characteristics. In the lung there are 3 different types of macrophages, i.e. the interstitial macrophages, the alveolar macrophages and the bronchial macrophages. Alveolar macrophages play an important role in orchestrating

inflammation by production of host of inflammatory cytokines, tissue regeneration and maintaining homeostasis. Any disturbance of tissue homeostasis causes rapid expansion of macrophages due to influx of blood monocytes. There is evidence to suggest that these newly recruited macrophages have distinct phenotypical and functional characteristics compared to the resident alveolar macrophages. They are smaller in size, have >150 fold increased expression of surface marker CD14 and are exclusively pro-inflammatory in nature with substantially increased expression of pro-inflammatory cytokines like TNF-α (Frankenberger, Menzel et al. 2004). In this study of patients with chronic obstructive pulmonary disease (COPD), there was an increase in the induced sputum small macrophage proportion in COPD patients compared to healthy controls (47.1 +/- 24.6% of all macrophages Vs. 6.9 +/- 6.7% of all macrophages respectively). The small macrophages were smaller in size, with high expression of receptors CD14 and HLA DR compared to the large macrophages. Further functional differences were also demonstrated; small macrophages exhibited constitutive TNF production, which increased 6-fold on stimulation with lipopolysaccharide (LPS). Whereas there was little constitutive TNF production from the large macrophages with only a modest 2-fold increase on stimulation with LPS.   These results would suggest that small macrophages have a pro-inflammatory profile and therefore may have an important role in recruiting other leukocytes and maintaining inflammation. In acute inflammatory conditions, once the stimulus is removed, there is a gradual shift in the macrophage population from smaller immature to more mature large alveolar macrophages suggesting a decrease in monocyte influx. This facilitates resolution of inflammation and aids in the repair of the tissue. However, in the presence of continuing stimulus, there appears to be a persistent increase in the pro-

inflammatory small macrophage population due to sustained monocyte recruitment and appears to contribute to a vicious circle of persistent inflammation and irreversible tissue damage. For instance, in ARDS (Rosseau, Selhorst et al. 2000), a decrease in the influx of monocytes after the initial phase of inflammation as evidenced by the transition of predominant macrophage population from small to large phenotype is associated with better prognosis. Only 25% of subjects survived when a sustained increase in smaller monocyte population was observed compared to 75% of subjects if there was a transition from smaller to more mature alveolar macrophages on serial BAL samples.

One could argue that the increase in small macrophage proportion is due to the expansion of resident macrophage population. However, in the above study of ARDS patients, Rousseau et al provided indirect evidence that small macrophages are in fact newly recruited blood monocytes. They showed that these airway macrophages had phenotypic appearance of the blood monocytes and that there was no significant local proliferation of airway macrophages as measured by nuclear staining of Ki67 antigen, a marker of cell proliferation. More convincing direct evidence is available from their further experiments in mice models. Maus et al (Maus, Herold et al. 2001) have demonstrated that monocytes are recruited from the intravascular compartment in response to intratracheal instillation of rJE/MCP-1 and the newly recruited macrophages show a monocytic phenotype but upregulate CD14 and therefore are primed for enhanced responsiveness to endotoxin.

In addition to their enhanced inflammatory profile, small macrophages are believed to amplify recruitment of other inflammatory cells such as neutrophils. In a murine model, Maus et al (Maus, von Grote et al. 2002) have shown that firstly, the recruitment of blood monocytes into the airway is strictly dependent on an airway-intravascular gradient of CCL2 (formally known as monocyte chemoattractant protein-1). Secondly, presence of monocytes expressing CCR2, the receptor for CCL2 resulted in 4 fold increase in lipopolysaccharide-stimulated airway neutrophil accumulation. Finally, they showed that macrophage recruitment during inflammation could be stopped by blocking chemokine CCL2 and this resulted in strong reduction of neutrophil recruitment into the lung.

In cystic fibrosis, the role of macrophages has been poorly studied. The macrophages appear to be inherently pro-inflammatory due to defective CFTR gene. In model of G551D CF mice (McMorran, Palmer et al. 2001), macrophages derived from bone marrow and therefore not exposed to inflammatory stimuli in the serum were hypersensitive to LPS stimuli and exhibited 4-fold higher production of TNF- compared to the wild-type cells. Similar results were observed in study of CF subjects by Pfeffer et al (Pfeffer, Huecksteadt et al. 1993). Further there appears to be infiltration of macrophage in CF airways before birth (Hubeau, Puchelle et al. 2001 Oct), which could play a role in initiating the later influx of neutrophils. However, phenotypic characterisation of macrophages in CF has not been performed so far. Specifically small macrophages have not been studied in CF patients, as yet. Given the persistent inflammation in the disease, it is possible that these cells are expanded in CF as well. If there were indeed an expansion of these cells then this would indirectly suggest that there is increased transmigration of monocytes in CF.

Therefore, in a pilot study of paediatric CF patients I studied if the proportion of the small airway macrophages was increased compared to controls. These results were further confirmed and extended by Mr. Adam Wright my co-researcher and has presented these data in his PhD thesis.

In the Paediatric age-group obtaining a reliable sputum sample using a non-invasive method is challenging. Towards that goal, the method of sputum induction has shown some success. In the following section, sputum induction as a technique of collecting lower airway sample has been appraised.

1.5   Sampling of macrophages and inflammatory cells from the lower airways: sputum induction

Induction of sputum using hypertonic saline has been used extensively for a variety of applications in clinical and research areas. This method involves subject inhaling hypertonic saline delivered as an aerosol by a nebuliser. The exact mechanism by which the hypertonic saline acts is not completely understood. It is believed that it has both direct and indirect mechanisms of action. Hypertonic saline directly increases the osmolarity of the airway surface fluid resulting in increased vascular permeability in the bronchial vasculature leading to more airway secretion production. Although there is evidence for this hypothesis in the animal models (Umeno, McDonald et al. 1990) this has not been confirmed in the clinical studies where conflicting results were observed when osmolarity of the induced sputum samples was measured (Louis, Shute et al. 1999). Whether hypertonic saline induces secretions or not there is evidence to suggest that it improves secretion clearance

from the airways (Pavia, Thomson et al. 1978). It has been suggested that hypertonic saline facilitates collection of pre-existing airway secretions and thereby improves clearance.

Although sputum induction is a simple and direct method of obtaining a sample from the lower airways, it has only recently gained the wider acceptance of clinicians and researchers. The main concerns about this method have mainly been about the safety of the procedure (Pizzichini, Pizzichini et al. 2002) and about the quality of sample obtained compared to bronchoalveolar lavage (BAL) (Paggiaro, Chanez et al. 2002). In cystic fibrosis and other infectious conditions, there are further concerns about whether patients with marked lung disease can tolerate the procedure and also about cross-infection of patients and technicians with lower airway pathogens.

A standardised method of sputum induction in asthma was first published by Pin et al in 1992 (Pin, Gibson et al. 1992). Since then there has been an enormous increase in number of publications in which researchers have used this method to study various aspects of inflammation initially in asthma but recently extending to other chronic inflammatory conditions such as chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF). Sputum induction has been shown to be comparable to BAL with respect to the cellular and cytokine profile of the samples obtained (Reinhardt, Chen et al. 2003 Sep), reproducible with minimal variability between the samples (in 't Veen, de Gouw et al. 1996; Pizzichini, Pizzichini et al. 1996), safe even in subjects with marked lung disease (Pizzichini, Pizzichini et al. 1997; Pizzichini, Pizzichini et al. 2002) and non-invasive. Therefore it has offered a

new range of applications that were not possible before. A wide range of inflammatory markers in various conditions can not only be studied but a close correlation of these findings with clinical picture is now possible. Particularly, the longitudinal studies on effect of intervention on various markers could not be performed before as it involved performing serial invasive bronchoscopies. However, such studies have now become feasible as repeated sputum inductions are more acceptable to both patients and clinicians, less invasive and easier compared to serial BAL. For the same reasons, it has also encouraged development of some interesting clinical applications. It has enabled better detection of lower airway infection in group of non-expectorating TB patients who would otherwise have required invasive bronchoscopy to obtain reliable sample (Cairns, Crockard et al. 2002 Mar). It has also enabled clinicians to measure various inflammatory markers to guide changes in therapy. For example, it has been shown in asthma that titrating steroid therapy based on the sputum eosinophils in induced sputum, results in better symptom control and fewer exacerbation rates compared to the standard therapy where the disease is monitored on symptoms and lung function (Green, Brightling et al. 2002). Therefore, in future, one could speculate that there will be even wider applications of this method especially in monitoring airway inflammation and using that knowledge to guide disease management.

1.5.1   Sputum Induction in Cystic Fibrosis

In this section, an attempt has been made to summarise the available evidence in sputum induction in cystic fibrosis. The main areas of this review in CF are similar to the previous section but certain specific points of particular relevance to CF are discussed in more detail. Recently, there has been a considerable increase in the

number of studies using sputum induction to sample lower airways in CF for various applications (Armstrong 2003). However, sputum induction in CF does require some important considerations and it is important to discuss these in more detail.

1.5.2 Safety of Induced sputum in CF: hypertonic saline induced bronchospasm

Hypertonic saline inhalation induces bronchospasm (Bickerman, Sproul et al. 1958) in subjects with pre-existing lung disease. It was first described by Bickermann et al(Bickerman, Sproul et al. 1958) in 1958 in their experiments involving saline between 3-15% and they concluded that the more hypertonic the saline more the risk of bronchospasm. Later in 1981, Scheoffel et al (Schoeffel, Anderson et al. 1981) demonstrated progressive bronchoconstriction with increasing osmolarity of the saline solution. In fact, hypertonic saline has been used to measure bronchial hyper-responsiveness (BHR) in asthma (Smith and Anderson 1990). The exact mechanism of this adverse effect is not known and it has been suggested that hypertonic saline could cause activation of mast cells (Gravelyn, Pan et al. 1988) or sensory nerve endings (Makker and Holgate 1993). Salbutamol inhibits the saline induced BHR (Boulet, Turcotte et al. 1989) and pre-treatment with salbutamol in some studies has shown to have a protective effect (Popov, Pizzichini et al. 1995) although it does not completely abolish it (Pizzichini, Pizzichini et al. 2002).

CF patients often have degree of lung impairment associated with increased BHR. Therefore there are concerns that saline induced bronchospasm might be more severe in this group of patients. However, hypertonic saline (up to 7%) is used as one of the treatment modalities to improve airway clearance in CF patients. A recent

Cochrane review has shown that it improves airway clearance associated with short-term benefit on the lung function. It appears that hypertonic saline inhalation in fact has protective rather than deleterious effect on CF airway. Further, safety of hypertonic saline inhalation during sputum induction has been the subject of number of studies and they conclude that it is indeed a safe procedure to perform even in patients with markedly reduced lung function. Suri et al (Suri, Marshall et al. 2003) showed that in a group of 45 children with moderate to severe lung disease (mean FEV1 48% predicted, range 14-77%) only two subjects developed clinically significant bronchoconstriction with fall in FEV1 >15%. There was in fact no correlation between the airway hyperreactivity and baseline lung function suggesting that even in subjects with severe lung disease, sputum induction is safe and not associated with severe adverse effects. However, nearly two thirds of the patients showed a fall in FEV1 with hypertonic saline inhalation which means that careful monitoring of the lung function and facilities for prompt reversal of bronchospasm are absolute requirements when inducing sputum in CF patients. The other contributing factor to this adverse effect could be the strength of the hypertonic saline; higher the strength of saline higher the incidence of bronchospasm. De Boeck at al (De Boeck, Alifier et al. 2000 Jul) have investigated this question in a study of relatively stable CF patients with inhalation of increasing saline concentrations and shown that the likelihood of developing bronchospasm after 3% saline inhalation remains the same as it is after 6% saline inhalation. Premedication with bronchodilator example salbutamol prior to saline inhalation is believed to have a protective effect. With pre-treatment with bronchodilator, the fall in lung function FEV1 >10% is seen only in 6% of children (Jones, Hankin et al. 2001). However, in CF, there is some evidence that bronchodilator therapy itself

could result in increased airway instability (Zach, Oberwaldner et al. 1985) which could in fact pre-dispose them more pronounced reaction. Therefore, premedication with salbutamol is recommended only for patients receiving bronchodilator therapy.

1.5.3 Safety of Induced sputum in CF: risk of cross-infection

Further, sputum induction is associated with an increased risk of cross-infection because of the aerosolisation of respiratory secretions containing pathogens and dissemination of these secretions by induction of cough. There is a dramatic increase in the airborne bacterial concentrations during and immediately after the procedure (Menzies, Adhikari et al. 2003) which could then infect other patients as well as technicians performing the procedure. The importance of other cough-inducing procedures in disseminating TB to technicians (McKenna, Hutton et al. 1996), trainees (Malasky, Jordan et al. 1990) and to other patients is well recognised. Recently a case of a technician contracting tuberculosis during sputum induction from a patient with undaignosed infection has been reported (van den Berg, Kerstjens et al. 2003). This complication is of particular importance in CF for two reasons - firstly there is a risk of cross-infection between the patients as well as of the technician performing the procedure. Cross-infection of bacteria such as *P.aeruginosa* in a previously well CF patient bears serious consequences. There is increased respiratory morbidity and mortality with acquisition of certain bacteria in CF (Chmiel and Davis 2003). In addition to respiratory secretions, the nebulisers themselves are a further source of cross-infection. Hence it is imperative to have strict infection-control procedures in place. There needs to be adequate ventilation in the procedure room for sufficient air exchange and strict disinfection of equipment as well as the environment should be undertaken during and after the procedure.

1.5.4 Induced Sputum in CF: reliability and reproducibility of measurement of cellular constituents and cytokines

There is substantial evidence to suggest that various inflammatory markers including cellular constituents and cytokine measurements can be reliably measured from the airway samples collecting by sputum induction. Reinhardt et al (Reinhardt, Chen et al. 2003 Sep) compared the constituents of paired BAL and induced sputum samples from CF patients and concluded that there were no significant differences. They also showed that prolonged inhalation results in sampling more and more distal airways. There are concerns that the hypertonic saline might itself induce an inflammatory reaction and hence measurements of these mediators might not be reliable. But Suri et al(Suri, Marshall et al. 2003) compared the neutrophil elastase, myeloperoxidase, IL-8 concentrations in induced sputum with paired spontaneously expectorated samples and demonstrated no difference between the groups. This would suggest that hypertonic saline does not induce significant airway inflammatory response. On the contrary, the cytokine concentrations in induced sputum were significantly lower than spontaneously expectorated sample possibly due to dilution of the samples by inhaled saline.

Several studies have used sputum induction to sample lower airways in CF and shown that there is a raised inflammatory response in CF patients compared with controls. Sagel et al (Sagel and Accurso 2002 Aug; Smountas, Lands et al. 2004 Nov) showed in their study an increase in the levels of sputum IL-8, elastase concentration in the CF population. Smountas et al (Smountas, Lands et al. 2004 Nov) studied the reproducibility of inflammatory markers within two weeks and found no significant differences in the neutrophil count, IL-8, elastase and TNF-α

concentrations between the samples and suggested that sputum induction could be used to evaluate therapeutic interventions. Similar results have been reported by Ordonez et al (Ordoñez, Henig et al. 2003 Dec 15). Sagel et al (Sagel and Accurso 2002 Aug) have further shown that IL-8, elastase and neutrophil counts in induced sputum correlate with lung function and therefore may be a useful surrogate marker to assess response in clinical trials. Ordonez et al (Ordoñez, Henig et al. 2003 Dec 15) in their study of 72 CF subjects have showed that it is indeed the case. They demonstrated that induced sputum is reliable enough in assessing the response to intravenous antibiotic treatment. In 55 subjects who concluded the study, treatment with intravenous antibiotics resulted in significant improvement in the sputum neutrophil counts, IL-8 and neutrophil elastase concentrations and this improvement correlated with a significant increase in FEV1. These data suggest that sputum induction is an accepted method of obtaining lower airway samples to measure various inflammatory markers in the airways.

1.5.5 Induced sputum in CF: limitations

Although sputum induction has several potential benefits, there are few important drawbacks. Importance of safety of the procedure has been discussed already. The second limitation is that it is not always possible to obtain a sputum sample particularly in healthy individuals. The success rate of this procedure varies between 68-100% depending on the studies (Clancy, Rowe et al. 2007). Children do not like the taste of the hypertonic saline and need constant encouragement which can often be time-consuming. There is also a requirement for trained technicians as well as a setting with appropriate infection control procedures. Other issues are with regard to the measurement of various inflammatory mediators.

The hypertonic saline does not influence the cellular or other markers but it is difficult to be certain about the compartment of airways/lungs that have been sampled from (Gershman, Liu et al. 1999). Similarly, processing the entire sputum instead of selecting the mucus plugs might mean that varying dilutions of sputum with inhaled saline and/or saliva within subjects can influence the results (Pizzichini, Pizzichini et al. 1996; Feng, Chen et al. 1999). Despite these limitations, the measurements as shown above are reproducible and hence reliable (Pizzichini, Pizzichini et al. 1996; Feng, Chen et al. 1999). Further, the above two problems are not just limited to the induced sputum samples but are also encountered with BAL specimens. In addition, the mucolytic, di-ethritiol (DTT) might have an influence on cell count and cytokine measurements. In asthmatic patients, use of DTT has been shown to increase the cell count but the cytokine levels were unaffected (Efthimiadis, Pizzichini et al. 1997). But the CF sputum is more viscous and purulent and hence this effect might be minimised. But the effect of DTT on various other cytokine measurements has not been studied at all.

In summary, sputum induction appears to be relatively safe, non-invasive, reliable and reproducible method of obtaining lower airway samples to measure infection and inflammation in cystic fibrosis patients.

1.6   Hypothesis

I hypothesised that monocytes/macrophages are one of the important drivers of persistent neutrophilic airway inflammation in CF. The derivatives of the above hypothesis are:

1.There is evidence of systemic monocyte activation in CF involving an expansion of CD14+CD16+ subpopulation of blood monocytes in CF.

2.There is increased systemic and airway chemoattractant activity for both subpopulations of blood monocytes in CF.

3.There is an expansion of airway small macrophages in CF. The findings of my pilot study in children was subsequently confirmed, with inclusion of adults by Dr. Adam Wright PhD thesis, University of Leicester.

4.Airway small macrophages in CF have altered inflammatory profile (Work of Dr. Adam Wright)

2 Methods and Materials

In this section, I describe the methods and subjects used in subsequent chapters.

2.1   Ethics

I obtained approval from the Leicestershire Local Ethics Committee (Reference: 7183). The initial application for children was subsequently extended to include CF adults and adult volunteers. As part of the application, I produced the invitation letter, information leaflets for the parents of CF and healthy children, and for CF and healthy adults (appendix 1).

Initial contact for both CF and healthy adult volunteers was through an invitation letter and for children via their parents. The contact details for healthy children were obtained from the list of subjects who had participated in a previous research project and had expressed willingness to participate in future projects. Informed consent was obtained from the subjects if they were over 16 years and from parents if they were under 16 years old.

2.2   Subjects

The CF patients were recruited from the Specialist Paediatric and Adult CF centres at Leicester. The Paediatric Centre was based at Leicester Royal Infirmary and cared for patients under 16 years age. The Adult Centre was based at the Glenfield Hospital. Most patients were identified on neonatal screening programme and confirmed with a positive sweat test (Chloride >60 mmol/L). All patients had confirmatory genetic testing for CF.

2.2.1 Estimation of sample size

At present, there is no published data on study of monocyte transmigration in CF. Specifically, there is no data available on monocyte subpopulations, sputum and serum CX3CL1 in CF. Furthermore, there are no studies comparing serum and sputum CCL2 between CF and healthy controls. Therefore power calculation to estimate sample size is not applicable for this research project. I have performed a retrospective sample size calculation for variables where there was no significant differences between the groups to clarify if the results were due to type II error.

I initially compared the absolute counts of different monocyte subpopulations, serum CCL2 and CX3CL1, chemokine receptor expression for CCL2 and CX3CL1 on monocytes subpopulations between CF and healthy control groups. To confirm that changes in chemokines in the blood reflected changes in the airway, I then measured the concentrations of CCL2 and CX3CL1 in induced sputum samples.

2.3 Characterisation of monocyte subpopulations and determination of absolute count

At least 5 millilitres of whole blood was obtained from the subjects in to a standard sterile syringe and transferred in to an ethylene-di-amine tetra-acetic acid (EDTA) tube. 100μl aliquot of the sample was then stained with fluorescent-labeled antibodies for CD14, CD16 and HLADR (5μl of MY4-FITC, 10μl of CD16 PE and 5μl of HLADR- PC5) and incubated for 20 minutes at 4C. Red cells were then lysed by adding 3 ml of ammonium chloride based erythrocyte lysis buffer and incubating at room temperature for 20 minutes. 100μl of flow-count

beads (Beckman Coulter, PN 7507992-E) added to determine absolute counts. The sample was acquired FACScalibur[TM] (Becton Dickinson, Wymcombe, UK) with the instrument settings as shown below (Figure 2-3.1). The machine is equipped with a 4-colour PMT, dual laser (488 nm 'blue' argon laser) and 633 nm red diode laser connected to Apple Macintosh computer. The samples were acquired and analysed using CellQuest[TM] Pro software. The gating strategy used is shown in the figure below (Figure 2-3.2). Absolute counts of CD14++ monocytes and CD14+CD16+ monocytes populations were calculated derived from the flow-count bead number.

```
Cytometer  Type:      FACSCalibur

Detectors/Amps:
Param    Detector  Voltage   AmpGain      Mode
P1        FSC       E-1        8.02        Lin
P2        SSC       265        1.00        Log
P3        FL1       659        1.00        Log
P4        FL2       544        1.00        Log
P5        FL3       592        1.00        Log
P6        FL2-A                1.00        Lin
P7        FL4       751                    Log

Threshold:
Primary Parameter:   FSC
Value:   108

Secondary Parameter:    None

Compensation:
FL1 - 0.6 % FL2
FL2 - 29.0 % FL1
FL2 - 0.0 % FL3
FL3 - 12.7 % FL2
FL3 - 0.0 % FL4
FL4 - 0.0 % FL3
```

Figure 2.3-1: Instrument settings for acquisition and analysis of blood monocyte subpopulations
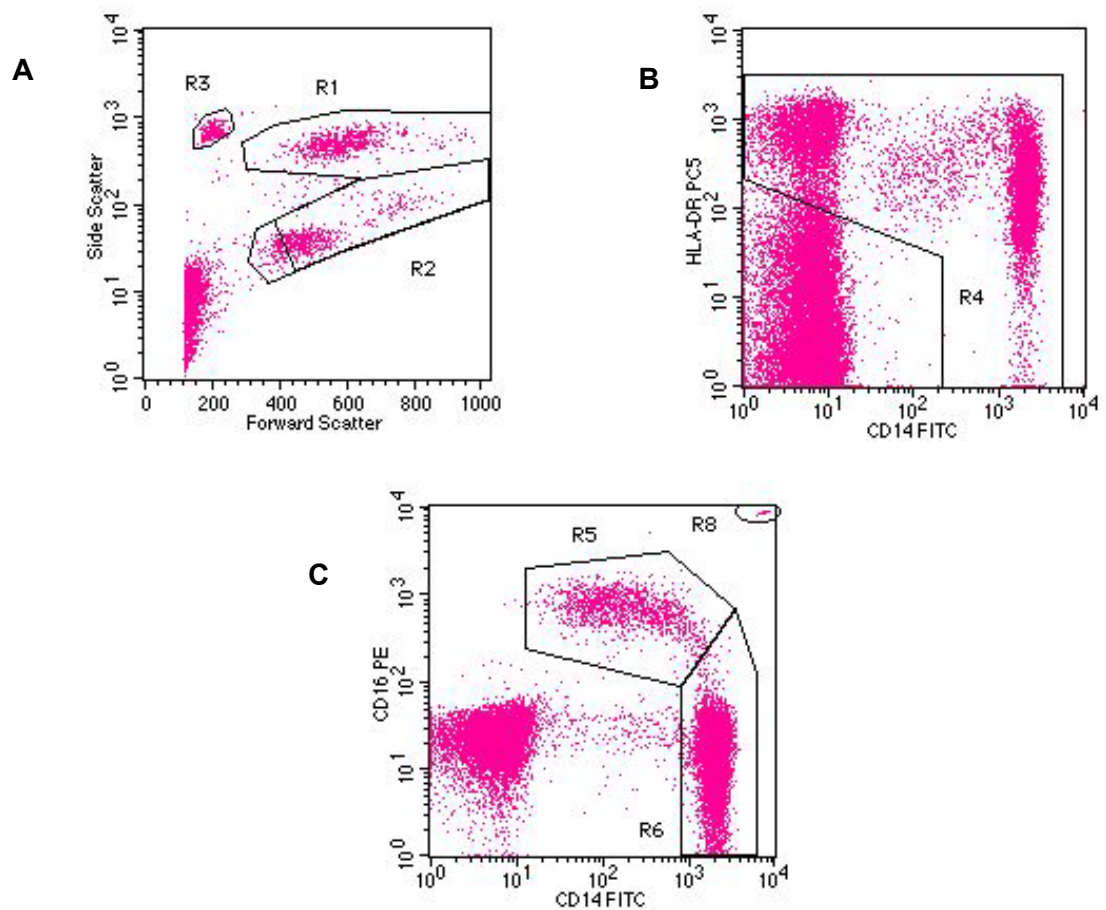
Figure 2.3-2: **Determination of absolute numbers of blood monocytes by flow cytometry**. **Peripheral blood leucocytes are identified using flow cytometry by light scatter characteristics. Mononuclear cells are identified in gate R2 (A). Gate R3 (A) is placed around flow-count beads. Events in R2 are gated onto a CD14/HLA DR dot plot. Gate R4 (B) defines events that express HLA DR and CD14. Events in R4 are then given in the CD14 *versus* CD16 dot plot (C). CD14++ monocytes are represented in R6 and CD14+CD16+ monocytes in gate R5. The absolute count is derived from the number of flow-count beads represented in gate R8.**

2.4 Determination of expression of chemokine receptor CCR2 on CD14++ and CD14+CD16+ monocytes

At least 1ml of whole blood was obtained in an EDTA tube from the subjects. 100µl of whole blood each was then pipetted in to 2 FACS tubes. The samples were then washed three times with 1ml of phosphate buffer solution (PBS) with 2% fetal calf serum (FCS) at 400g for 5 minutes per wash. The samples were then stained as below and incubated at 4C for 20 minutes:

Tube 1:

10 µl of CCR2 PE

10µl CD16 FITC

5µl CD14 APC

5µl HLADR PerCP

Tube 2:

10 µl of IgG$_{2b}$ PE (isotype control for CCR2 PE)

10µl CD16 FITC

5µl CD14 APC

5µl HLADR PerCP

Red cells were lysed by adding 3ml of ammonium chloride based erythrocyte lysis buffer incubated at room temperature for 20 minutes. The samples were then spun at 400g for 5 minutes at room temperature and 2ml of the supernatant was aspirated. At least 1000 events were acquired in the region R8 which corresponds to CD14+CD16+ monocytes. Monocyte cell populations were initially

characterized by using appropriate gating strategy on the flow cytometer as shown above (Figure 2.4-1). The median fluorescence intensity for the chemokine receptor for each monocyte sub-population was then determined by comparing with respective isotype controls. Geometric median was used to compare the fluorescence intensities as the median is less affected by the outliers (Figure 2.4-2). The instrument settings used for the acquisition and analysis is given in figure 2.4-3.

The remaining blood sample was then spun at 400g for 5 minutes and the serum separated was stored at -70^0C for further analysis.
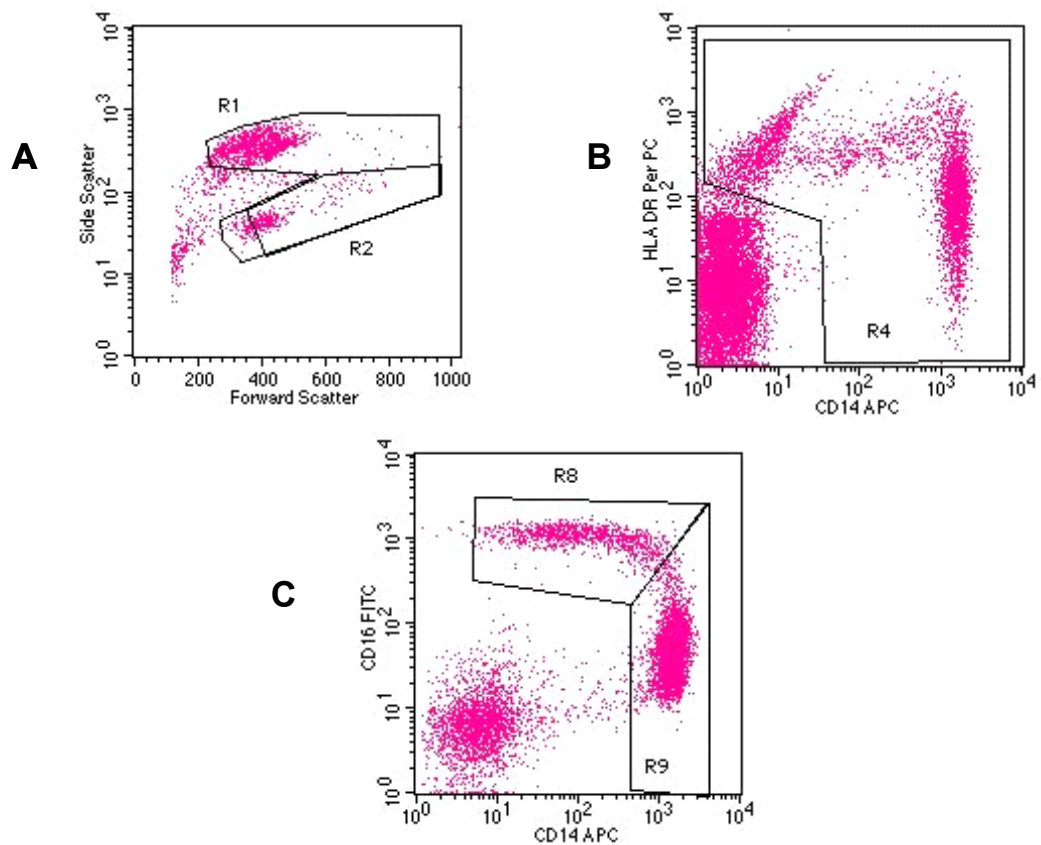
Figure 2.4-1: Characterisation of blood monocytes by flow cytometry. Peripheral blood leucocytes are identified using flow cytometry by light scatter characteristics. Mononuclear cells are identified in gate R2 (A). Events in R2 are gated onto a CD14/HLA DR dot plot. Gate R4 (B) defines events that express HLA DR and CD14. Events in R4 are then given in the CD14 *versus* CD16 dot plot (C). CD14++ monocytes are represented in R9 and CD14+CD16+ monocytes in gate R8.

CD14++CD16- Monocytes

CD14+CD16+ Monocytes
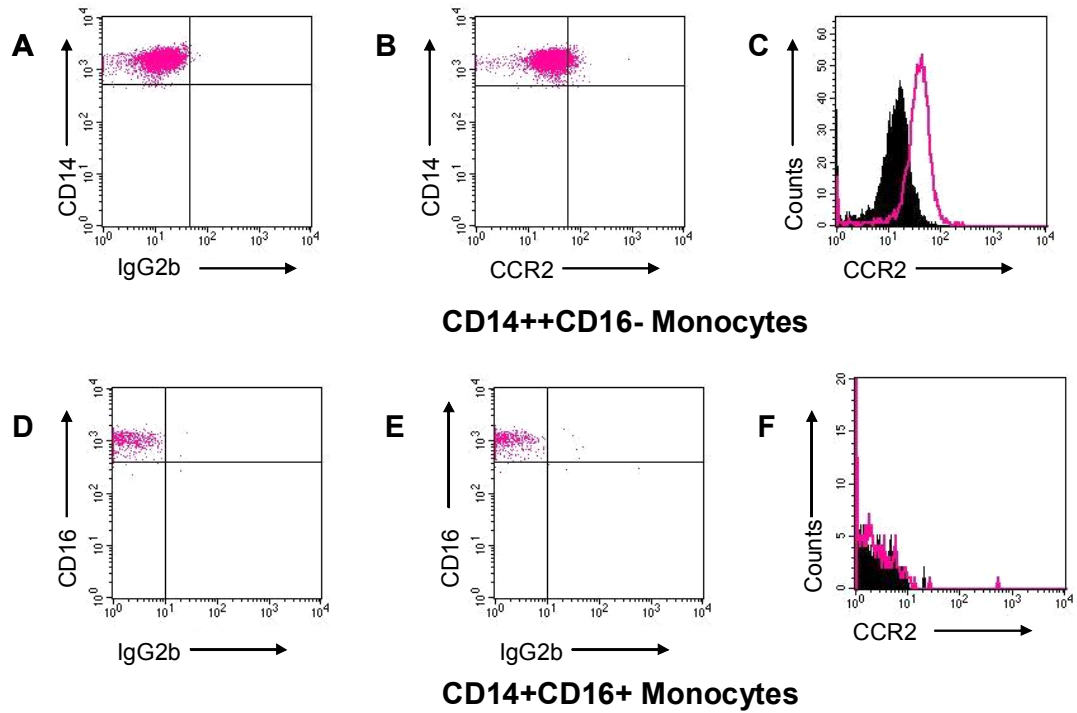
Figure 2.4-2: Determination of CCR2 expression on blood monocytes.
Specific CCR2 staining, the receptor for CCL2, by CD14++CD16- and CD14+CD16+ monocyte subsets is given in (B) and (E), while (A) and (D) denote non specific background staining respectively. Median fluorescence intensity is calculated from the difference between specific staining (open purple histogram) and non-specific staining (black histogram) for CD14++ (C) and CD14+CD16+ monocytes (F). CCR2 was expressed in CD14++ cells but there was no expression of CCR2 in CD14+CD16+ monocytes.

```
Cytometer Type:    FACSCalibur

Detectors/Amps:
Param    Detector   Voltage    AmpGain      Mode
P1          FSC        E-1        8.02        Lin
P2          SSC        265        1.00        Log
P3          FL1        600        1.00        Log
P4          FL2        496        1.00        Log
P5          FL3        660        1.00        Log
P6          FL2-A                 1.00        Lin
P7          FL4        620                    Log

Threshold:
Primary Parameter:    FSC
Value:   108

Secondary Parameter:    None

Compensation:
FL1 - 0.6 % FL2
FL2 - 29.0 % FL1
FL2 - 0.0 % FL3
FL3 - 0.0 % FL2
FL3 - 10.8 % FL4
FL4 - 0.0 % FL3
```

Figure 2.4-3: Instrument settings for acquisition and analysis of chemokine receptor CCR2 in monocyte subpopulation

I used 4 different fluorochromes for these experiments which posed challenges. Each fluorochrome has specific excitation and emission spectra. There is considerable spectral overlap between dyes which results in 'spillover' when multiple dyes are used together (Figure 2.4-4).
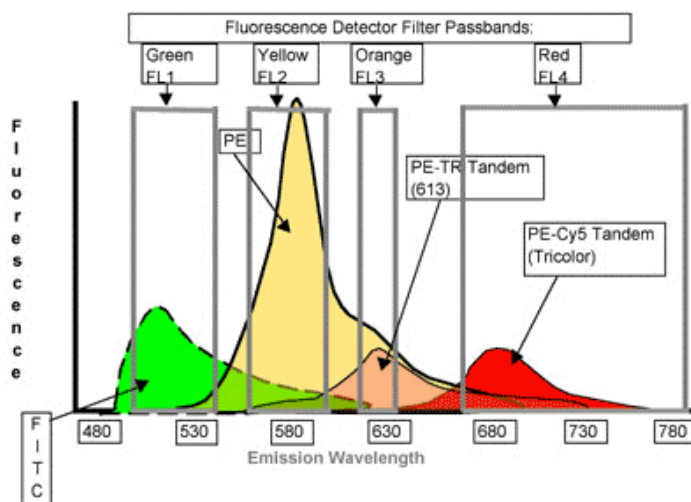
Figure 2.4-4: Schematic representation showing spectral overlap between different fluorochromes. (Baumgarth et al 2000)

However, this can be minimised in 3 ways; firstly, by selecting fluorochromes with minimal overlap (Figure 2.4-4), exciting fluorochromes with specific wavelength laser and finally by adjusting the optical filters to ensure optimal light collection and minimal 'spillover'. However, despite these adjustments, each fluorochrome will still contribute signal to several detectors. Therefore contributions to those detectors which are not assigned for the particular fluorochrome will have to be subtracted from the total signal in these detectors. This process is called 'compensation'.

In my initial experiments, I used HLA DR PC-5 and CD14 APC which have significant spectral overlap leading to poor definition of the monocyte

subpopulations. This overlap could not be adequately 'compensated'. Therefore I changed the HLA antibody to HLA DR PerCP which resulted in better definition of the monocyte subpopulations (Figure 2.4-5).
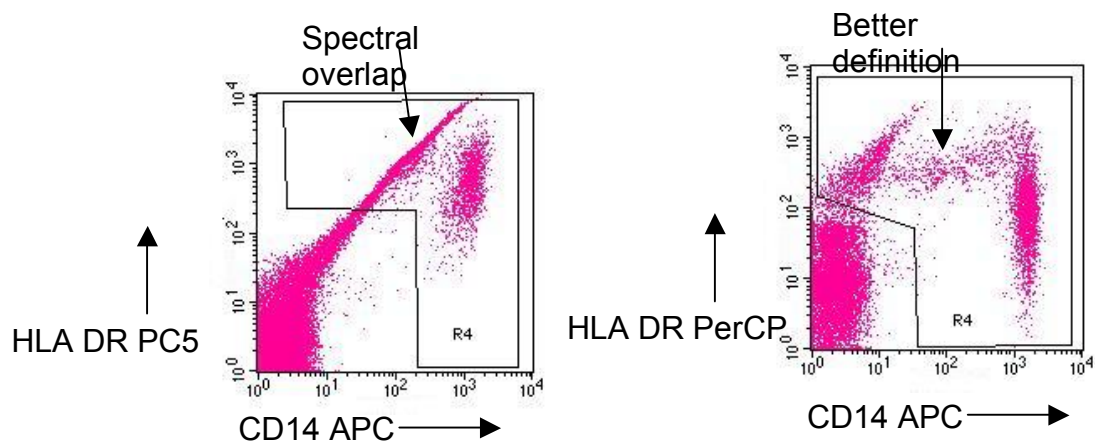
Figure 2.4-5: Illustration of spectral overlap with use of fluorochromes with similar emission spectra. In the dot plot on the left side, fluorochromes with similar spectra, PC5 and APC are used which results in crowding of cells due to 'spillover' (left). This is corrected by using PerCP instead of PC5 with APC, which have less spectral overlap. This had led to better definition of cell population (right).

2.5 Determination of expression of chemokine receptor CX3CR1 (fractalkine) on CD14++ and CD14+CD16+ monocytes

At least 1ml of whole blood was obtained in an EDTA tube from the subjects. 100μl of whole blood each was then pipetted in to 2 FACS tubes. The samples were then stained as below and incubated at 4C for 20 minutes:

Tube 1:

   20 μl CX3CR1 FITC

   10μl CD16 PE

   5μl CD14 APC

   5μl HLADR PerCP

Tube 2:

   20μl Rat IgG2b FITC (isotype control for CX3CR1)

10μl CD16 PE

   5μl CD14 APC

   5μl HLADR PerCP

The samples were then washed once with 1ml of phosphate buffer solution (PBS) with 2% fetal calf serum (FCS) at 400g for 5 minutes. Red cells were lysed by adding 3ml of ammonium chloride based erythrocyte lysis buffer incubated at room temperature for 20 minutes. The samples were then spun at 400g for 5 minutes at room temperature and 2ml of the supernatant was aspirated. At least

1000 events were acquired in the region R8 which corresponds to CD14+CD16+ monocytes. The instrument settings are given in the figure 2.5-3. Monocyte cell populations were initially characterized by using appropriate gating strategy on the flow cytometer as described above Figure 2.5-1. The median fluorescence intensity for the chemokine receptor for each monocyte sub-population was then determined by comparing with respective isotype controls as shown in figure 2.5-2.

The remaining blood sample was then spun at 400g for 5 minutes and the serum separated was stored at -70^0C for further analysis.
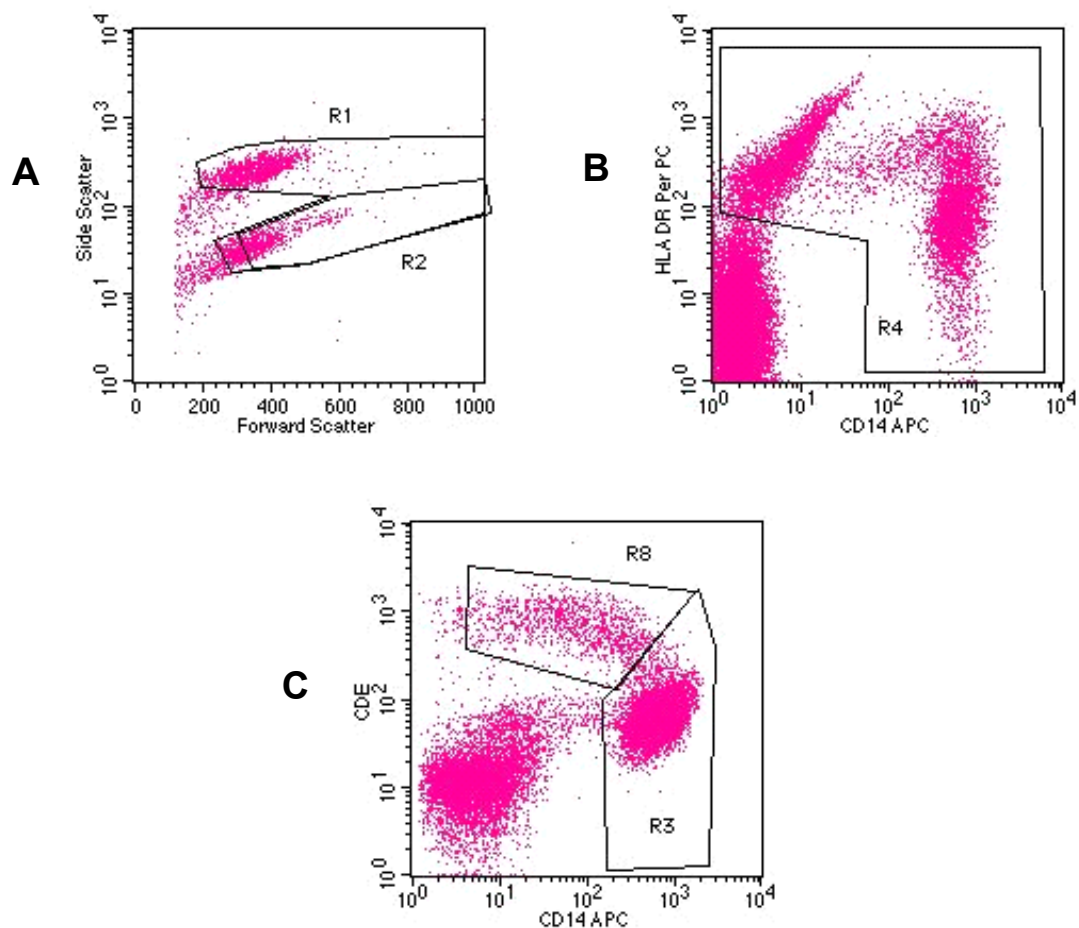
Figure 2.5-1: **Characterisation of blood monocytes by flow cytometry**.
Peripheral blood leucocytes are identified using flow cytometry by light scatter
characteristics. Mononuclear cells are identified in gate R2 (A). Events in R2 are
gated onto a CD14/HLA DR dot plot. Gate R4 (B) defines events that express
HLA DR and CD14. Events in R4 are then given in the CD14 *versus* CD16 dot
plot (C). CD14++ monocytes are represented in R9 and CD14+CD16+ monocytes
in gate R8.
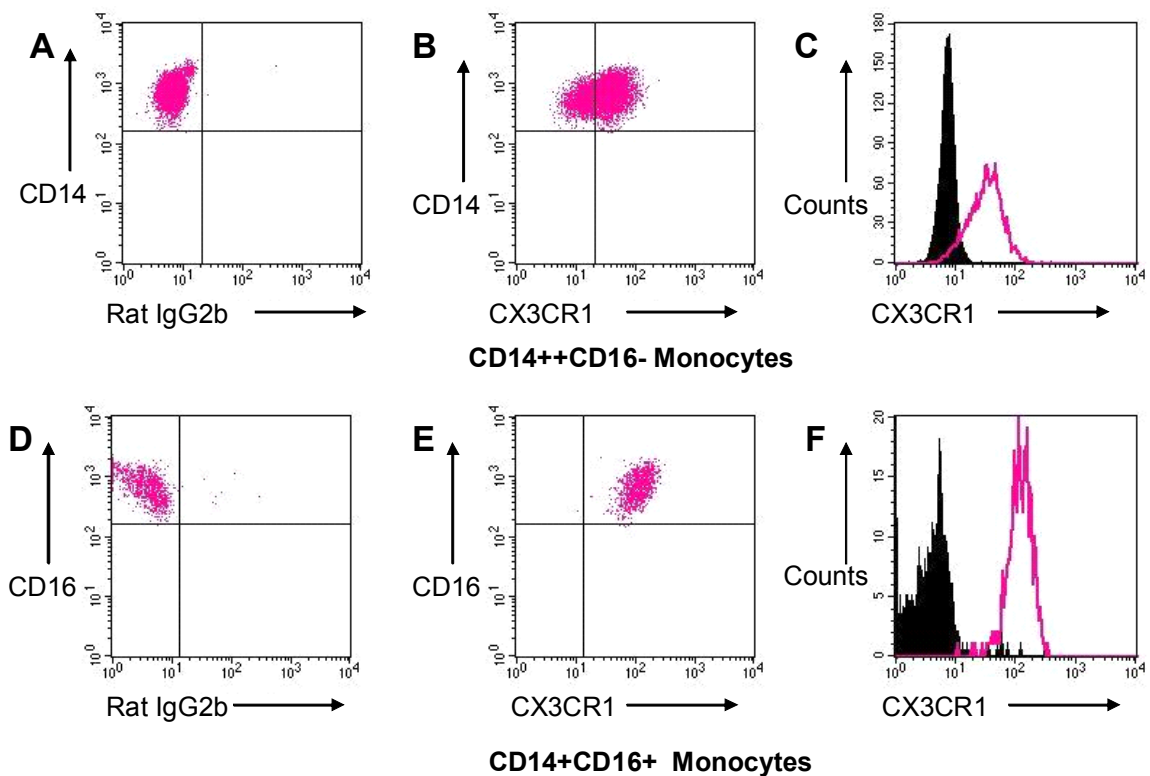
Figure 2.5-2: Determination of CX3CR1 expression on blood monocytes. Specific CX3CR1 staining, the receptor for CCL2, by CD14++CD16- and CD14+CD16+ monocyte subsets is given in (B) and (E), while (A) and (D) denote non specific background staining respectively. Median fluorescence intensity is calculated from the difference between specific staining (open purple histogram) and non-specific staining (black histogram) for CD14++ (C) and CD14+CD16+ monocytes (F). CX3CR1 was expressed in CD14++ cells but there was much stronger in CD14+CD16+ monocytes.

```
Cytometer Type:    FACSCalibur

Detectors/Amps:
Param    Detector  Voltage    AmpGain        Mode
P1          FSC       E-1        8.02         Lin
P2          SSC       265        1.00         Log
P3          FL1       600        1.00         Log
P4          FL2       601        1.00         Log
P5          FL3       660        1.00         Log
P6          FL2-A                1.00         Lin
P7          FL4       641                     Log

Threshold:
Primary Parameter:    FSC
Value:   108

Secondary Parameter:    None

Compensation:
FL1 - 0.6 % FL2
FL2 - 0.0 % FL1
FL2 - 0.0 % FL3
FL3 - 0.0 % FL2
FL3 - 10.8 % FL4
FL4 - 0.0 % FL3
```

Figure 2.5-3: Instrument settings for acquisition and analysis of chemokine receptor CX3CR1 in monocyte subpopulation

2.6   Sputum Induction

2.6.1   Subjects (General information)

Children were over 6 years of age as they were needed to be able to perform lung function tests reliably. Children were required to be clinically stable without obvious respiratory distress. There was no lower limit for lung function test but the baseline spirometry should have been stable over last few weeks. Appropriate infection control procedures were followed as detailed below.

2.6.2   Infection control procedures

The infection control measure was discussed with the infection control team. P.aeruginosa and non P.aeruginosa infected children were strictly isolated. The nebuliser handset *(multisonic Profi, Schill)* was disinfected by washing in a dishwasher or [by immersing the medication chamber in mild detergent followed by thorough drying by alcowipes as discussed with Dr. Jenkins, 27/09/04]. The impact plate, nebuliser head, mouthpiece and the valves were autoclaved after each use. The work-surfaces and spirometer were wiped with 70% ethanol after each induction. Appropriate disposable mouthpieces for spirometer were used for each patient. Appropriate ventilation was maintained in the Wheeze lab. The air-exchange rate was assessed and found to be satisfactory. Volumatic spacers were disinfected by immersion in Virkon (overnight) in between patients. Also each cystic fibrosis patients were encouraged to bring their own spacers. Disposable plastic aprons and masks were worn by the Lab personnel during the sputum induction.

2.6.3 Medications

Hypertonic saline (5.4%) (#G005027, Nova Laboratories, UK) is dispensed by the pharmacy and stored in the refrigerator. Unused saline was be discarded a week after each bottle is opened. Strict aseptic precautions were taken whilst using the saline.

2.6.4 Procedure

A history of recent health and symptoms was obtained from the subject or their parent. A clinical assessment of the fitness was undertaken. Baseline spirometry *(Vitalograph 2120)* was performed and forced expiratory volume in 1 second (FEV1) was recorded. 2-4 puffs of salbutamol preferably through the volumatic spacer were administered to protect against bronchoconstriction. After 15 minutes, repeat spirometry was performed and taken as the reference for future comparisons. The subject then asked to blow the nose, rinse the mouth to clear any nasopharyngeal and oropharyngeal secretions and was encouraged to cough up any sputum spontaneously. This was followed by 5-minute (X 3) nebulisations of 5.4% hypertonic saline using ultrasonic nebuliser (Schill, #MN 80180, Germany). Prior explanation and demonstration of using nebuliser and recognising broncho-constriction were given and they were encouraged to cough during and in between nebulisations in to a sterile petri dish. In between each nebulisation repeat spirometry was performed and compared with the post-salbutamol reference. If FEV1 dropped by >10% but <20%, further 2-4 puffs of salbutamol was administered. Procedure was continued if the FEV1 improved to within 10% of the reference. If the drop in FEV1 exceeded 20% then the procedure was discontinued and salbutamol was administered as needed. Similarly, the procedure stopped if child becomes symptomatic with wheeze and

shortness of breath and salbutamol was administered as appropriate. If there was more than 10% drop in FEV1or child was symptomatic, they were monitored until FEV1 improved to within 10% of the baseline or the child became asymptomatic. Additional salbutamol was administered as appropriate.

2.6.5 Sputum Processing

Sputum was expectorated into a large 14mm Petri dish and examined with the naked eye. The sample from the CF patients was often homogenous and therefore the whole sample was taken for analysis. However, the sputum sample from healthy controls were often contaminated by saliva and sputum selection method described by Kelly et al (Kelly, Efthimidias et al. 2001) was used as a guide. White plaques which are made of leucocytes and bound by mucin were separated from the saliva by sterile forceps and used for analysis. These plaques are rich in non-squamous epithelial cells and thereby contamination by the salivary epithelial cells is minimised. The sample was thus selected and transferred to a pre-weighed Corning centrifuge tube. The weight of the sputum sample was determined and 4 times the volume of 10% dithiothritol (DTT) was followed by a vigorous vortex. 10% DTT was prepared by diluting concentrated DTT (Sputolysin^R #560000, Calbiochem, California, USA) with distilled water (1 in 10). The resulting dilution yielded 6.5mM DTT in 100mM phosphate buffer at pH 7.0. DTT reduces the disulphide bonds between cysteine domains of the sputum mucin and facilitates homogenisation. The sample was then placed on a bench rocker (ThermoDenley 'Spiramix' – Shandon) until homogenised (for 15mins) on ice. An equal volume of phosphate buffer solution (composed of NaCl, KCl, Na_2HPo_4, $KH2PO_4$ and adjusted to pH 7.0) was added to the sputum/DTT mixture and subjected to

vigorous vortex. Large nonhomogenous aggregates of mucina and epithelial cells were separated by passing the sample over pre-wetted non-sterile cotton medical guaze (#18508, Lohmann Rauscher, Germany) in a powder funnel (#FPH-455-010Y, Fisher Scientific, UK) followed by 48mn nylon (#03-48/31 Serofar, UK) twice. The filtrate was centrifuged at 420g (2000rpm) at 4°C for 10 mins brake off. The supernatant was stored in -70^{0}C for analysis of CCL2 and CX3CL1 concentrations as described in further sections.

The cell pellet was then re-suspended in 1ml of PBS. 10 µl of the sample was mixed with 10 µl of Trypan blue (0.4%) in an eppendorf, leaving to stand for 1-2 minutes to ensure equilibration. The sample was then pipetted under a Neubauer haemocytometer and count squamous and non-squamous cells, and bronchial cells.

% Squamous cells = No. squamous cells / Total cells x 100

% Cell viability = No. of alive or dead cells / Total number of non-squamous cells

Cells/ml = No. of cells / No. Large squares counted (Usually 4)

x 2 (Dilution factor) x 10^4 (Vol. each large square)

2.6.6 Preparation of cytospin

75 µl of cell suspension was transferred to the cyto-centrifuge cap and was centrifuged (ThermoFisher Scientific) on to a slide at 450rpm for 6 minutes. The slides were then stained using DiffQuick (#130832, Medion Diagnostics Germany). Each slide was dipped in fixative solution (Fast green in methanol) followed by stain solution 1 (Eosin G in PBS pH 6.6) and then in stain solution 2 (Thiazine dye in PBS pH 6.6). The slides were washed with dH2O and allowed to air dry. Thiazine binds to negatively charged nuclear materials whereas eosin binds to cytoplasmic proteins.

2.6.7 Identification of sputum small macrophages

The protocol for identification of small macrophages was developed by Dr. Adam Wright. It is described below. 100µl sample each of sample was transferred to 4 tubes and stained as below.

Tube 1 5µl CD66b FITC; 20µl CD16b FITC

  5µl HLA-DR PC5

  5µl CD14-APC

  (20µl CD68 PE)

  Tube 2 5µl CD66b FITC; 20µl CD16b FITC

  5µl HLA-DR PC5

  5µl CD14 APC

  (20µl PE Isotype)

  Tube 3 5µl CD66b FITC; 20µl CD16b FITC

5µl PC5 Isotype

5µl CD14 APC

(20µl CD68 PE)

Tube 4 5µl CD66b FITC; 20µl CD16b FITC

5µl HLA-DR PC5

5µl APC Isotype

(20µl CD68 PE)

This was followed by intracellular staining for CD68 PE following incubation with above antibodies for 15-20 minutes. The samples were then washed with 1ml PBS + 2% FCS at 400g for 5 minutes. The pelleted was resuspended in 250µl Cytofix™ and incubated in the fridge for 20 minutes. The samples were then washed with 1ml of Permwash buffer (1 to 10 PBS) at 400g for 5 minutes and re-suspended in 100µl permwash (Saponin keeps cells permeable). Intracellular stain (CD68 PE) is then added, followed by 500µl PBS for cell acquisition. The gating strategy is shown below (figure 2.6-1).
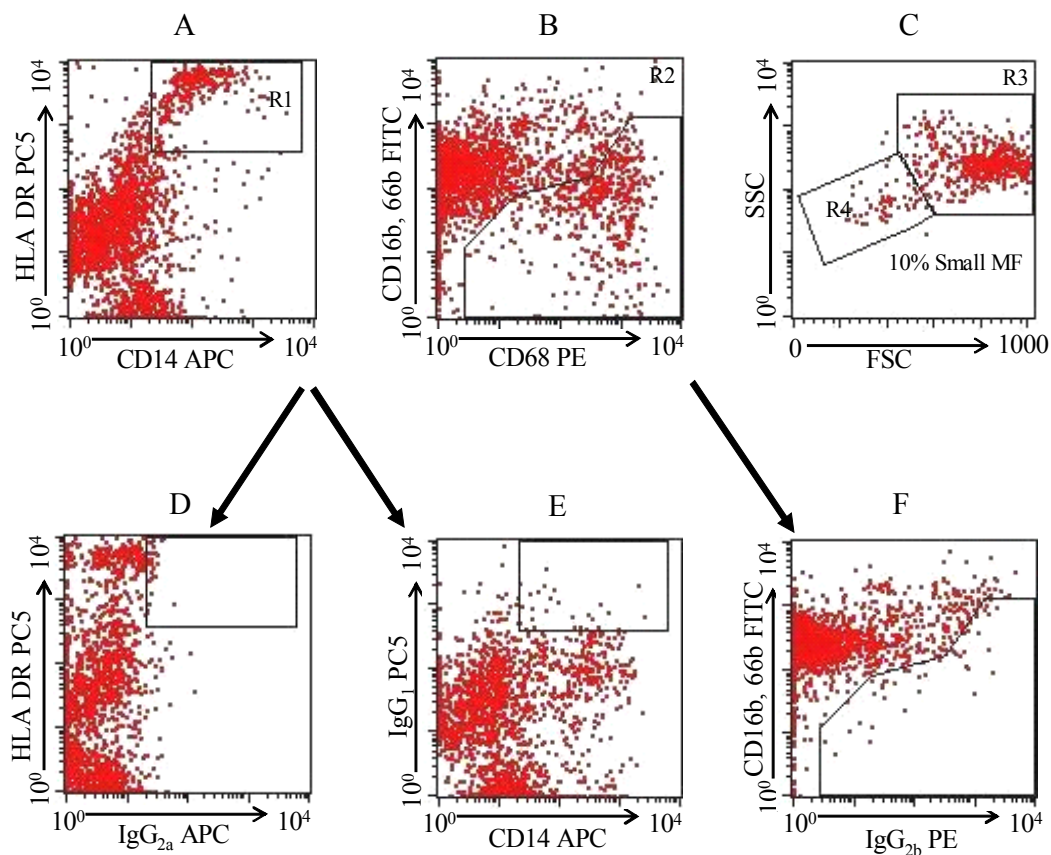
Figure 2.6-1: Sputum cells were stained with anti-CD14-APC, anti HLA-DR-PC5, anti-CD16b-FITC plus anti-CD66b-FITC and anti-CD68-PE. Positive cells were gated by comparison with the appropriate isotype controls. The CD14+ and DR+ events gated in R1 (A) were analysed for CD68 expression (B). In B a gate was set around the CD68+ cells (R2) with exclusion of CD16b+ CD66b+ neutrophils. The CD68+ events were then analysed for light scatter properties (C). Gate R3 defines large macrophages, gate R4 small macrophages. In D the isotype control for CD14 (IgG_{2a}) is combined with the specific antibody for DR and in E the isotype for DR (IgG_1) is combined with the specific antibody for CD14. In F the isotype control for CD68 (IgG_{2b}) is combined with the anti-granulocyte antibodies. The arrows connect the specific antibody to the respective isotype controls. (Provided by Dr. Adam Wright).

2.7   Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to measure the chemokines concentrations in sputum supernatant samples and serum samples. The technical assistance for performing and interpreting the samples was provided by Mr. Will Monteiro with advice from Dr. Debbie parker based at the Department of Infection, Institute of Lung Health,, Glenfield Hospital Leicester.

ELISA has been used extensively in immunology for quantitative measurements of various antigens and antibodies. Briefly, a known quantity of antigen is affixed to a 96 micro-titter well. These samples will constitute the standard curve used to calculate antibody concentrations from the unknown samples. The plates are first coated with the serum of the samples and specific detection antibody is added to these samples after washing the plates. The specific detection antibody binds to the immobilized antigen on the well surface. A secondary antibody against the detection antibody is then added. These secondary antibodies are conjugated with substrate-specific enzyme. Unbound antibody and enzyme are washed off. The substrate is then applied which emits a fluorogenic signal, which can then be quantified using the standard curve. The standard curves for various measurements are shown below.

The standard curves for serum and sputum CX3CL1 shows negative absorbance values. While correcting for absorbance due to the zero standard/ assay buffer the value can be slightly higher than some actual samples. Therefore with low concentration standards when the correction is done, it can show negative absorbance value which means the absorbance is effectively zero.

Serum/Sputum CCL2 Standard Curve

$y = 0.0026x + 0.0066$
$R^2 = 0.9996$

Absorbance at 450nm

MCP-1or CCL2 (pg/ml)
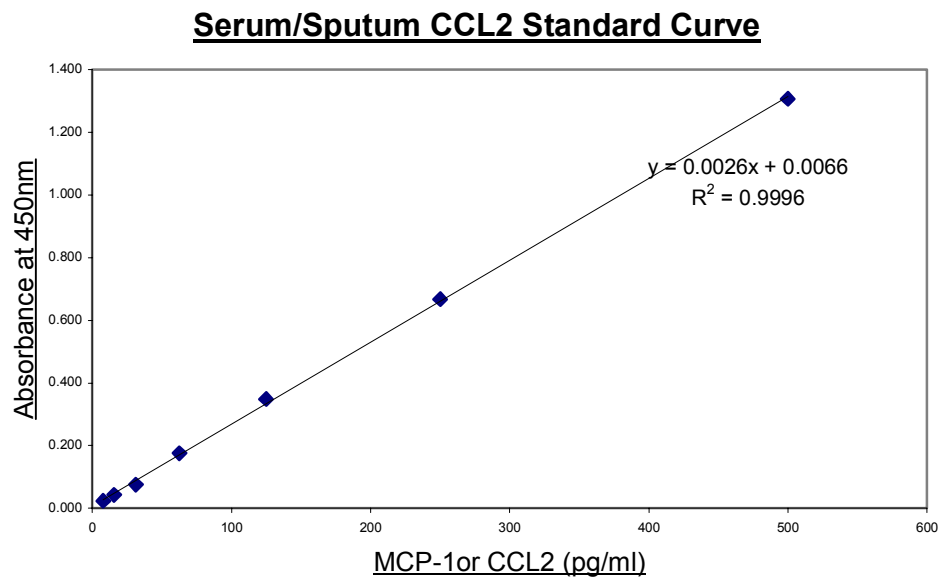
Figure 2.7-1: Standard curve for measurement of sputum and serum CCL2 concentration

MCP-1 Standard Curves +/- DTT 26-1-06

$y = 0.0039x + 0.0703$
$R^2 = 0.9994$

$y = 0.0036x + 0.063$
$R^2 = 0.9996$

Absorbance @ 450nm

MCP-1 or CCL2 pg/ml

- No DTT
- 0.004%DTT
- Linear (No DTT)
- Linear (0.004%DTT)
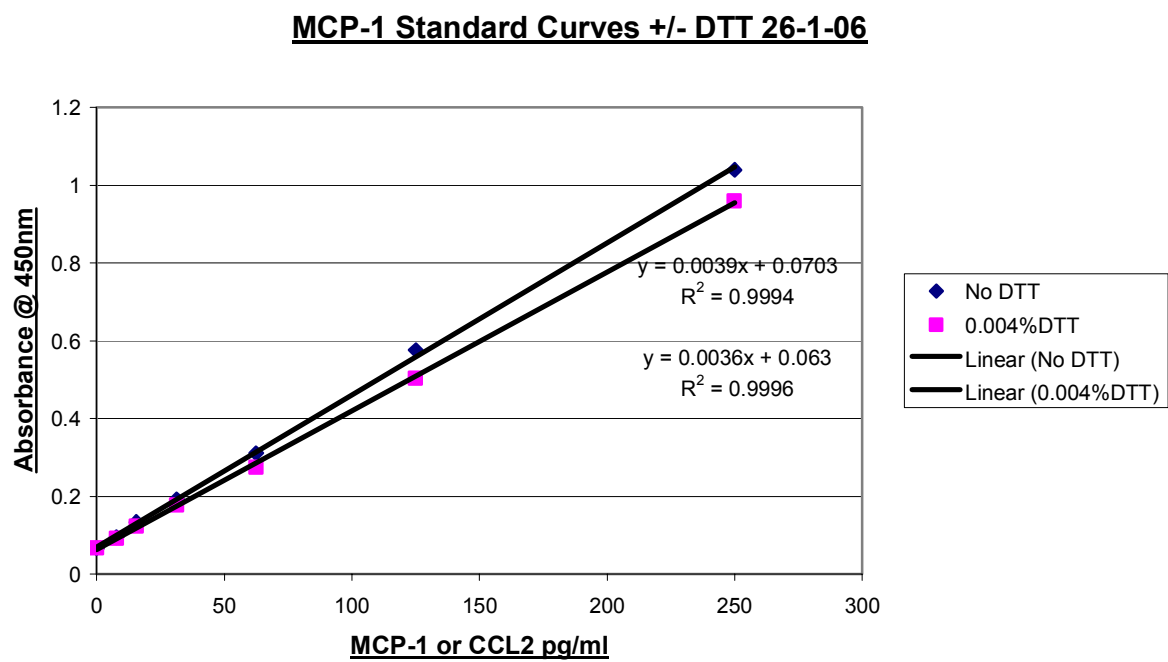
Figure 2.7-2: Standard curve with and without addition of DTT

Serum CX3CL1 Standard Curve

$y = 0.0054x - 0.0146$
$R^2 = 0.9898$

Absorbance (y-axis)

Fractalkine or CX3CL1 (ng/ml)

Sputum CX3CL1 Standard curve

$y = 0.0001x - 0.0016$
$R^2 = 0.9902$

Absorbance (y-axis)

Fractalkine or CX3CL1 (ng/ml)
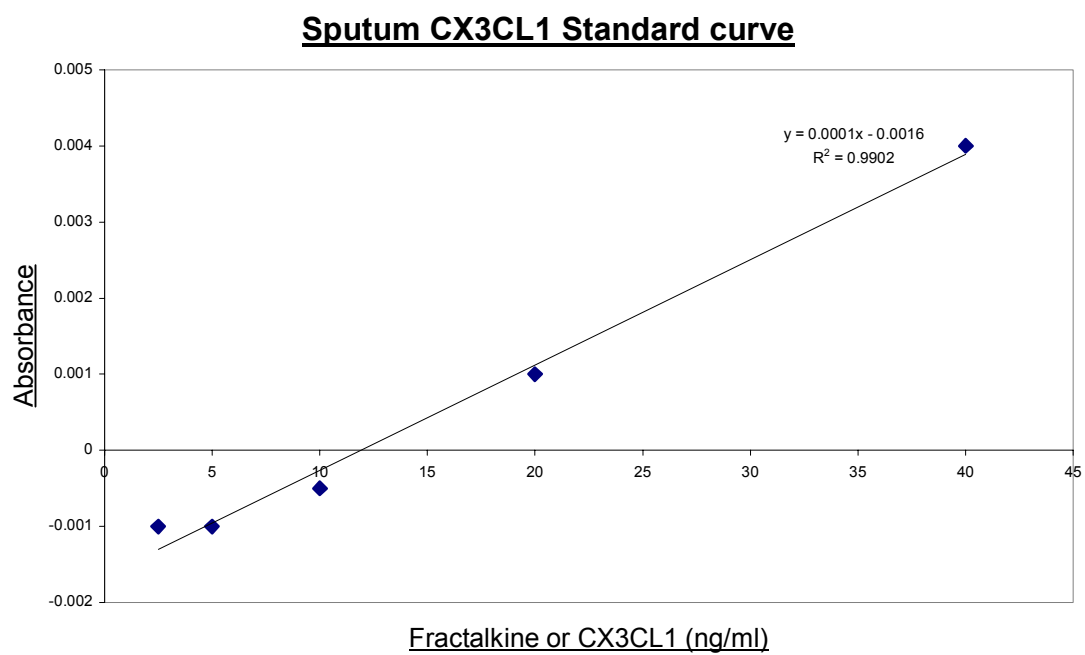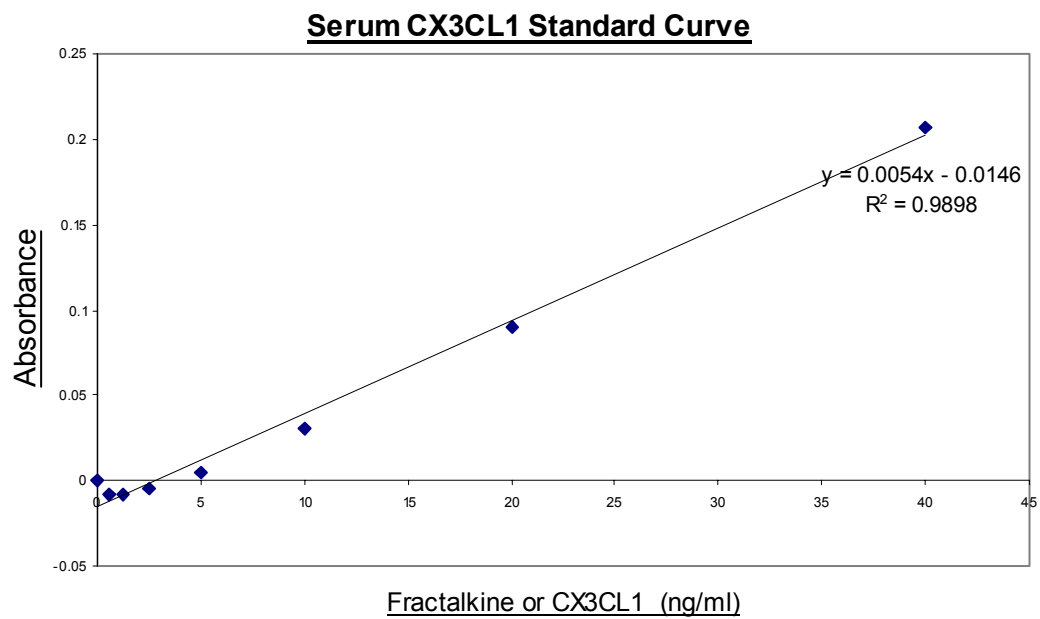
2.8   Statistics

Data are presented as median and range. Comparison of data was performed using the Mann-Whitney test. Correlations were determined by Spearman rank coefficient (Rs). Statistical analysis was performed using GraphPad Prism software (version 3.00, GraphPad Inc. California, USA). A p value of <0.05 was considered statistically significant. Graphs (except flow cytometry plots) were created using GraphPad Prism software (version 3.00, GraphPad Inc. California, USA).

Analysis of all the measured variables showed that they were not normally distributed. One approach to normalise is to converting the data into a logarithmic scale (Altman 1991). Only some measurements like serum CCL2, Sputum CCL2 and serum CX3CL1 could be normalised by this approach. In order to maintain uniformity with analysis of the variables, they were all analysed using non-parametric tests. However, for those variables that could be normalised, difference in mean was compared with t test and showed similar results to the non-parametric tests (not shown).

3 Results- Serum CCL2 and not CX3CL1 is raised in CF. There is no systemic activation of monocytes in CF.

My hypotheses were in CF:

1. There is systemic activation of monocytes, i.e. there is expansion of CD14+CD16+ monocytes in blood.

2. There is increased systemic and airway monocyte chemoattractant activity for both sub-populations of monocytes.

3. There is expansion of airway small macrophages

I initially compared the absolute counts of different monocyte subpopulations, serum CCL2 and CX3CL1, chemokine receptor expression for CCL2 and CX3CL1 on monocytes subpopulations between CF and healthy control groups. In this chapter, I present the findings of the above experiments. To confirm that changes in chemokines in the blood reflected changes in the airway, I then measured the concentrations of CCL2 and CX3CL1 in induced sputum samples which is presented in the following chapter. Finally, I present the findings of the pilot study of small airway macrophages in paediatric CF and healthy control groups.

As discussed before (section 2.2) the CF patients were recruited from the Specialist paediatric and adult CF centres at Leicester. The Paediatric Centre was based at Leicester Royal Infirmary and cared for patients under 16 years age. The Adult Centre was based at the Glenfield Hospital. Most patients were identified on neonatal screening programme and confirmed with a positive sweat test (chloride >60 mmol/L). All patients had confirmatory genetic testing for CF.

3.1 Introduction

In the earlier chapter (sections 1.2, 1.3 and 1.4), I presented the recent advances in our understanding of monocytes and macrophages, their role in chronic inflammation and preliminary evidence of their role in CF. I also highlighted gaps in our knowledge of possible role of monocytes and macrophages in sustaining airway inflammation in CF. Broadly, we know that there are different populations of monocytes and lung macrophages with distinct phenotypic, transmigration and pro-inflammatory properties. In CF, there is some evidence that suggests that macrophages might be affected by CFTR defect and have enhanced pro-inflammatory properties. In this study, I sought to determine if there was expansion of the monocyte subpopulations and if there was indeed an increase in the systemic chemoattractant activity for these subpopulations.

The CF patients were enrolled for the study during their routine outpatient visits or hospital admissions for intravenous antibiotic therapy. Informed consent was obtained from parent or patient as appropriate. Clinical details including age at diagnosis, genotype, respiratory symptoms, treatment and lung function test at the time of sampling were recorded. At least 1ml of blood was then obtained from the patients in an ethylene-di-amine tetra-acetic acid (EDTA) tube. The CD14+CD16+ count was determined as described below. The control population was derived from group of volunteers or siblings of patients who agreed to participate in the study.
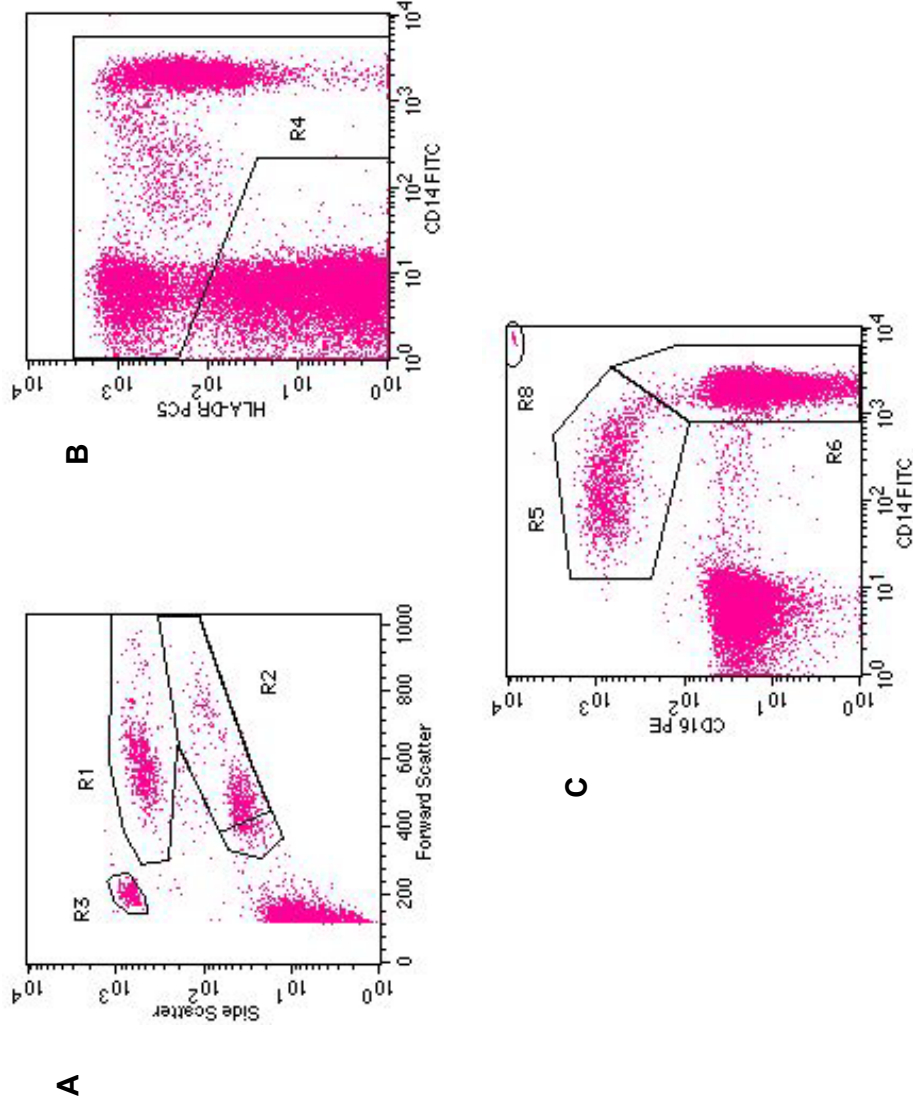
Figure 3.1-1: Determination of absolute number of monocyte subpopulations. The monocyte population is first defined on the forward and side scatter and gate R2 is placed (A). R3 is placed around flow-count beads. Events in R2 are then shown in CD14/HLADR dot plot and R4 defines events that express both HLA DR and CD14 (B). Events in R4 are then illustrated in CD14/CD16 dot plot (C). The CD14++ monocytes are represented in R6 and CD14+CD16+ monocytes in R5. The absolute count can be derived by the number of flow-count beads represented in R8.

3.2 Characterisation and determination of total monocyte, CD14++ monocyte and CD14+CD16+ counts

The samples obtained from the CF patients and healthy volunteers were processed as described in the previous chapter.

3.2.1 Subjects

36 CF and 25 healthy control samples were available to determine the absolute counts of total monocytes and two main monocyte subpopulations. The comparison of characteristics between the two groups is summarised in the table 3.2-1. The male/female distribution was similar in both groups (p=1.00). However, there were differences in the age, ethnicity and FEV1 between the groups. The control population was significantly older compared to CF patients (mean age 24 years and 16 years, respectively, p=0.01). 8 controls were of Asian origin compared to 2 in the CF group (p=0.004). Further, the lung function expressed as FEV1 (% predicted) was significantly lower in the CF group [Mean % predicted (range) CF vs. Control- 65% (24-103) and 90.64% (77- 110) respectively, p=0.0001)].

The characteristics of the CF group are provided in the Table 3.2-2. Within this group, the age ranged between 4 and 34 years (mean age 16yrs). 19 (57%) were aged less than 16 years and were being looked after in the Paediatric CF clinic. Over half of the patients (n=19, 57%) were homozygous for delta F508 CF mutation and further 4 were compound heterozygotes for delta F508 and another CF mutation. The lung function data measured as FEV1 (predicted %) was available for 26 patients and it ranged from 24% to 103%. Lung function data was not available 6 children who were too young to perform reliable spirometry. There

was evidence of moderate lung disease with FEV1 between 50-70% of predicted value in 8 patients, three of whom were in the Paediatric age group. Further, 4 patients had severe lung disease with FEV1 less than 50% predicted value. All the patients had insufficiency of pancreatic exocrine function and were on regular pancreatic enzyme supplements. Furthermore eleven patients had other complications of CF; five- CF related diabetes, five- CF related liver disease and one patient with both liver disease and diabetes.

Just over half of (58%) of the patients were experiencing physician diagnosed exacerbation of respiratory symptoms at the time of sampling and were receiving additional antibiotic or anti-inflammatory therapy. In majority of these patients (76%), the exacerbation was significant enough to require intravenous antibiotic therapy. Further, 13 (40%) were on regular inhaled steroid therapy and 4 of them (3 children and 1 adult) were also taking additional oral prednisolone. Since oral steroids are known to deplete the CD14+CD16+ monocyte population, these patients were excluded from the analysis. Therefore 32 CF patients were available for the analysis.

24/25 of control group did not have any documented illness at the time of study and were not receiving any regular medications. However, one subject gave history of hayfever, intermittent asthma and had required bronchodilator therapy for acute wheeze on the day of the study. Since, abnormalities in the CD14+CD16+ population have been described in active asthma, this subject was excluded from the analysis.

	CF	Controls	p value*
Total Number	32	24	ns
Male: Female (n)	16:16	13:12	0.01**
Age, yr, Median (range)	16 (4-34)	24 (7-35)	
FEV$_1$ % predicted, median (range)	65 (24-103)	82.5 (72-110)	0.001**
Respiratory exacerbation	21	0***	<0.0001

*by Mann Whitney test
**significant difference between the groups p<0.05
***One control with hay-fever was excluded from the study

Table 3.2-1: Study of absolute counts of monocyte subpopulations, chemokine receptor expression (CCR2 and CX3CR1), serum chemokines (CCL2 and CX3CL1): Comparison of characteristics of CF Patients and Healthy Controls. Data are given as median unless otherwise specified

Age: Mean years (range)	16 (4-34)
Sex: M:F	18:18
Race: Caucasian, Asian	34, 2
Genotype (deltaF508 +)	23
FEV1 % predicted: Median (range) [n=26]*	65 (24-103)
Symptomatic (%)	21 (58%)
Additional Antibiotics: (Intravenous, Oral)	21 (16, 5)
Steroids: (Oral, Inhaled)	17 (4, 13)
Liver Disease (%)	5 (15%)
CF Related Diabetes (%)	5 (15%)

*6 children were too young to perform lung function and data was not available for four adults

Table 3.2-2: Characteristics of CF Patients. Data are given as median unless otherwise specified

3.2.2 Comparison of total monocytes, CD14++ monocytes or CD14+CD16+ monocytes

The total monocyte count was similar in both groups [Median (range) cells/μlitre: CF 468 (97-1001) Vs. Control 381 (142-844), p=0.38)] (figure 3.2-1). There was no significant difference in the CD14++ monocyte number between the between groups (figure 3.2-2). Similarly there was no difference in the CD14+CD16+ counts between the groups (figure 3.2-3); the median (range) cells/μlitre for CD14+CD16+ monocytes in the CF and control groups were 36 (8-117) and 35 (2-98) respectively (p=0.3114) (table 3.2-3).

Also there was no correlation between total monocyte and CD14++ monocyte and CD14+CD16+ monocytes with age, chest symptoms, antibiotic, inhaled steroid therapy. Further, there was no relationship between monocyte counts and severity of lung disease expressed as FEV1 (figures 3.2-4 and 3.2-5) in the CF population.

In a subgroup of CF patients over 17 years of age, there was a non-significant increase in the CD14+CD16+ monocyte number [Median (range) cells/μlitre: CF 38 (8- 97) Vs. Control 27 (2- 98), p=0.085)]. Although the lung function in this subgroup of patients was comparable to the younger patient group, there was however a higher proportion of patients with multi-system complications such as liver disease and CF related diabetes (7/14 vs. 3/16) in this group.

Blood monocyte subset		CF	Controls	p value*
CD14++	Cell count (/μL blood) median (range)	426 (79 to 982) (n=29)	362 (140 to 765) (n=21)	0.35
	CCR2 (median fluorescence intensity)	21.45 (4.86 to 156.4) (n=14)	22.85 (4.22 to 82.31) (n=15)	0.84
	CX3CR1 (median fluorescence intensity)	51.77 (17.11 to 92.83) (n= 12)	31.49 (17.81 to 78.24) (n= 13)	0.24
CD14+CD16+	Cell count (/μL blood) median (range)	36 (8-117) (n= 29)	35 (2-98) (n=21)	0.31
	CCR2 (median fluorescence intensity)	Not expressed (n=14)	Not expressed (n=15)	Not done
	CX3CR1 (median fluorescence intensity)	168.6** (83.73 to 242.4) (n= 12)	198.8** (34.94 to 274.1) (n= 13)	0.38
Serum CCL2 pg/ml, median (range)		1081 (149.8 to 2340) (n=14)	295.5 (40.07 to 1301) (n=15)	0.006*
Serum CX3CL1 pg/ml, median (range)		25.5 (11 to 75.5) (n=14)	16.00 (8.5 to 44) (n=15)	0.15

Table 3.2-3: Absolute monocyte count, and expression of CCR2 and CX3CR1 on CD14++CD16- and CD14+CD16+ monocytes and serum CCL2, CX3CL1 concentrations in CF patients and controls. Data are given as median unless otherwise specified

* significant difference between groups $p<0.05$, Mann Whitney test

**significant difference between CD14++ and CD14+CD16+ monocytes ($p<0.0001$), Mann Whitney test
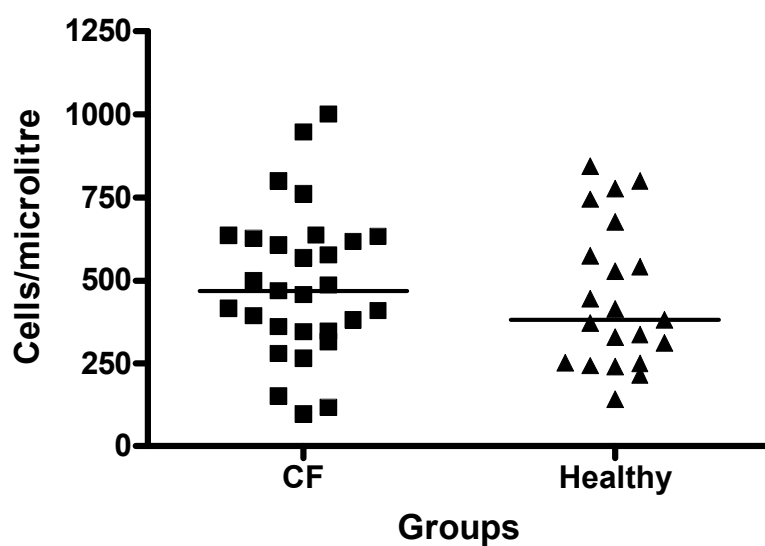
Figure 3.2-1: Dot plot showing total monocyte number in CF patients and healthy controls. There was no significant difference between the groups (p= 0.38). The horizontal bars denote the median value. (Table 3.2-3)
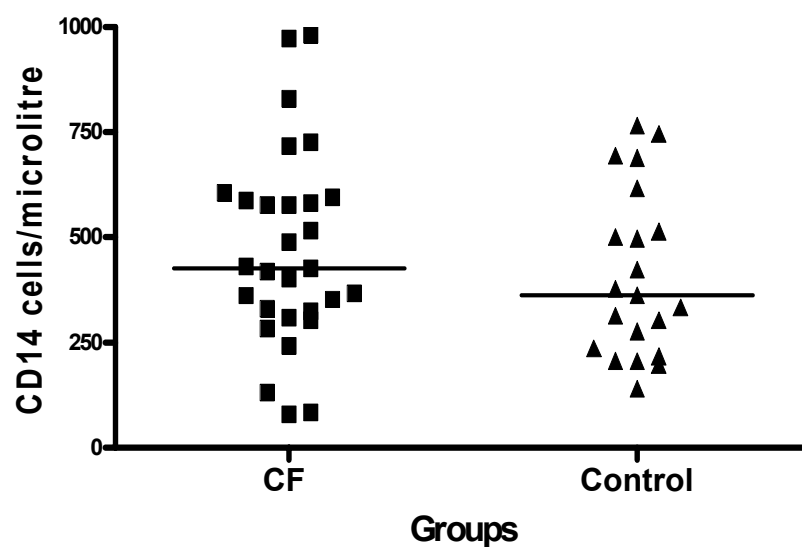
Figure 3.2-2: Dot plot showing CD14++ monocyte number in CF patients and healthy controls. There was no significant difference between the groups (p=0.35). The horizontal bars denote the median value. (Table 3.2-3)
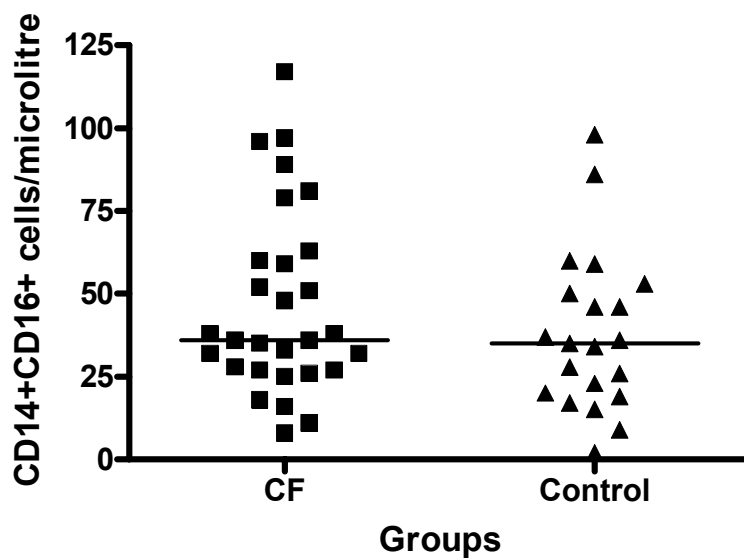
Figure 3.2-3: Dot plot showing the CD14+CD16+ monocyte number in CF patients and healthy controls. There is no significant difference between the groups (p=0.31). The horizontal bars denote the median value. (Table 3.2-3)
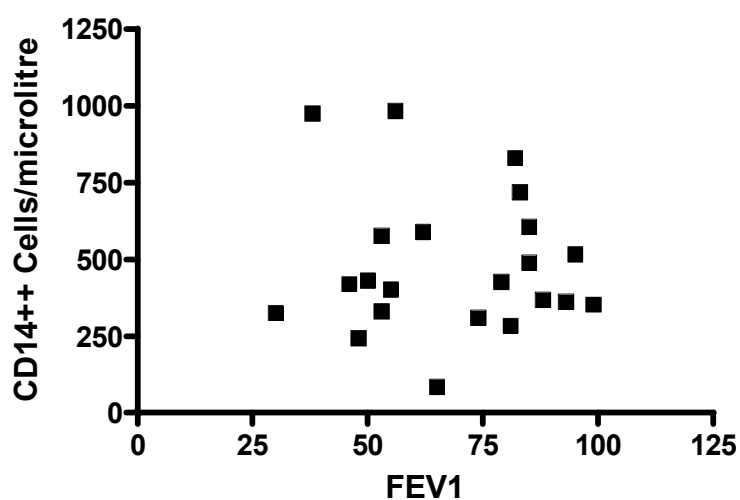
Figure 3.2-4: Scatter plot of CD14++ monocyte count Vs. FEV1 in CF patients. The figure shows no significant correlation between the parameters (r=0.06, p=0.78). (Table 3.2-3)
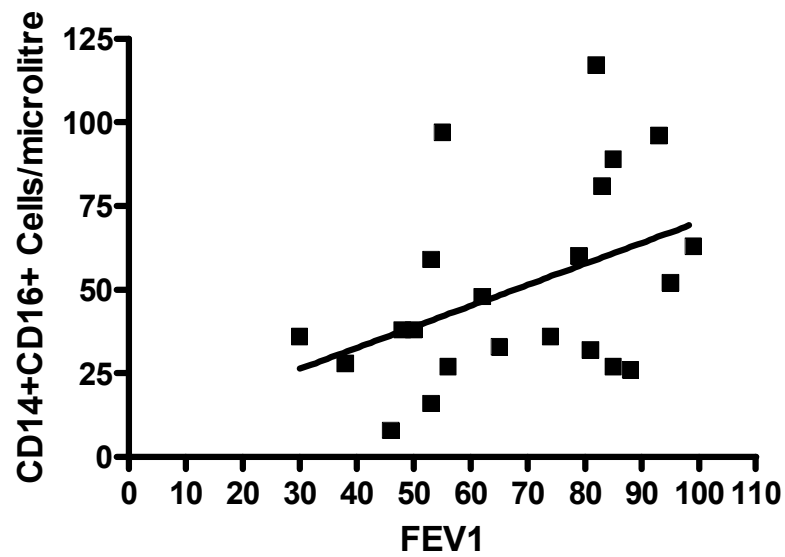
Figure 3.2-5: Scatter plot of CD14+CD16+ monocyte count Vs. FEV1 in CF patients. The figure shows no significant correlation between the parameters (r=0.37, p=0.087)

3.3   Comparison of serum CCL2 and CX3CL1concentration

My hypothesis was that both serum CCL2 and CX3CL1 are raised in CF.

Serum CCL2 concentrations were available from 14 CF patients and 15 healthy controls. There was a significant increase in the CCL2 concentration in CF patients (Figure 3.3-1) [median concentration of CCL2 picograms (pg)/ml of supernatant fluid (range) CF and healthy respectively: 1081 (149.8-2340) vs. 295.5 (40.07-1301), 95% CI 686.4-1515; p=0.0064 (Mann Whitney test)]. There was no correlation between CCL2 concentration and CD14++ monocytes (figure 3.3-2), symptoms (figure 3.3-4), inhaled steroid therapy or lung function (figure 3.3-3) in the CF group.

However, there was no significant difference in serum CX3CL1 concentration between the groups [Median concentration picograms (pg)/ml of supernatant fluid (range)] in CF and healthy were 25.5 (11-75.5) vs. 15.58 (8.5-44) respectively [95% CI 18.44-39.7; p=0.145 (Mann Whitney test)] (figure 3.3-5). There was no correlation between CD14+CD16+ count, FEV1 (figure 3.3-6) and CX3CL1 levels.

In summary, serum CCL2 and not CX3CR1 is raised in CF.
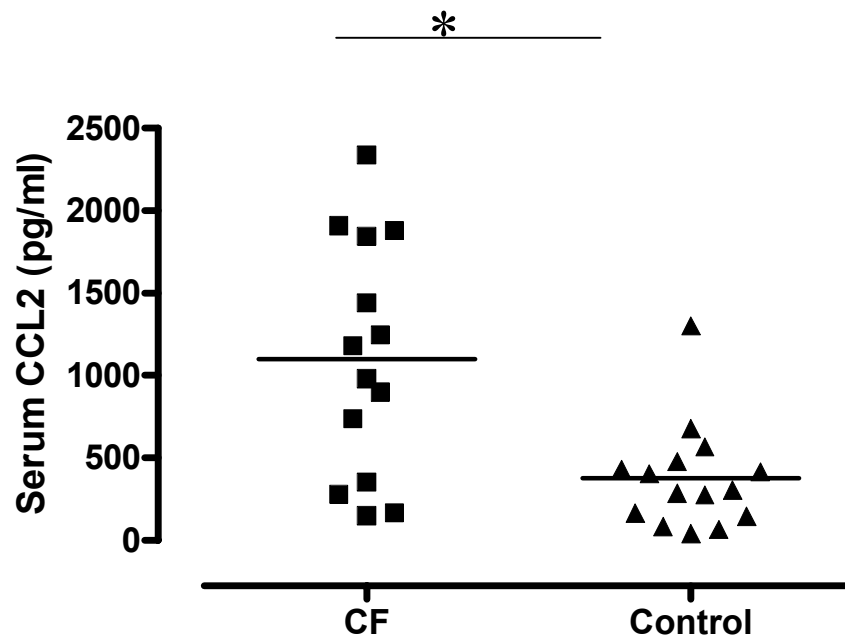
Figure 3.3-1: Dot plot showing concentrations of CCL2 (formally called monocyte chemoattractant protein-1) blood from cystic fibrosis patients compared with healthy controls. The horizontal bar represents median value. *p<0.01 by Mann Whitney test.(Table 3.2-3)
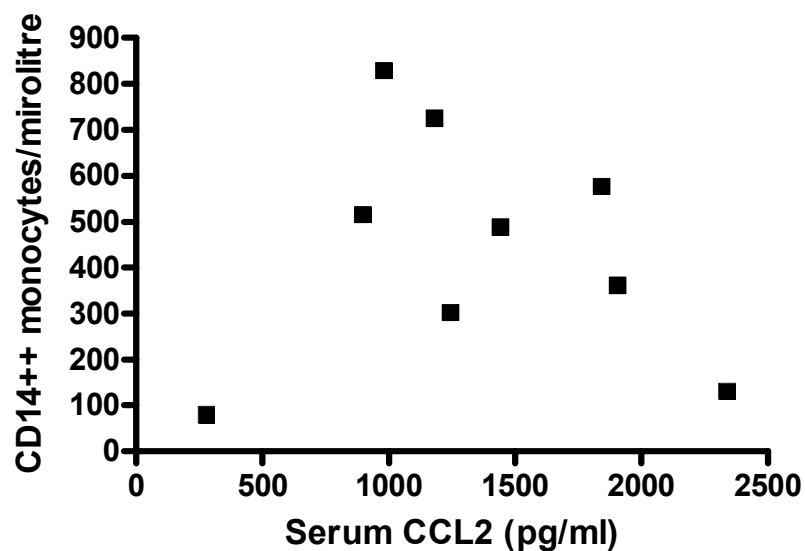
Figure 3.3-2: Scatter plot showing lack of correlation between serum CCL2 concentration and CD14++ monocyte number (Pearson r=-0.08, p=0.83) (Table 3.2-3)
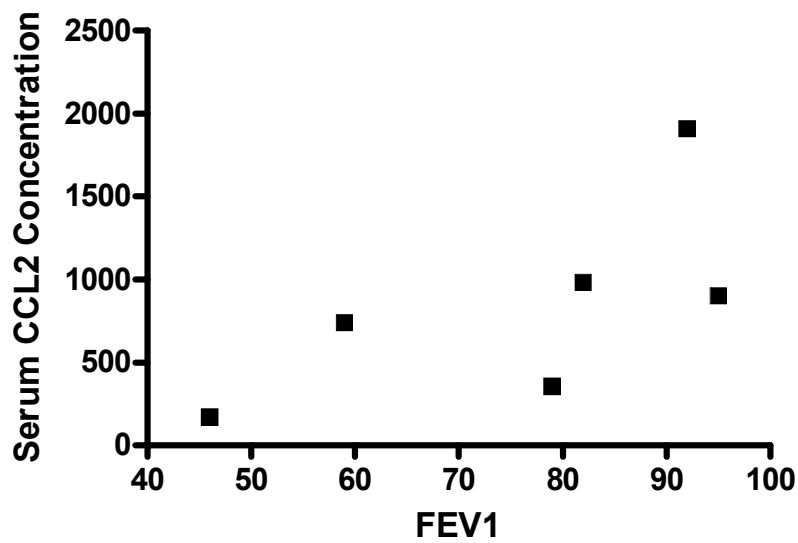
Figure 3.3-3: Scatter plot showing no significant relationship between serum CCL2 concentrations and lung function in cystic fibrosis r=0.77, p=0.10). (Table 3.2-3)
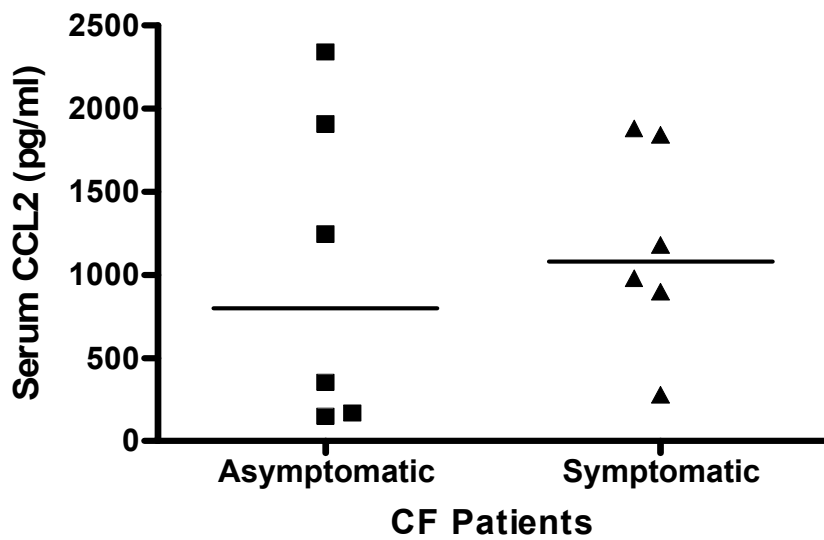
Figure 3.3-4: Comparison of serum CCL2 concentration between symptomatic and asymptomatic CF patients. Horizontal bar represents the median value. There was no difference between the groups (clinical status for this experiment not available for 2 patients) (Table 3.2-3)
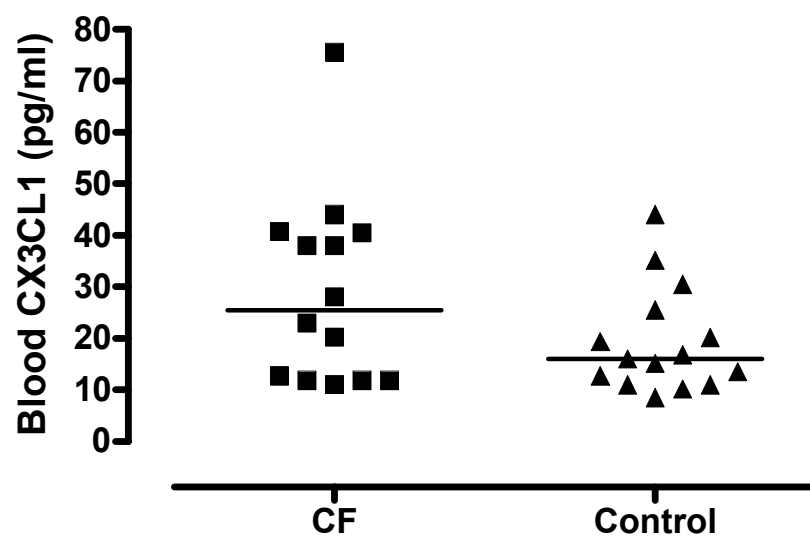
Figure 3.3-5: Dot plot showing concentrations of CX3CL1 (formally called fractalkine) in blood from cystic fibrosis patients compared with healthy controls. The horizontal bar represents median value. There is no significant difference between the groups by Mann Whitney Test. (Table 3.2-3)
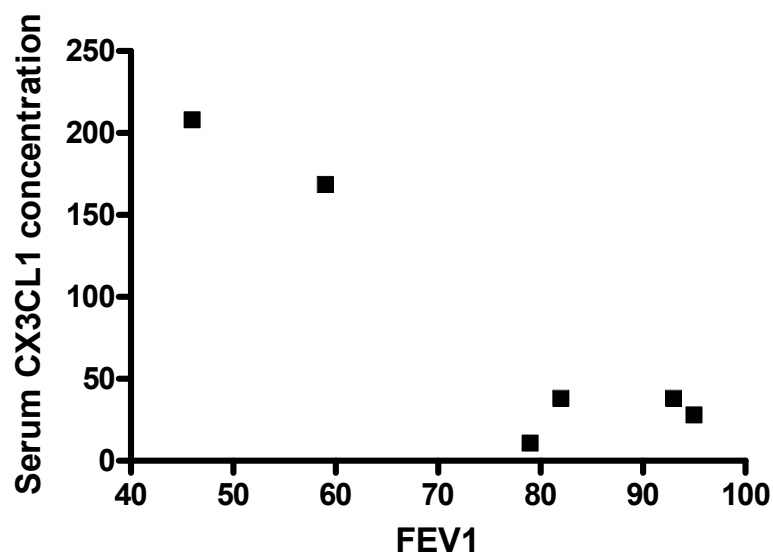
Figure 3.3-6: Scatter plot showing no significant correlation between serum CX3CR1 concentration and FEV1 in cystic fibrosis group (r=-0.64, p=0.18). (Table 3.2-3)

3.4 Blood monocytes and chemokine receptor expression

Transmigration of monocytes is influenced by the chemokines and their respective receptor. It is well recognized that CCR2 is the principal chemokines receptor for CCL2 and CX3CR1 for CX3CL1. In the next set of experiments I investigated if these receptors are expressed on the CD14++ classical monocytes and CD14+CD16+ monocytes subpopulations. I also investigated if there was a difference in the intensity of expression of these receptors between the CF and healthy control groups.

I have detailed the method that I developed to study the expression of CCR2 and CX3CR1 receptor expression in previous chapter. I have used median fluorescence intensity as the measure of receptor expression. Figures provide a summary of the methodology and also representative histograms for the CF and the healthy control populations.

3.4.1 CD14++ monocytes but not CD14+CD16+ express CCR2 receptor

Consistent with previous reports (Geissmann, 2003 #261), CD14++ monocytes expressed CCR2, whereas CD14+16+ monocytes did not express this receptor for CCL2. There was no difference in CCR2 expression by CD14++ cells between CF patients (n=14) and controls (n=15) (table 3.2-3) (figures 3.4-1, 3.4-2 and 3.4-3).

3.4.2 CD14+CD16+ monocytes have significantly higher expression of CX3CR1 compared with CD14++ monocytes

CX3CR1 was strongly expressed on CD14+CD16+ monocytes compared to CD14++ monocytes in both groups [CF (n=12) and control (n=13)] (table 3.2-3). However there was no significant difference in the median fluorescence intensity of CX3CR1 expression on CD14+CD16+ monocytes between the groups [median fluorescence intensity CF Vs. control respectively: 168.6 vs. 198.8, p=0.384, Mann Whitney test] (figure 3.4-5). Similarly, there was no difference in CX3CR1 expression on CD14++ monocytes between the study groups [median fluorescence intensity CF Vs. control respectively: 51.77 Vs. 31.49, p=0.243, Mann Whitney test] (figures 3.4-1, 3.4-4).
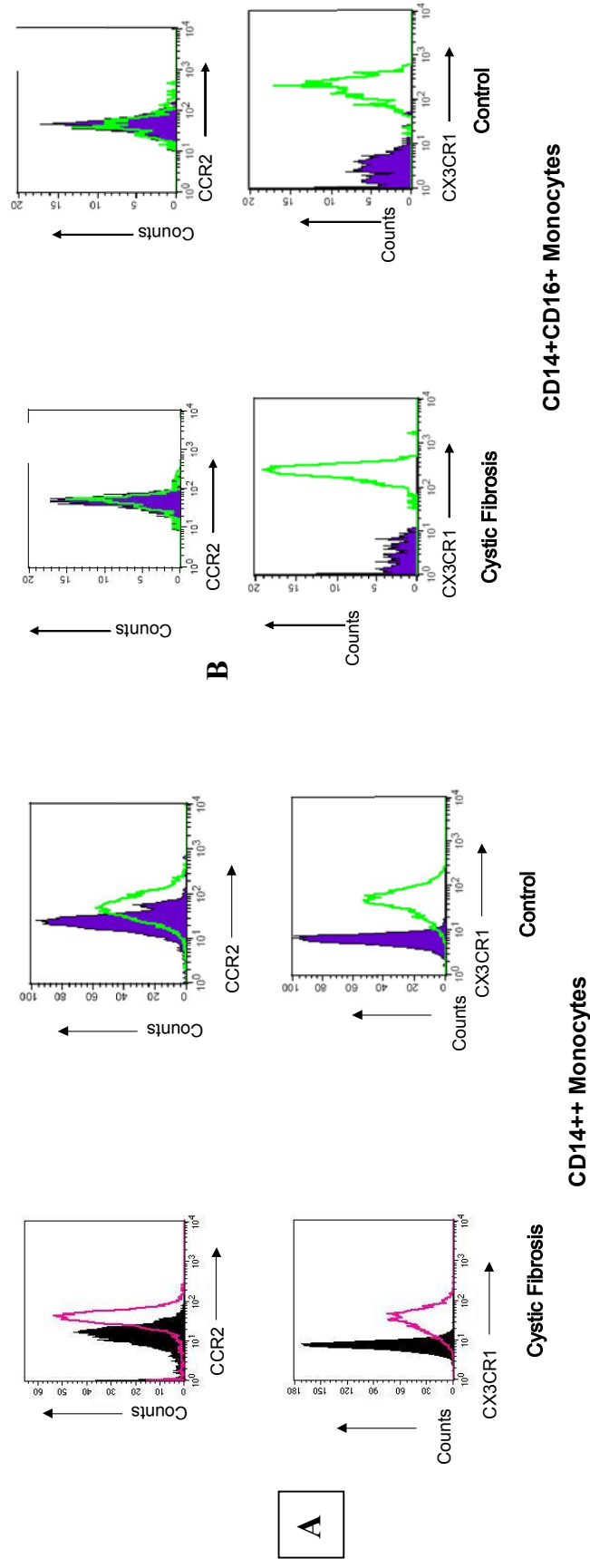
Figure 3.4-1: Representative histograms from CF and control subjects. The shaded histogram represents non-specific background staining and the open histogram specific antibody staining. The histograms show that the CD14++ monocytes express both CCR2 and CX3CR1 (A) whereas the CD14+CD16+ cells donot express CCR2 but have significantly stronger expression of CX3CR1 compared with CD14++ monocytes (B). There is however no significant difference in CCR2 and CX3CR1 on both populations of monocytes between the CF and control groups.
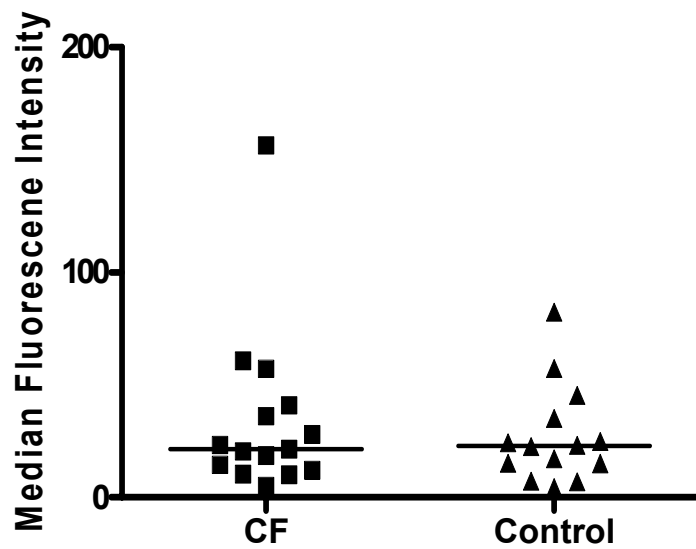
Figure 3.4-2: Dot plot of median fluorescence intensity of CCR2 expression on CD14++ monocytes. There is no significant difference between the groups, p=0.843. The horizontal bars denote the median value. (Table 3.2-3)
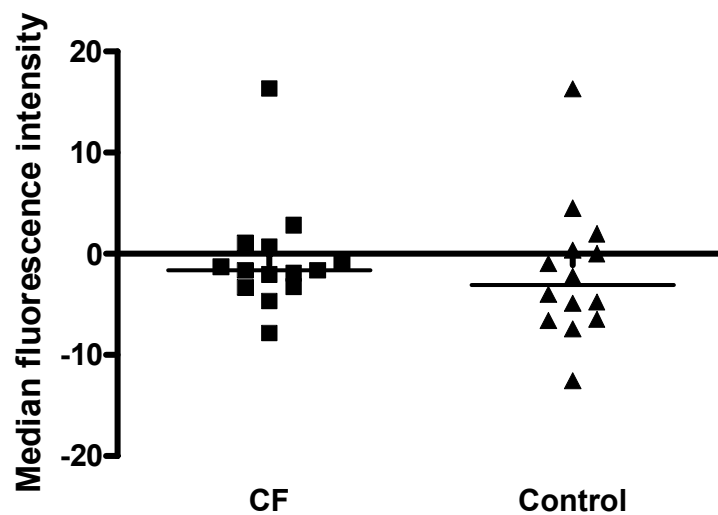
Figure 3.4-3: Dot plot of median fluorescence intensity of CCR2 expression on CD14+CD16+ monocytes. There is no significant difference between the groups, p=0.58. The horizontal bars denote the median value. (Table 3.2-3)
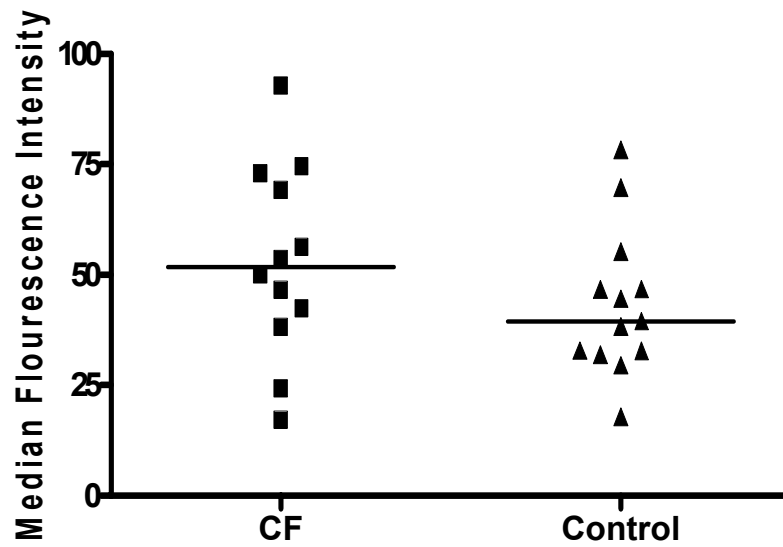
Figure 3.4-4: Dot plot of median fluorescence intensity of CX3CR1 expression on CD14++. There was no significant difference between the groups, p=0.243. The horizontal bars denote the median value.
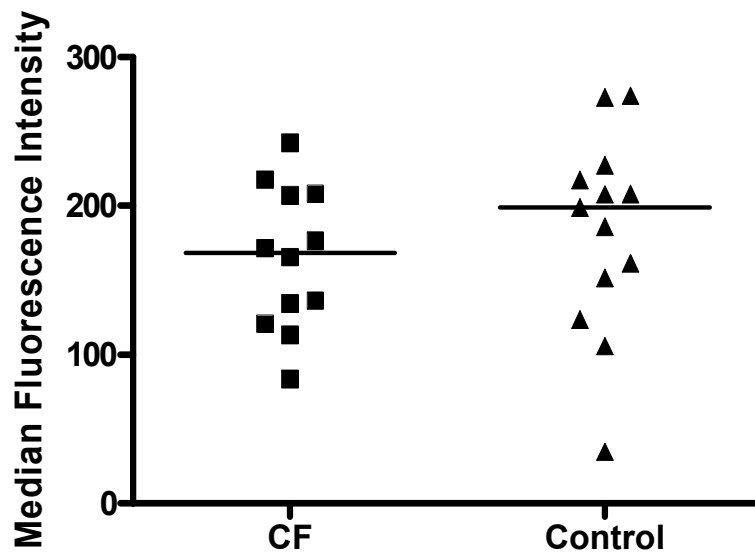
Figure 3.4-5 Dot plot of median fluorescence intensity of CX3CR1 expression on CD14+CD16+ monocytes. There was no significant difference between the groups, p=0.38.The horizontal bars denote the median value.

3.5   Conclusion

In this chapter, I have shown that

1. There is no systemic monocyte activation in CF.

2. There is significantly raised serum CCL2 but not CX3CL1 concentration in CF.

3. CCR2 is expressed on CD14++ and not CD14+CD16+ monocytes. CX3CR1 is expressed on both CD14++ and CD14+16+ monocytes but the expression is stronger on CD14+CD16+ monocytes compared to CD14++ monocytes. However, there is no difference in degree of expression of either CCR2 or CX3CR1 on both monocytes subpopulation between the CF and control groups.

I studied different aspects involved in monocyte transmigration, which have not been previously described in CF. I have shown that the serum CCL2 concentration but not CX3CL1 is raised in CF patients and that CCR2 is expressed on CD14++ classical monocytes. Although raised serum CCL2 has been described before (Augarten, Paret et al. 2004), I have extended these results by firstly demonstrating that there is indeed an increase in CCL2 levels in serum of CF patients by comparing with healthy controls. Secondly, I have studied the 2 main populations of blood monocytes in CF for the first time. I have demonstrated that CCR2 is expressed on CD14++ monocytes in CF thereby implying that CCR2/CCL2 interaction is possible in CF. On the other hand, I have shown that there is no significant increase in the levels of CX3CL1, suggesting that CD14+CD16+ monocytes might not play an important role in monocyte transmigration in CF.

The above findings are further supported by lack of evidence of systemic monocyte activation in cystic fibrosis. The CD14+CD16+ monocyte number in the CF patients was similar to that in healthy controls. This finding would suggest that CX3CR1/CX3CL1 interaction does not play a role in monocyte transmigration in CF. However, this finding could also be due to type 2 error, lack of adequate sample size to demonstrate the difference. As there is no previous data on monocyte subpopulations in CF, it was not possible for me to do the power calculation and estimate sample size before conducting the experiments. Another approach is to calculate the minimum difference in means between the groups that could be detected with the current sample size. With the current sample size, mean and standard deviations within the CF and control groups, I would be able to detect a difference of 21.69 cells/microlitre with 80% power between the means of control and CF groups. In this experiment, the difference between the mean in CF and control group was 8.78 [Mean cells/microlitre CF Vs. control: 46.83 Vs. 38.05 respectively (note the data in Table 3.2-3 is expressed as median and not mean)]. This would suggest that I have ruled out statistically significant difference between the two groups. However, it should be noted that the clinical significance of difference of 21.69 cells/microlitre (55% increase in CF) between the groups is unknown. Furthermore, majority of patients had exacerbation of respiratory symptoms, likely to be associated with increased inflammation in the airways and therefore monocyte activation if present would be more evident in this stage of clinical presentation. Similar argument can be made that there is indeed no statistical difference in the CX3CL1 concentration between the groups. I would have been to detect difference of 15.62pg/ml between the groups whereas the

actual difference in my experiment was 9.7 pg/ml [Mean pg/ml CF Vs. control: 29.07 Vs. 19.3 respectively (note the data in Table 3.2-3 is expressed as median and not mean)]

Similarly, I have ruled out statistically significant difference in CD14++ monocytes between the two groups. The sample size, mean and standard deviations would allow detection of a difference of 178 cells/microlitre between the means of CF and control groups with 80% power. The measured difference was 58.5 [Mean cells/microlitre CF Vs. control: 468.6 Vs. 410.1 respectively (note the data in Table 3.2-3 is expressed as median and not mean)].

There was trend to a raised CD14+CD16+ number in older patients with higher proportion of multi-system CF complications, but this was not statistically significant. The number of older patients in this group would not have been sufficient to detect a small but yet significant difference between the groups.

 The next step was to ascertain if the changes in serum were mirrored by similar changes in the airways of CF patients. Therefore, in the next chapter, I studied airway CCL2 and CX3CL1 concentrations.

4 CF airway chemokines mirror changes in serum: sputum CCL2 and not CX3CL1 concentration is raised

24 CF patients and 17 healthy controls were recruited for sputum induction. CF patients undergoing sputum induction also had lower FEV_1 compared with controls (Table 4.1-1). 10/24 CF patients had an exacerbation of respiratory symptoms at the time of sampling of which 3 were receiving intravenous antibiotic treatment (table 4.1-2). 14 patients were receiving regular inhaled steroids and none on oral prednisolone.

4.1 Comparison of sputum CCL2

24 CF samples and 17 samples from healthy controls were available for analysis. In 8 CF patients, where more than 2 sputum samples at different time points were available (n=8), the first sample was selected for the analysis. CF patients had significantly raised concentrations of CCL2 in induced sputum samples [median concentration of CCL2 picograms (pg)/ml of supernatant fluid (range) CF and healthy respectively: 190.6 (0-7551) vs. 77.31 (0-504.5), 95% CI 0-1131; p=0.0294 (Mann Whitney test)] (Figure 4.1-1). When data from CF and control subjects were analysed together, there was a trend to negative correlation between the CCL2 concentration and FEV1 but this was not statistically significant (r=0.3, p=0.07). However, there was no significant correlation between CCL2 concentration and FEV1 in the CF group (Figure 4.1-2). Further there was no correlation with CCL2 concentration and age or chest symptoms (Figure 4.1-3) in the CF group. CCL2 concentrations were similar whether or not sputum culture results identified any micro-organisms (p=0.33 by Mann Whitney test) (Figure 4.1-4).

	CF	Controls	p value by Mann Whitney Test
Total (n)	24	17	
Male: Female (n)	9:15	9:8	
Age (yr)	13 (7 to 47)	22 (9 to 50)	
FEV$_1$ % predicted	69 (46 to 97)	94 (81 to 113)	<0.05*
Respiratory exacerbation	10	Not applicable	
Sputum CCL2 (pg/ml)	190.6 (0 to 7551)	77.31 (0 to 504.5)	0.029*
Sputum CX3CL1 (ng/ml)	17.68 (0 to 36.3)	18.25 (16.8 to 24.73)	0.36

Table 4.1-1: Subject demographics for induced sputum, and CCL2, and CX3CL1. Data are given as median unless otherwise specified

*significant difference between the groups (Mann Whitney test)

Age: Mean years (range)	16.9 (7-47)
Sex: M:F	9:15
Race: Caucasian, Asian	22, 2
Genotype (deltaF508 +/+)	19
FEV1 % predicted: Median (range) [n=24]*	67.4% (46-97)*
Symptomatic (%)	10 (42%)
Positive microbiological culture	11 (7 *P.aeruginosa*, 4 *S.aureus* aureus, 1 *H.influenzae*, 1 mycobacterium abscesses, 1 aspergillus)
Additional Antibiotics: (Intravenous, Oral)	4 (3, 1)
Steroids: (Inhaled)	14
Liver Disease (%)	5 (15%)
CF Related Diabetes (%)	5 (15%)

Table 4.1-2: Sputum CCL2 and CX3CL1 Concentration: Characteristics of CF patients
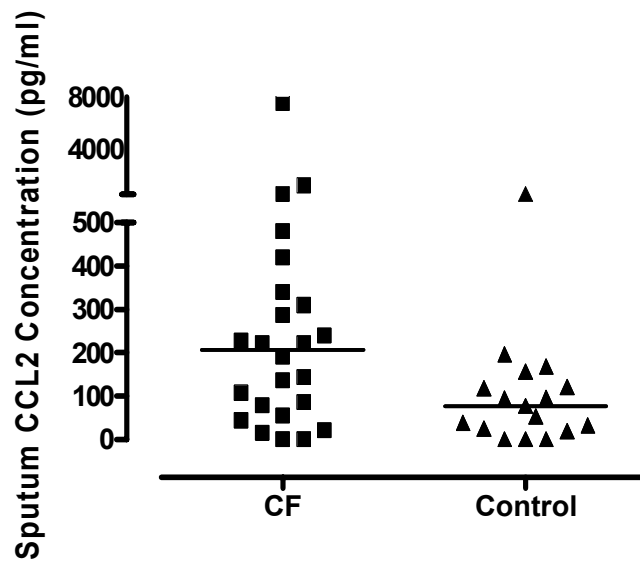
Figure 4.1-1: Dot plot showing sputum CCL2 concentrations in CF and control subjects. The CCL2 concentration is significantly raised in CF compared to controls (p=0.029). The horizontal bars denote the median value. (Table 4.1-1)
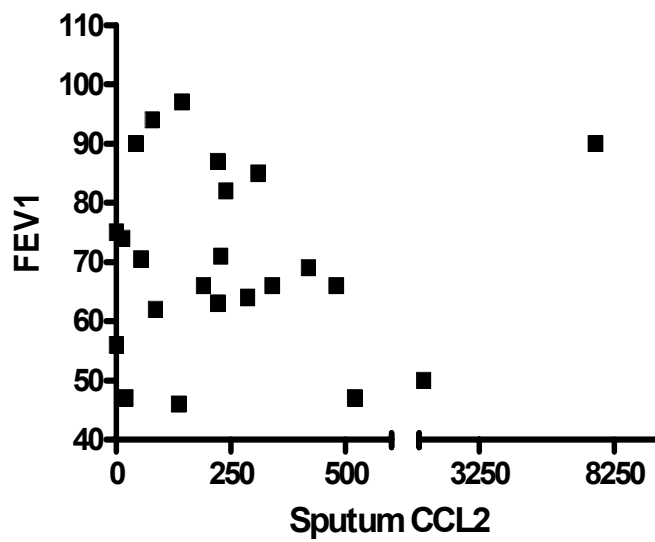
Figure 4.1-2: Scatter plot showing no correlation between sputum CCL2 concentration and FEV1 in CF groups (Pearson r=-0.05, p=0.79). (Table 4.1-1)
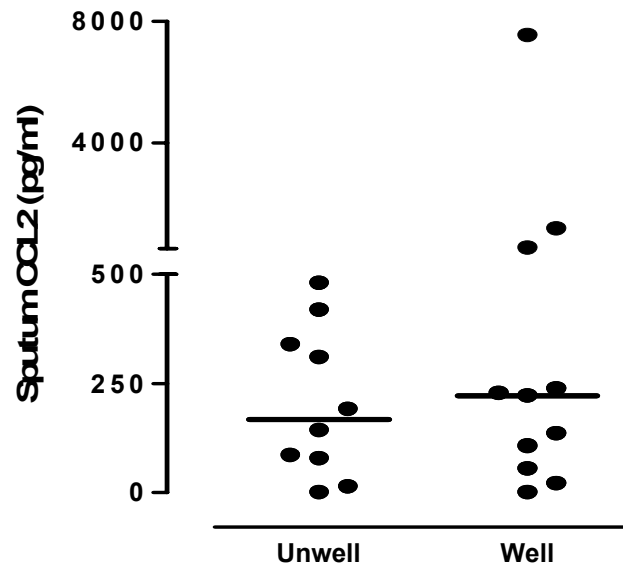
Figure 4.1-3: Sputum CCL2 concentration in well and unwell CF patients showing no correlation with symptoms (p=0.34). The horizontal bars denote the median value. (Table 4.1-1)
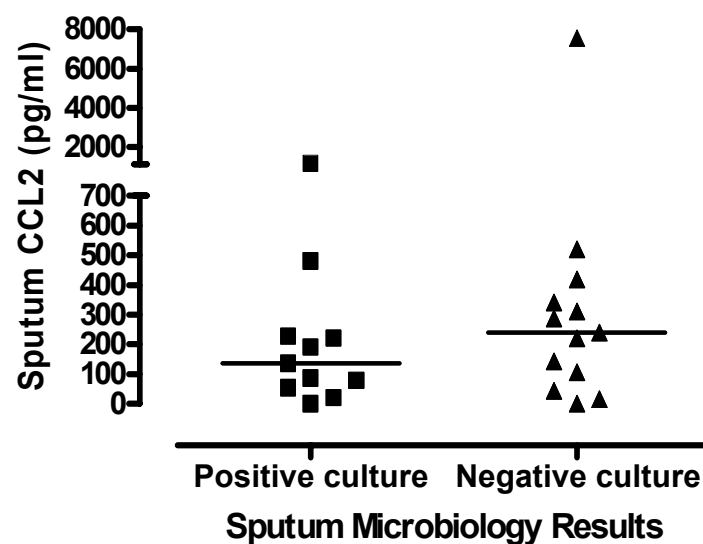
Figure 4.1-4: Comparison of sputum CCL2 in CF patients with and without identifiable micro-organism in the sputum. The horizontal bars denote the median value. There was no significant difference between the groups (p=0.33). (Table 4.1-1)

4.2   Comparison of sputum CX3CL1 concentration

There was no significant difference in the sputum CX3CL1 concentration between

the CF (n=27) and healthy control groups (n=14) [median concentration of

CX3CL1 nanograms (ng)/ml of supernatant fluid (range) CF and healthy

respectively: 17.68 (0-36.3) vs. 18.25 (16.8- 24.3) (Mann Whitney test)] Figure

4.2-1. There was no correlation between the CX3CL1 concentration and chest

symptoms, severity of lung disease or therapy in the CF group.
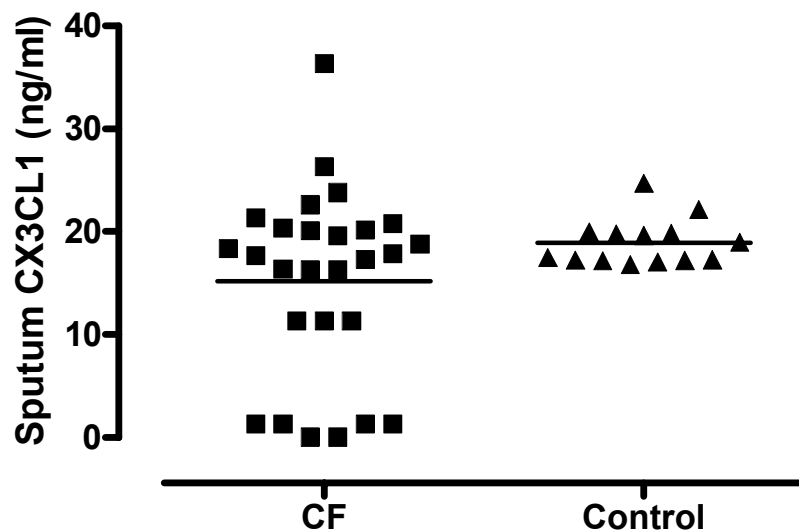
**Figure 4.2-1: Dot plot showing no significant difference in sputum CX3CL1 concentrations
between CF and healthy controls (p=0.36). The horizontal bars denote the median value.
(Table 4.1-1)**

4.3   Conclusion

The sputum CCL2 and CX3CL1 is raised in the CF airways mirroring the changes in the serum. Raised CCL2 is consistent with earlier reported studies (Augarten, Paret et al. 2004; McAllister, Henry et al. 2005). However this is the first study to my knowledge airway to measure airway CX3CL1 levels in CF. However, I was unable to demonstrate significant relationship between the sputum CCL2 levels and lung function or presence of respiratory exacerbations in CF group unlike some of the earlier studies. Possible explanations are further discussed in chapter 6.

Sputum CX3CL1 is not raised in CF patients. These results are consistent with the findings in the earlier chapter and would further suggest lack of role for CX3CR1/CX3CL1 in CF monocyte transmigration. The mean in the CF group was in fact lower than that in the control group [Mean (ng/ml) CF Vs. control: 15.16 Vs. 18.95 respectively) when the sample size would was powered to detect a difference of 7 ng/ml with 80% power. Therefore, one can conclude that the lack of difference between the groups is not due to the small sample size.

5 There may be an expansion of small macrophages in CF airways

The next step was to demonstrate if there was indeed an increase in the transmigrated monocytes into CF airways. I tested this hypothesis in a pilot study of characterisation on macrophages in paediatric CF airways. This pilot study contributed towards the establishment of sputum induction procedure towards obtaining sputum samples to enable macrophage characterisation. The protocol for airway macrophage characterisation was developed by Mr. Adam Wright, who was one of my colleagues working on this project. Adam continued the work on phenotypic and functional characterisation of airway macrophages in adult CF patients.

Expansion of the small macrophage population in the airways would be indirect evidence of increased monocyte recruitment into the airways (Rosseau, Selhorst et al. 2000). Due to the time constraints and difficulty in obtaining samples, I was unable to measure serum CCL2, CX3CR1, sputum CCL2, CX3CR1 and small macrophages in CF patients at a single time point. Therefore, even if small macrophage population were increased in CF airways, it would not be possible to conclude that this was as a direct result of raised CCL2 in blood and airways. However, I would argue that since this was a proof-of-principle study, demonstration of small macrophages in CF airways could then lead to more specific experiments *in vivo* or *in vitro* to obtain further evidence as to the role of the CCR2/CCL2 axis in monocyte transmigration in CF.

5.1 Results

14 CF children and 18 healthy controls were enrolled in this study. The characteristics of the two groups are presented in the table 5.1-1. There were no significant differences in the age and sex between the groups. CF children had significantly lower FEV1 compared with controls (p=0.005). None of the healthy controls reported any recent chest symptoms, whereas 8/15 CF children had exacerbation of chest symptoms at the time of obtaining sputum sample (p<0.0001).

Approximately half the children in both groups were unable to provide adequate sputum sample for FACS analysis. However, there were no significant differences in the characteristics of these patients compared with those who were able to provide adequate sputum samples in both groups (table 5.1-2). One CF child was excluded from the study as she developed significant bronchospasm with sputum induction and further sputum induction in that patient was not deemed to be safe.

The small macrophage proportion was significantly increased in the CF group (Figures 5.1-1 and 5.2-2). I have included five out of 6 samples that were available for CF patients. The data from the excluded sample belongs to Dr. Adam Wright and has been used in his PhD thesis. Inclusion of this sample does not alter the results. In this pilot study there was no correlation between the small macrophage population and lung function and chest symptoms in the CF group. As reported previously, the small macrophage population was smaller in size, had higher expression of surface receptor CD14 and lower expression of HLA-DR compared with large macrophages (not shown).

	Cystic Fibrosis (n=14)	Controls (n=18)	p value
Male: Female	6:8	9:9	
Age years: median (range)	11.5 (8-16)	14 (8-16)	0.08
Race: Caucasian, Asian	13, 1	14, 4	
Chest Symptoms	8	0	<0.0001*
FEV1 % predicted Median (range)	72 (47-102)	95 (66-117)	0.005[*]
Adequate Sputum sample, n (%)	6 (43)	8 (57)	0.29

Table 5.1-1: Comparison of characteristics of CF patients and Controls

* p value significant (Mann Whitney test)

		Adequate sputum sample	Inadequate sputum sample	p value
Cystic Fibrosis (n=14)	n (percentage)	6 (43)	8 (57)	
	% FEV1 predicted median (range)	75 (53-102)	72 (47-94)	0.94
	Symptomatic	3	5	
Healthy control (n=18)	n (percentage)	8 (44)	10 (56)	
	Age, years median (range)	14 (10-16)	13 (10-16)	0.38
	% FEV1 predicted median (range)	98 (77-117)	91.5 (66-109)	0.35

Table 5.1-2: Comparison of characteristics of adequate and inadequate sputum producers in CF and control groups.
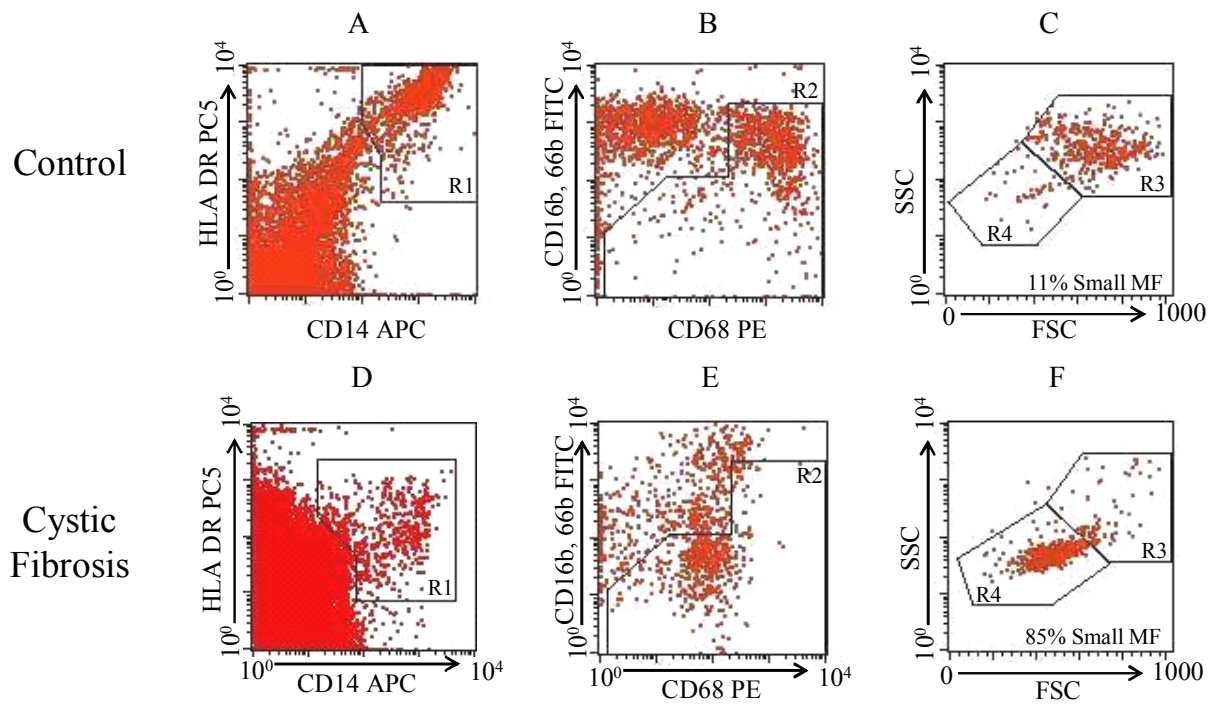
Figure 5.1-1: Sputum cells from a healthy control donor (A-C) and from a CF patient (D-F) were stained as detailed in the legend to Figure 1. CD14+ and HLA-DR+ events gated in R1 (A and D) were analysed for CD68 expression (B and E) and the CD68+ cells in R2 (B and E) were analysed for light scatter properties (C and F). The percentage of small macrophages in R4 (C and F) was 11% for the control donor and 85 % for the CF donor. (Provided by Dr. Adam Wright).
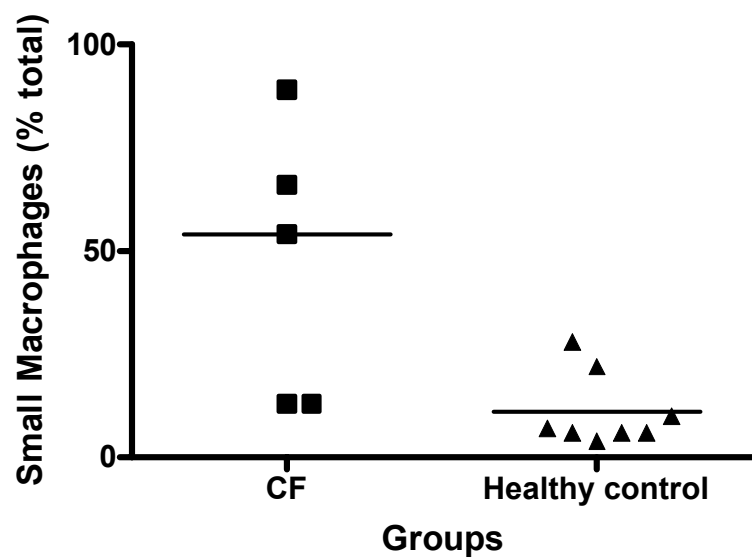
Figure 5.1-2: Dot plot showing increased small macrophage proportion in CF children (p=0.018, Mann Whitney test)

5.2   Conclusion

In this pilot study I demonstrated that characterisation of airway macrophages in CF and healthy children is feasible. I further demonstrated that there could be an increased small macrophage population in CF airways. However, these results would need to be confirmed by a larger study. Nevertheless, this finding would suggest an important role for small macrophages in regulation of inflammation in CF. These results are consistent with the published literature in both acute and chronic inflammatory lung conditions. Loems et al (Frankenberger, Menzel et al. 2004) in their study in adult COPD showed similar findings and were further able to demonstrate that the small macrophages have a pro-inflammatory profile. Similarly in adult ARDS, Rosseau et al demonstrated an increase in the small macrophage population and they also showed that the proportion correlated with the clinical severity.

There are several limitations to our study. Firstly, adequate sputum samples were obtained in only half of the recruited subjects. It is possible that I have selected out the patients with higher small macrophage proportion. However, there were no significant differences in the clinical status, FEV1 in patients or infection status between children that could and could not produce enough sputum. However, this finding will need to be confirmed with a larger study. Secondly, there did not appear to be any correlations between the small macrophage proportion and clinical parameters such as symptoms, FEV1 and infective stage. This could be as a result of small sample size which could make detection of such meaningful correlation impossible. Therefore, these results need to be confirmed with a larger study.

Thirdly, the presence of these small macrophages could just be an epi-phenomenon as I was unable to demonstrate the clinical relevance of this finding. A further longitudinal study of pre and post interventions in CF patients (for instance antibiotics or anti-inflammatory therapy example statins) will help us to further our understanding of the role of these cells in airway inflammation in CF.

In conclusion, characterisation of airway macrophage is possible in healthy and CF children. Preliminary results indicate that there could be an expansion of the small macrophage population in CF airways suggesting a role for these cells in sustaining airway inflammation.

6   Discussion

Cystic fibrosis is characterised by persistent neutrophilic airway inflammation, which is exaggerated to the degree of infection and may occur in absence of any detectable airway infection. In recent years there has been recognition that drivers other than airway infection may play an important role in maintaining inflammation. However, the role of airway macrophage in CF has been poorly studied even though it is well recognised that macrophages play a key role in other chronic inflammatory lung conditions such as COPD, asthma and interstitial lung disease. Therefore in this study I sought evidence for the pathways that could stimulate transmigration of systemic monocytes into the airways of patients with cystic fibrosis, including the CCL2/CCR2 axis and the CX3CL1/CX3CR1 axis. I initially compared the absolute counts of different monocyte subpopulations, serum CCL2 and CX3CL1, chemokine receptor expression for CCL2 and CX3CL1 on monocytes subpopulations between CF and healthy control groups. To confirm that changes in chemokines in the blood reflected changes in the airway, I then measured the concentrations of CCL2 and CX3CL1 in induced sputum samples. In addition, in a pilot study, I aimed to investigate if there is an increase in the proportion of pro-inflammatory small macrophages in the CF airways, as an indirect evidence for increased transmigration of monocytes in to CF airways.

My key finding is that CCL2, the most potent chemoattractant for classical CD14++ monocytes (Gerard and Rollins 2001) is increased in both the induced sputum and serum in CF, and that blood monocyte from CF patients have normal levels of expression of CCR2, the CCL2 receptor. Conversely, I found no increase

in CX3CL1, the major chemoattractant for migration of non-classical (CD14+CD16+) monocytes (Zlotnik and Yoshie 2000), in either sputum or serum from CF patients. Further there appeared to be a suggestion of increased small macrophage proportion in the CF airways. These findings would support the hypothesis that there may be an increased transmigration of monocytes in to the airways and suggests a key role for monocyte/macrophage in maintaining airway inflammation in cystic fibrosis.

The findings of raised CCL2 are consistent with other studies. CCL2 has been studied in various other inflammatory conditions of lungs including COPD (de Boer, Sont et al. 2000), bronchitis (Capelli, Di Stefano et al. 1999) and interstitial lung disease (Suga, Iyonaga et al. 1999). In cystic fibrosis, various inflammatory indices (including CCL2, interleukin-8, neutrophil elastase) have been studied extensively. Further, Augarten et al (Augarten, Paret et al. 2004) reported increased levels of serum CCL2 levels in patients with severe lung disease and similarly McAllister et al (McAllister, Henry et al. 2005) detected CCL2 in induced sputum samples of hospitalised CF patients. CCL-2 was increased at day 1 of hospitalization for an exacerbation and reduced by day 20. Normal controls were not included in either of these studies. I have extended the results of these studies by firstly demonstrating that there is indeed an increase in CCL2 levels in both airway and serum of CF patients by including the healthy controls. Secondly, I have also demonstrated that there is no significant increase in the levels of CX3CL1, a major chemoattractant for the non-classical monocytes CD14+CD16+ therefore the CX3CL1/CX3CR1 axis might not play an important role in monocyte transmigration in CF.

In contrast to the above studies, I did not find association between the level of serum and sputum CCL2 concentrations and lung function and symptoms of exacerbation. Augarten et al reported that the levels of CCL2 were higher in patients with severe disease compared to those with less severe disease. McAllister et al showed that the sputum CCL2 level was high in symptomatic patients and improved with anti-microbial treatment. Similarly in other chronic lung inflammatory conditions (Capelli, Di Stefano et al. 1999; Suga, Iyonaga et al. 1999), raised CCL2 correlated with increase in other inflammatory indices and poor clinical status. However, I did not find any association between the CCL2 concentration and markers of severity of lung disease such as FEV1, bacterial culture results or exacerbation status. The cross sectional nature of my study, may not have provided sufficient power to detect subtle changes during exacerbations and to determine association between CCL2 and severity of lung disease. Nearly half in blood group and about 40% in the sputum group were experiencing an exacerbation. A longitudinal study before and after treatment of respiratory exacerbation would address some of the above questions.

My findings however are consistent with other studies. For example Traves et al (Traves, Smith et al. 2004 Aug) reported increased levels of CCL2 in induced sputum from patients with COPD compared with healthy controls, but found no association between CCL2 and FEV_1 within the COPD group. Indeed, a much larger study of 269 CF patients only found modest inverse correlations (Pearson coefficient <-0.37) between lung function and sputum biomarkers of inflammation (Mayer-Hamblett, Aitken et al. 2007). One could argue that absence of a

correlation between CCL2 and lung damage, does not exclude an important role for monocyte transmigration in modulating neutrophilic airway inflammation. In fact this could be due to the dissociation between lung inflammation and measurable lung damage. In recent years, numerous studies have shown that there is lack of strong relationship between lung inflammation, lung damage and chest symptoms. Significant lung inflammation appears to be present even in the absence of significant lung damage or symptoms. Studies of inflammation in the early, asymptomatic CF patients diagnosed on neonatal screening have shown that the inflammation could be present in absence of any detectable airway infection and even in the absence of any symptoms. Khan et al (Khan, Wagener et al. 1995 Apr) in their study showed that the airway neutrophil count in 7 infants without any detectable airway infection was significantly higher than the normal controls. Similar results were found in other longitudinal studies of clinically asymptomatic young cystic fibrosis patients (Balough, McCubbin et al. 1995 Aug; Grosse, Rosenfeld et al. 2006). In fact, in a recent study, Upham et al reported that in their group of young stable CF patients undergoing annual surveillance bronchoscopy, the concentration of CCL2 in BAL was significantly elevated in the absence of overt bacterial and viral infection. In our study, there was significant increase in the serum CCL2 concentrations even in children as young as 4 years of age, consistent with the above findings. However, the younger children were more likely to have an exacerbation of chest symptoms in my study and therefore this might well be a transient increase associated with an exacerbation. Taken together, these results would suggest that significant inflammation could occur without evidence of significant lung damage or airway infection. In other words, there is

no strong correlation between the degree of inflammation and lung function and presence of airway infection and these results are consistent with my findings.

MCP-1/CCL2 belongs to CC families of chemokines and is expressed by epithelial cells, endothelial cells and macrophages and believed to be one of the principal chemoattractants for classical monocytes (Geissmann, Jung et al. 2003). It is possible that that increase in the levels of CCL2 in CF is non-specific and as a result of generalised pro-inflammatory state. However, several human studies (Szalai, Duba et al. 2001; Viedt and Orth 2002; Yao, Kuo et al. 2006) and animal models (Maus, Waelsch et al. 2003) have illustrated the specific role of CCL2/CCR2 interaction for monocyte transmigration in to tissues and its importance in maintaining inflammation. I was able to demonstrate normal expression of CCR2 receptor on CD14++ monocytes in CF patients and this could imply that such an interaction is possible in CF. Increased levels of CCL2 could potentially lead to increased transmigration in to the airways. From this study, however, I have not shown whether airway CCL2 in CF stimulates monocyte transmigration *in vivo*. But the levels of CCL2 (100 pg/mL) detected in IS samples from CF patients in this study, have previously been reported to stimulate the migration of human peripheral blood monocytes across an artificial membrane *in vitro* (Traves, Smith et al. 2004 Aug).

Further indirect evidence for increased transmigration is available from my pilot study of characterisation of airway macrophages in Paediatric CF population. There was an expansion of pro-inflammatory small macrophages with increased expression of CD14 compared with healthy controls. Following this pilot study,

these findings have since been confirmed by our group in the adult CF population (Mr. Adam Wright, PhD thesis, University of Leicester, 2008). The proportion of small macrophage in IS of CF patients was significantly raised compared to healthy controls (73 +/- 18% vs. 16 +/- 8%, p < 0.0001, Mann Whitney test). In the CF group, raised small macrophage proportion correlated with reduced lung function (Pearson correlation, r= -0.59, p=0.0082). One could argue that increase in small macrophage population could be the result of expansion of local alveolar macrophages. However, evidence from other studies would suggest that the expansion of small macrophage population is in fact due to increased transmigration of blood monocytes in to the airways and unlikely to be due to expansion of resident alveolar macrophages (Rosseau, Selhorst et al. 2000; Maus, Herold et al. 2001). Rosseau et al (Rosseau, Selhorst et al. 2000) in their study of ARDS have shown that the increased BAL CCL2 concentration was associated with increase in small airway macrophages expressing CD14+ and CD11b+ exhibiting monocytic phenotype (distinct to resident large CD14- alveolar macrophages) suggesting transmigration from the peripheral circulation in to the lungs. This was further supported by the fact that there was no evidence of local proliferation of airway macrophages. More direct evidence is available from a mouse model where the macrophages resembling monocytes (i.e. small macrophages) were recruited from blood following intratracheal administration of rJE/MCP-1 (Maus, Herold et al. 2001). The same group recently have provided us with more evidence that an inflammatory state results in an accelerated recruitment of monocytes in to the lungs. From their bone marrow chimeric CD45.2 mice model, they have demonstrated that whilst only 40% of the resident macrophages were replaced by immigrating monocytes in constitutive state in a

years time; more than 80% were replaced in just 2 months following treatment with *E coli* endotoxin (Maus, Janzen et al. 2006). Based on the results of my study, one could conclude that the increased transmigration is mostly likely due to CCL2 and its receptor (CCR2) on classical CD14++ monocytes and not CD14+CD16+ monocytes because of differential expression of CCR2 on classical monocytes and lack of expression on CD14+CD16+ monocytes (consistent with earlier studies (Frankenberger, Menzel et al. 2004).

Once recruited into the airways, these monocytes may then release a myriad of chemoattractants and pro-inflammatory cytokines further exacerbating the inflammation. Recently, Frankenberger et al have characterised these small CD14++ airway macrophages further and demonstrated that they are pro-inflammatory compared to resident large CD14- macrophages with an increased expression of TNF-α on exposure to LPS. The small macrophages role might not be limited to sustaining of inflammation but might even extend to initiation of the inflammatory process. In a murine model of allergic asthma (Gonzalo et al), exposure to the allergen resulted in early increase in BAL macrophage which peaked at day 15 and then declined. In contrast, the lymphocytes and eosinophils increased progressively beyond that period. Moreover, neutralisation of the CCR2 receptor thereby blocking monocyte recruitment during the early sensitisation and allergen challenge phase resulted in a markedly reduced airway eosinophilia subsequently. Similar results are available from a mouse model where presence of CCR2+ macrophages in the airways resulted in a pronounced neutrophilic influx (Maus, Waelsch et al. 2003 Mar 15). In a more convincing study, Maus et al showed that (Maus, von Grote et al. 2002) there is a 4 fold increase in

lipopolysaccharide-stimulated airway neutrophil accumulation in the presence of monocytes expressing CCR2, the receptor for CCL2. Further, blockade of macrophage recruitment during inflammation of the lung in the mouse model can be achieved by blocking the action of the chemokine CCL2. More importantly, this blockade also strongly reduced the recruitment of neutrophils to the lung. These results support the speculation that early infiltration of macrophages could lead to initiation and maintenance of subsequent chronic inflammation. Indeed, infiltration of macrophage in CF airways just before birth (Hubeau, Puchelle et al. 2001 Oct) has been described. Study of fetal CF lung tissue at various stages of development has shown an increase in the macrophage population in the airways of CF fetuses at later stage of development when compared with non-CF controls (Hubeau, Puchelle et al. 2001 Oct). This might suggest that the early increase in the macrophage cell population might result in initiation of neutrophilic inflammation but there is currently no direct evidence of this and hence this needs to be studied further.

In CF, the phagocytic ability of the macrophages might also be defective. A number of studies have described reduced phagocytic capability in CF macrophages (Thomassen, Demko et al. 1980; Thomassen, Demko et al. 1982; Knight, Kollnberger et al. 1997; Alexis, Muhlebach et al. 2006). In a parallel study, our group has extended the findings of these studies and demonstrated reduced phagocytic ability specifically in the CF small macrophage population compared to large macrophages (Mr. Adam Wright, PhD thesis, University of Leicester, 2008). Small macrophages exhibited significantly reduced expression of CD206 and MARCO PPRs compared to large macrophages. The combination of

pro-inflammatory profile together with reduced phagocytic activity in small macrophages could result in an exaggerated and persistent inflammatory response.

The CFTR genetic defect could alter the inflammatory profile of the macrophages *per se*. Pfeffer et al (Pfeffer, Huecksteadt et al. 1993 Nov) in their study of macrophages derived from peripheral blood monocytes of CF patients found that CF patient derived cells exhibited 2-4 fold higher expression of mRNA for TNF-α for a given level of LPS compared to healthy controls. Similarly, Zaman et al (Zaman, Gelrud et al. 2004 Sep) showed that presence of even a single allele CFTR mutation could be sufficient to augment IL-8 secretion in response to LPS stimulation. These studies seemed to suggest that defective CFTR results in an intrinsic pro-inflammatory profile for macrophages independent of exposure to infection. However, our group in another study has failed to confirm these results; the mRNA expression for TNF-α in monocyte-derived macrophages in CF patients was similar to that seen in healthy controls (Mr. Adam Wright, PhD thesis, University of Leicester, 2008).

The other key finding of my study is that there was no increase in systemic and airway levels of CX3CR1 and there was no expansion of CD14+CD16+ monocytes population in CF. These are in contrast to findings in other acute and chronic inflammatory conditions such as Kawasaki disease and rheumatoid arthritis respectively (Katayama, Matsubara et al. 2000; Kawanaka, Yamamura et al. 2002 Oct; Umehara, Tanaka et al. 2006). In the above studies, selective expansion of CD14+CD16+ monocytes correlating with disease severity and response to treatment has been described suggesting a key role for this sub-group

of monocytes. CD14+CD16+ monocytes are believed to represent a distinct sub-group of blood monocytes with different migratory and pro-inflammatory properties, but there is considerable uncertainty regarding their role in inflammation. Some studies would in fact suggest that their key role is in maintaining normal homeostasis in non-inflamed tissue and that they are incapable of migrating in to inflamed tissues (Geissmann, Jung et al. 2003; Sunderkötter, Nikolic et al. 2004; Tacke and Randolph 2006), whereas other studies (Maus, von Grote et al. 2002) would contradict these findings. Even in conditions where there is an expansion of CD14+CD16+ monocyte population, such as rheumatoid arthritis, there is still lack of evidence that expansion in tissue macrophage population in inflammation is due to increased recruitment of CD14+CD16+ monocytes. In rheumatoid arthritis the increase in proportion of CD16+ synovial macrophages could still be (Iwahashi, Yamamura et al. 2004), derived from transmigrated CD14++ monocytes under the influence of cytokine such as transforming growth factor- β (TGF- β) and not from CD14+CD16+ monocytes (Wahl, Allen et al. 1992). However my data and that of others suggests that in CF the CD14+CD16+ monocytes are unlikely to play a major role in airway inflammation.

Another explanation for lack of evidence for CX3CR1/CX3CL1 interaction in CF in my study could be that the sample size might not have been sufficient to detect small changes between the groups. However, I would argue that I have ruled out statistically significant differences between the groups for CD14+CD16+ number, serum CX3CL1 and sputum CX3CL1. I found differences between the groups that were less than the minimum difference my study was powered to detect for each

measurements. However, whether the difference my study was powered to detect is clinically important is not known.

The clinical significance of increased monocyte transmigration in lung inflammation in humans has been poorly studied. In study of ARDS patients where serial BAL samples were obtained, the raised BAL CCL2 levels correlated with clinical severity and survival. Better survival (76% vs. 25%) was seen in patients in whom there was a reduction in BAL CCL2 levels and decrease in CD14++ airway macrophages compared to the group where CCL2 level remained high. But the evidence for therapeutic benefit of blocking monocyte migration is derived from other chronic inflammatory such as rheumatoid arthritis where reduced CCL2 expression resulted in improvement of joint inflammation (Dawson, Miltz et al. 2003; Yao, Kuo et al. 2006). I have not addressed the clinical significance of raised CCL2 concentrations and increased sputum small macrophages in CF. The cross-sectional design of my study has some limitations in this regard. The cross-sectional design enabled me to study a wide group of patients more easily and is suited for proof-of-principle studies. As the inflammatory process is dynamic and dependent on various clinical parameters such as presence or absence of infection, duration of infection, timing of anti-microbial treatment and other anti-inflammatory treatments, it was not possible to examine the independent effect of each of the confounding variables without recruiting large number of subjects. Because of the intense resources and time needed to conduct these types of studies I would argue that the proof-of-principle needs to be established first. A longitudinal study of patients before and after anti-

inflammatory treatment would give more information on the relevance of the small macrophages in the inflammatory process.

The other limitation of the study is the possibility of selection bias. The CF patients were predominantly recruited in to the study when they were attending out-patient clinics or when they were inpatients receiving antibiotic therapy raising the possibility of over-representation of patients with chest exacerbations. CF patients have numerous hospital visits with significant impact on their lifestyle. Therefore every attempt was made to minimise the number of hospital visits. However, the proportion of patients with and without exacerbation was similar therefore it is unlikely that the significance of the results has been over-estimated. Further, there was no difference in the serum/airway concentrations of CCL2 between these groups suggesting that the concentration of CCL2 was raised irrespective of symptom status. Further, the patients in whom the blood sample was obtained were much younger. This was because it was difficult to recruit young healthy children for taking blood sample because of parental anxieties about the procedure whereas in the CF group the additional blood sample was taken for the study when the child was having the procedure for clinical indications. In both CF and healthy control groups, there did not appear to be any correlation with age and various parameters measured. Lastly, I was unable to obtain the blood and sputum samples to measure all variables at single time point for each patient and healthy control. Although there is an overlap of subjects between the blood and sputum group, I could not obtain paired samples. The reasons for this include inability of younger patients to produce sputum sample and often lack of time on part of the subjects to often participate in both steps.

Older subjects consented to providing the blood sample and frequently did not participate in sputum induction as the sputum induction would take longer time. Equally, younger patients were happy to participate in sputum induction but were reluctant to volunteer for venepuncture. In this age group blood sample was often obtained when they had venepuncture for a clinical reason. With more time and resources, I would have liked to obtain paired-samples from a sub-group of patients and controls so that correlation of all the variables at a single time point could be done.

Further understanding of clinical significance of monocyte trafficking in to lungs is important as it could potentially enable us to develop a novel anti-inflammatory strategy that would complement the existing therapies. The life expectancy and quality of life of CF patients has improved considerably over the last 20 years largely due effective chest physiotherapy, appropriate nutritional supplementation and aggressive antibiotic therapy (Konstan 1998). In addition, anti-inflammatory treatment is an effective adjuvant in certain groups of CF patients. Non specific anti-inflammatory agents such as oral steroids are known to be effective in arresting the decline of lung function in CF patients (Cheng, Ashby et al. 2000). Unfortunately they are associated with increased incidence of adverse effects such as diabetes and so are restricted for use in a small group of patients with rapidly progressing lung disease. Inhaled steroids, though safer, have not proved to be as effective as oral steroids in reducing inflammation or improving lung function (Balfour-Lynn, Walters et al. 2000). Similarly some benefit has been noted with ibuprofen (non-steroidal anti-inflammatory agent) but its use is limited due to the concerns of gastro-intestinal side-effects (Lands and Stanojevic 2007). Recent

studies with rhDNAase in CF children with mild lung disease suggested that early institution of DNAase might arrest the progression of neutrophilic inflammation and decline of lung function (Ratjen, Paul et al. 2005). However, there was significant drop-out rates in both treatment and non treatment groups which means that conclusions should be drawn with caution. The evidence for anti-inflammatory effect was inconclusive- there was no increase in the neutrophil count in BAL of the treatment group over 36 month period but the treatment did not reverse neutrophilia. Furthermore, the difference in lung function was modest and evident only with FVC and not FEV1. Other therapies such as with macrolides (example azithromycin) in some studies show an improvement in lung function of children not responding to conventional therapy but this was not associated with corresponding changes in inflammatory indices and the mechanism of action is still not clear (Jaffé, Francis et al. 1998) (Southern, Barker et al. 2004). These studies nevertheless suggest that there is undoubtedly a role for developing new strategies of anti-inflammatory therapy in CF.

The options for selective intervention are expanding all the time. Neutralisation of specific secreted mediators can now be achieved therapeutically, example a fully human monoclonal IgG_2 antibody directed against human IL-8 (ABX-IL-8), when given for 3-months to adults with chronic obstructive airways disease, improves shortness of breath scores (Capelli, Di Stefano et al. 1999). Similarly phase II clinical trials are now testing the efficacy of inhaled interferon-G and leukotriene B4 receptor antagonist BIIL 284 in CF. Targeting of molecules common to multiple signalling pathways could be another option. For example, administering lipoxin LXA4 analog increases its activity which then reduces neutrophilic

inflammation in mice infected with *P.aeruginosa*. Genistein, an isoflavanoid tryrosine kinase inhibitor together with 4-phenylbutyrate are known to increase the activity of CFTR whilst inhibiting IL-8 production by their effect on other pathways. The combination is now being tested in phase 2 trials in CF (Koehler, Downey et al. 2004 Oct).

To use more selective inhibitors of monocyte trafficking in inflammation in CF will require detailed understanding on the cells and mediators in the CF airway, and their relevance to lung injury. Proper understanding of the clinical significance of small macrophages in CF would enable us to develop a novel strategy of modulating airway inflammation. It might be possible to use monocyte migration blockers such as CCR1 antagonists (Haringman, Kraan et al. 2003 Aug) to arrest monocyte trafficking in to the lungs and thereby minimising lung damage. However, the anti-inflammatory treatment might also attenuate the useful effects of inflammation. A phase 2 clinical trial of leukotriene B4 antagonist BIIL 284 BS was stopped prematurely because of increased pulmonary exacerbations in the treatment group (Konstan, Doring et al 2005).

In summary, for the first time, the pathways of monocyte transmigration have been studied in cystic fibrosis. In this thesis, I have demonstrated that there is an increased CCL2 chemoattractant activity in blood and airways of CF patients suggesting an important role for CCL2/CCR2 axis in monocyte transmigration in CF. I have further demonstrated that the CX3CL1/CX3CR1 axis is unlikely to play a significant part in CF airway inflammation. Finally, I have provided early indirect evidence that there might indeed be increased monocyte transmigration in

to CF airways by demonstrating a raised small macrophage population is a pilot study, however, this needs to be confirmed further in larger studies.

7 Future work

Future work should concentrate on demonstrating the clinical relevance of the above findings.

1. As discussed before, raised systemic and airway CCL2 concentration could just be a reflection of the inflammatory state in CF and may not have any clinical relevance. Further work should involve longitudinal study pre and post intervention.

2. Use of monocytes blockers and its impact on the airway inflammation would help us to further understand the relevance of monocytes in inflammation.

3. Assessment of this aspect of the inflammatory cascade in infection-naïve CF children would help us understand if increased monocyte transmigration is indeed one of the early steps in CF airway inflammation,

8 Appendix 1: Publications and Presentations

Publications:

1. **Rao, S.** and J. Grigg (2006). "New insights into pulmonary inflammation in cystic fibrosis." <u>Arch Dis Child</u> **91**(9): 786 – 8

2. **Rao, S**, Wright AKA, Monteiro W, Ziegler-Heitbrock L, Grigg J. "Monocyte Chemoattractant Chemokines in Cystic Fibrosis." Journal of Cystic Fibrosis in press. http://dx.doi.org/10.1016/j.jcf.2008.09.009

3. Wright AKA, **Rao S**, Range S, Eder C, Hofer T, Frankenberger M, Kobzik L, Brightling C, Grigg J, Ziegler-Heitbrock L. "Expansion of Small Sputum Macrophages in CF: Failure to express MARCO and Mannose-Receptors". Journal of Leucocyte Biology in press

Abstracts and Presentations:

1. **Rao S,** Wright AKA, Range S, Ziegler-Heitbrock L, Grigg J, "CD14+CD16+ blood monocytes in cystic fibrosis". Thorax 2005;60(suppl 2):ii93. Presented at **CF session**, British Thoracic Society Winter Meeting, London, 2005

2. **Rao S**, Wright AKA, Ziegler-Heitbrock L, Grigg J. "Sputum Monocyte Chemoattractant Protein-1 Concentration in Cystic Fibrosis." Presented as poster discussion at European respiratory Society Conference, Munich, 2006.

3. **Rao, S**, Wright AKA, Monteiro W, Ziegler-Heitbrock L, Grigg J. "Systemic monocyte chemoattractant concentrations and receptor expression in cystic fibrosis". Presented as poster discussion at American Thoracic Society Conference, San Francisco, 2007.

4. Wright AKA, **Rao S**, Frankenberger M, *et al.* "Small Macrophages in Cystic Fibrosis Airways". Presented at the European Macrophage and Dendritic Cell Conference, Innsbruck, 2007.

5. Wright AKA, **Rao S**, Frankenberger M, *et al.* "Small Macrophages in Cystic Fibrosis Airways". E-poster presentation at European respiratory Society Conference, Berlin, 2008.

6. Hofer TPG, Frankenberger M, Wright AKA, **Rao S** et al. "Decreased Expression of HLA-DQ on Blood Monocytes and Sputum Macrophages in Patients with Cystic Fibrosis versus Healthy Subjects". Presentation at European Macrophage and Dendritic Cell Society Conference, Brescia, 2008.

9 Appendix 2- Information leaflets and consent forms

9.1 Information leaflets

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.
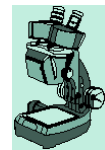
Do I have to take part? What happens if I do not wish to take part in this study or wish to withdraw from the study?
It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. You are still 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 example 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.

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.

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.

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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.
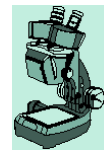
Do I have to take part? What happens if I do not wish to take part in this study or wish to withdraw from the study?

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.

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
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, the monocytes during and after exacerbations of chest problems in

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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.
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.

Patient Identification Number for this trial:

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. 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, 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 is a normal healthy child. It would be important to compare the activity of this cell in a normal child with that in a child with long standing chest infection.

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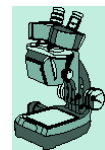
What will be involved if my child takes part in the study?
You and your child will be asked to attend the lung function laboratory of the Leicester Children's Asthma Centre (at the Leicester Royal Infirmary). We will ask you and your child about the general health and any medications. We will then obtain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

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 10 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.

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 and adults. The only known side effect is mild wheezing which is very unlikely in normal children. 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 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 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.

Patient Identification Number for this trial:

We are trying to find out why some children get poorly with chestiness

Here is a story of a child called Tom.

Tom's mum and dad have agreed for Tom to help us.

Tom comes to our Asthma Lab at the Children's Hospital.

His height and weight are measured by one of our doctors/nurses. The doctor will speak to mum and dad to find out how Tom has been over the last few days. He will be asked to blow hard in to a device called spirometer.

Then Tom takes a medicine (called salbutamol) to open up the tubes inside his lungs. Now Tom's breathing in a salty mist through a mask, which tastes a little salty, but not too bad at all. After 5 minutes we will ask him to cough into a pot. If nothing comes up he will continue to breathe the mist for another 5 minutes. The test finishes after 15 minutes.

We then do a variety of tests on what is coughed up including looking under a microscope.

Tom can tell us how he feels at any time. We will stop the test if he is not feeling too well.

Then, a blood sample will be taken from the back of his hand after applying the magic cream to numb the back of his hand. However this will be done if only Tom agrees to it.

Thank you very much for reading this leaflet and you can ask us anything that you do not understand.

We are trying to find out why some children get poorly with chestiness

As it is important to find out what happens inside the lungs of healthy children, we have asked you and your parents to help us. This will help us work out what is different in children with repeated chestiness.

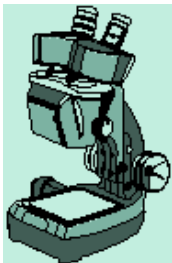**Here is a story of a child called Tom.**

Tom's mum and dad have agreed for Tom to help us.Tom comes to our Asthma Lab at the Children's Hospital.

His height and weight are measured by one of our doctors/nurses. The doctor will speak to mum and dad to find out how Tom has been over the last few days.
He will be asked to blow hard in to a device called spirometer.

Then Tom takes a medicine (called salbutamol) to open up the tubes inside his lungs.

Now Tom's breathing in a salty mist through a mask, which tastes a little salty, but not too bad at all. After 5 minutes we will ask him to cough into a pot. If nothing comes up he will continue to breathe the mist for another 5 minutes. The test finishes after 15 minutes.

We then do a variety of tests on what is coughed up including looking under a microscope.
Tom can tell us how he feels at any time. We will stop the test if he is not feeling too well.

Then, a blood sample will be taken from the back of his hand after applying the magic cream to numb the back of his hand. However this will be done if only Tom agrees to it.

Thank you very much for reading this leaflet and you can ask us anything that you do not understand.

9.2 Consent Forms

**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 [……………………..] 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;

<center>**PARENT CONSENT FORM for Healthy Children**</center>

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 [……………………..] 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

<center>1 for patient; 1 for researcher;</center>

10   References:

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