4

Circulating leucocytes and COPD

4.1 Introduction

The COPD lung is characterized by infiltration of inflammatory cells, mainly neutrophils and T lymphocytes, throughout the airways, lung parenchyma and pulmonary vasculature. The neutrophils are present in the epithelium and the lumen of the airways as well as in the sub-mucosal glands and smooth muscle of the peripheral airways (Baraldo et al 2004). The CD8 cells are found in the mucosa and the sub-mucosa of the entire bronchial tree as well as in the lung parenchyma, smooth muscle and the pulmonary vasculature. The number of CD8 cells in the airways is related to the severity of the airway obstruction (O'Shaughnessy et al 1997, Saetta et al 1999). T cells play a key role in the defence against infection.

The inflammation in the lungs is accompanied by systemic inflammation which can be quantified by measuring the circulating levels of C-reactive protein (CRP), fibrinogen, interleukin-6(IL-6), tumour necrosis factor alpha(TNF-α) and it's receptors (Gan et al 2004) and many other biomarkers (Hurst et al 2006). Patients with COPD also have significantly higher circulating leucocytes than healthy controls (Gan et al 2004). Epidemiological studies performed in large samples of the general population show that increased peripheral blood leucocytes are associated with reduced lung function, increased decline in lung function and increased all cause mortality, independent of age and cigarette smoking (Weiss et al 1995, James et al 1999). A recent study in a large cohort of COPD patients shows that increased circulating leucocytes are associated with more frequent exacerbations (Hurst et al 2010).

Patients with COPD experience frequent exacerbations which are associated with increased morbidity, mortality and impaired quality of life (Seemungal et al 1998). Exacerbations are associated with increased systemic inflammation as measured by plasma endothelin-1 (Roland et al 2001), fibrinogen (Wedzicha et al 2000), serum IL-6 (Wedzicha et al 2000), C-reactive protein (Dentener et al 2001, Hurst et al 2006) and soluble ICAM-1, PARC, MPIF, ACRP-30 (Hurst et al 2006). Circulating leucocytes can also be increased at exacerbation.

The lower respiratory tract of patients with COPD is frequently colonized by bacteria in the stable state (Monso et al 1999, Zalacin et al 1999), and this is considered an independent stimulus for airway inflammation (Sethi et al 2001). The number of bacteria and the species contribute to airway inflammation (Hill et al 2000). The lower airway bacterial load in stable COPD is linked to exacerbation frequency and lung function decline (Patel et al 2002, Wilkinson et al 2003). Isolation of a primary pulmonary pathogen in the sputum of stable COPD patients is associated with a higher level of plasma fibrinogen and a worse health status (Banerjee et al 2004). The exact mechanisms leading to lower airway colonization are incompletely clarified. A defect in the host immunity is one of the potential mechanisms. Alveolar macrophages of COPD patients show an impaired response to H influenza outer-membrane P6 and lipooligosacharide (Berenson et al 2006). Another recent study

shows that TH1-mediated adaptive immune response to bacterial infections is impaired in COPD patients (Knobloch et al 2011).

At the time we conducted the present study, only a few publications had examined circulating T cells in COPD patients (De Jong et al 1997, Majori et al 1999, Kim et al 2002 and Hodge et al 2003). These studies included small numbers of patients and their findings were variable. In the last 5 years several studies have demonstrated abnormalities in the circulating T lymphocytes in smokers and COPD patients (Hodge et al 2005, Pons et al 2005, Barcello et al 2006, Smyth et al 2007, Zhu et al 2009). However, to the best of our knowledge no study has examined the relationship between the number of bacteria present in the lower airways in patients with stable COPD and the number of the different circulating leucocytes.

4.2 Aim

The aim of our study was to assess the relationship between the different types of circulating leucocytes and the lower airway bacterial load in patients with well characterized COPD. We sampled the patients in the stable state and followed them up prospectively to evaluate the changes occurring at exacerbation.

4.3 Method

4.3.1 Study protocol

Patients were recruited and sampled from the London COPD Cohort whilst in the stable state and at least 6 weeks away from their last exacerbations. They were then followed prospectively with daily diary cards where they recorded any change in their respiratory symptoms and the best of 3 morning PEFRs. They were asked to contact the study team via a dedicated phone line if they noticed any change in their respiratory symptoms. They were generally seen within 48 hours. The presence of an exacerbation was confirmed, symptoms and lung function were recorded. Samples of

sputum and blood were collected and patients were treated with antibiotics, steroids or both depending on the severity of the episode. Patients were also seen routinely every 3 months and lung function was recorded and diary cards reviewed. This study was conducted between July 2003 and July 2004.

4.3.2 Study subjects

Fifty eight patients were recruited from the London COPD cohort. Patients had COPD defined as a ratio of post bronchodilator forced expiratory volume in 1 second (FEV_1) /forced vital capacity (FVC) <70 % and a β2-agonist reversibility of FEV_1<15% or 200 ml. Patients with a history of asthma, atopy, allergic rhinitis, clinically significant bronchiectasis, or lung cancer were excluded.

At recruitment the following information was recorded for each patient: daily respiratory symptoms, smoking history, drug history, age, sex, height, weight, FEV_1, FVC, peak expiratory flow rate(PEFR) [measured by rolling seal spirometer SensorMedics Ltd, Yorba Linda, California, USA] and ear lobe capillary blood gas tension (measured on a Model 278 Blood Gas Analyser, Ciba-Corning, Medfield, Maine, USA).

4.3.3 Skin testing

All patients underwent skin prick testing to the following common inhaled allergens: house dust mite, 3 trees, 6 grasses, cat, dog and Aspergillus fumigatus. The skin tests were considered positive if the wheal to any allergen was larger than the positive control histamine. Patients with positive skin tests were excluded from this study. Skin tests were performed in the stable state, at least six weeks away from any infection or treatment with antibiotics. No patients were taking oral steroids or anti-histamines when the skin tests were performed.

4.3.4 Peripheral blood and sputum sampling

The blood and sputum samples used in the present study are different from those used in the time course study presented in chapter 3.

A sample of peripheral venous blood was collected into an EDTA tube. A spontaneously produced sputum sample was also collected into a clean sputum pot. The baseline sample was collected at least six weeks away from the last exacerbation

and treatment with antibiotics and/or oral steroids. The exacerbation sample was collected at the exacerbation visit prior to starting treatment. Blood and sputum samples were processed within 4 hours of collection.

4.3.5 Blood processing

Blood samples were divided into two portions. One portion was used to obtain a differential white cell count using a Coulter Gen S analyser in the hospital laboratory. The second portion was used to assess the lymphocyte subsets using a flow cytometer according to the process detailed below.

T cell phenotypes determination

We carried out dual-colour staining with fluorescein isothiocyanate (FITC) and R-phycoerythrin (RPE)-conjugated monoclonal antibody pairs (DakoCytomation, Glostrup, Denmark) on anticoagulated whole blood. The antibody pairs $CD3^+/CD4^+$ (helper/inducer), $CD3^+/CD8^+$ (suppressor/cytotoxic) and $CD3^-/CD16^+$ (natural killer (NK)) were used to detect the different sub-populations. A dual-colour isotype control was included and $CD45^{FITC}/CD14^{RPE}$ was used as a gating control. B cells were detected using FITC-conjugated CD20 (Becton Dickinson Biosciences Immunocytometry Systems, San Jose, CA, USA).

One hundred micro-litre blood samples were kept on ice for 60 minutes in the presence of 10 µl antibody reagent and red blood cells were then lysed for 10 minutes at room temperature in 2 ml FACS Lysing solution (BDIS). Cells were washed and acquired immediately onto a FACSort flow cytometer(BDIS). At least 15000 events were collected within a gate set around the lymphocyte population on the forward scatter versus side scatter dot plot and data were analysed using CELLQuest software.

$CD3^+$, $CD3^+/CD4^+$, $CD3^+/CD8^+$, $CD3^+/CD^{16+}$ and $CD20^+$ lymphocytes were expressed as the percentage of total lymphocytes and the absolute number of each subset was calculated from the white blood cell count(WBC) by multiplying the WBC by the percentage that was lymphocytes and by the percentage of lymphocytes that was the subset.

4.3.6 Sputum processing

Quantitative sputum bacteriology was performed according to our previously validated methodology (Patel et al 2002). In summary a portion of sputum was incubated for 30 minutes at 37°C with an equal weight of 0.1% dithiothreitol(DTT). Ten fold serial dilutions were then made in Brain Heart infusion broth, and 100 µl aliquots plated on to the surface of a range of culture media including blood, chocolate, MacConkey and cysteine lactose electrolyte deficient agars. These were incubated for 18 hours at 37°C in air enriched to 5% CO_2 and bacterial colonies were counted and sub-cultured for identification using standard morphological and biochemical assessments. The bacteriology results are expressed as the logarithm of the number of colony forming units per millilitre of sputum (log cfu/ml).

4.3.7 Statistical analysis

SPSS-11 for Windows was used for the statistical analysis. The One-Sample Kolmogorov-Smirnov Test was performed to assess the data distribution. Normally distributed data is expressed by mean and standard deviation. Skewed data is expressed as median and interquartile ranges. Pearson's and Spearman's correlation were used to correlate the lung function data with the different white cell subsets and the latter with the bacterial load. An unpaired *t* test and Wilcoxson Sign Ranked test were used to compare the different white cells between current and past smokers with COPD. The changes between baseline and exacerbation in the 18 paired samples were compared with paired *t* test.

4.3.8 Ethics

The study was approved by the East London and City Health Authority Ethics Committee and all patients gave written informed consent.

4.4 Results

4.4.1 Patient characteristics

The patients mean (SD) age was 69.2(7.3) years and they had moderate to severe COPD according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria. They had a significant smoking history with a mean (SD) of 53.3 (40.8) pack years. Eighty seven per cent of the patients were on an inhaled corticosteroid. The patient characteristics are shown in **Table 16**. The different white cell subsets and the bacterial load at baseline are presented in **Table 17.**

Table 16: Baseline characteristics of the 58 patients sampled in the stable state

Characteristic	Mean (SD)
Female:male (numbers)	24:34
Age (years)	68.2 (7.3)
FEV_1 (litres)	1.05 (0.52)
FEV_1 (percentage predicted)	42.73 (17.22)
FVC (litres)	2.25 (0.89)
FEV_1/FVC ratio	0.47 (0.14)
pH	7.41 (0.04)
pCO_2 (kPa)	5.74 (0.74)
pO_2 (kPa)	8.84 (1.19)
Pack-years of smoking	53.3 (40.8)
Number of active smokers (%)	18 (31%)

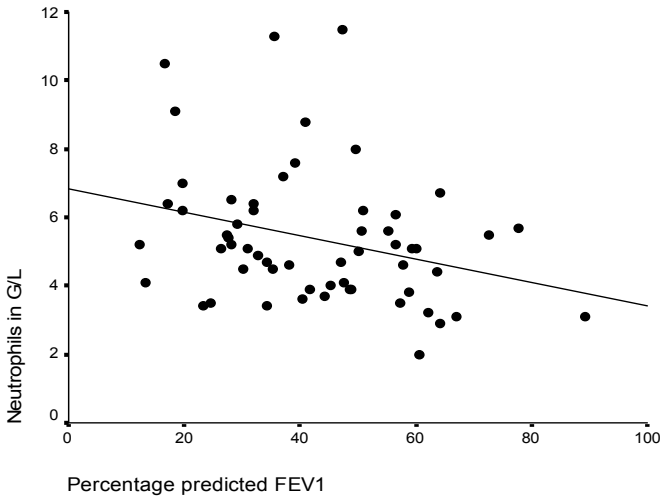
Table 17: Cell count and bacterial load at baseline in the 58 patients

Type of cells	Mean (SD)
White cells x 10^9/mm^3	7.91 (2.26)
Neutrophils x 10^9/L	5.37 (1.97)
Eosinophils x 10^9/L	0.20 (0.10-0.30)
Basophils x 10^9/L	0.10 (0-0.10)
Monocytes x 10^9/L	0.50 (0.40-0.60)
Lymphocytes x 10^9/L	1.75 (0.59)
B cells x 10^9/L	0.15 (0.08)
NK cells x 10^9/L	0.22 (0.15)
CD3 cells x 10^9/L	1.27 (0.50)
CD 4 cells x 10^9/L	0.80 (0.29)
CD 8 cells x 10^9/L	0.49 (0.34)
Bacterial load log cfu /ml sputum	7.79 (0.59)

4.4.2 Relationship between circulating leucocytes and lung function indices

In the stable state, patients with a lower percentage predicted FEV_1 had a significantly higher number of circulating neutrophils, r=-0.29, p=0.02, as shown on **Figure 19**.

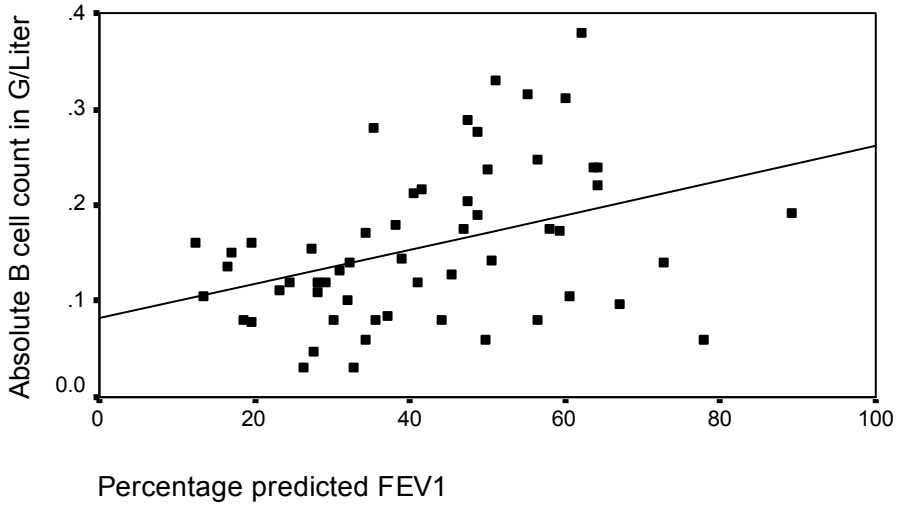
Figure 19: Relationship between circulating neutrophils and %FEV$_1$



The FEV$_1$/FVC ratio was inversely related to the number of circulating white cells (r=-0.329, p=0.01), neutrophils (r=-0.347, p=0.008) and monocytes (r=-0.39, p=0.003).

Patients with a higher percentage predicted FEV$_1$ had a higher absolute number of circulating B cells (r= 0.384, p=0.003) as demonstrated on **Figure 20**.

Figure 20: Relationship between the number of circulating B cells and %FEV$_1$



There was no significant correlation between the FEV$_1$, percentage predicted FEV$_1$ or FEV$_1$/FVC ratio and the circulating T cells, the CD4/CD8 ratio, the number of circulating eosinophils or basophils.

4.4.3 Effects of smoking on circulating leucocytes

Current smokers with COPD showed a trend towards an increased number of circulating total lymphocytes (1.95 x 10^9 /L vs 1.66 x 10^9 /L ,p=0.08), CD4 T cells (0.54 x 10^9 /L vs 0.46 x 10^9 /L ,p=0.06) and B cells (0.18 x 10^9 /L vs 0.14 x 10^9 /L, p=0.09).

Current smokers with COPD had a lower monocyte count than the ex-smokers with COPD (0.40 x 10^9 /L vs 0.50 x 10^9 /L,p=0.019). There was no significant difference in the other white cell subsets between current smokers and past smokers with COPD. There was no significant difference in the baseline bacterial load between the current and past smokers.

4.4.4 Relationship between circulating leucocytes and lower airway bacterial load in the stable state

Quantitative bacterial cultures were performed on 45 patients who provided a spontaneously produced sputum sample. The sputum bacterial load in the stable state was inversely related to the number of circulating total white cells (r=-0.56, p<0.01), neutrophils (r=-0.46, p=0.001), total lymphocytes (r=-0.46, p=0.001) and the circulating T lymphocytes (r=-0.48, p=0.001). The relationship between circulating T cells and bacterial load is illustrated in **Figure 21**. Patients with a higher sputum bacterial load had a higher percentage of circulating B cells (r=0.31, p=0.04) as shown in **Figure 22**. There was no significant relationship between the CD4/CD8 ratio and the bacterial load in the stable state (p=0.84).

Twenty patients grew a primary pulmonary pathogen (PPM) in the sputum. However, there was no significant difference in the white cell subsets between the patients colonized and not colonized by a PPM. In these 20 patients, a higher sputum bacterial load was associated with a higher percentage (r=0.54, p=0.01) and absolute number of circulating B cells (r=0.51, p=0.02).

Figure 21: Relationship between circulating T lymphocytes (G/L) and sputum bacterial load (CFU/ml)

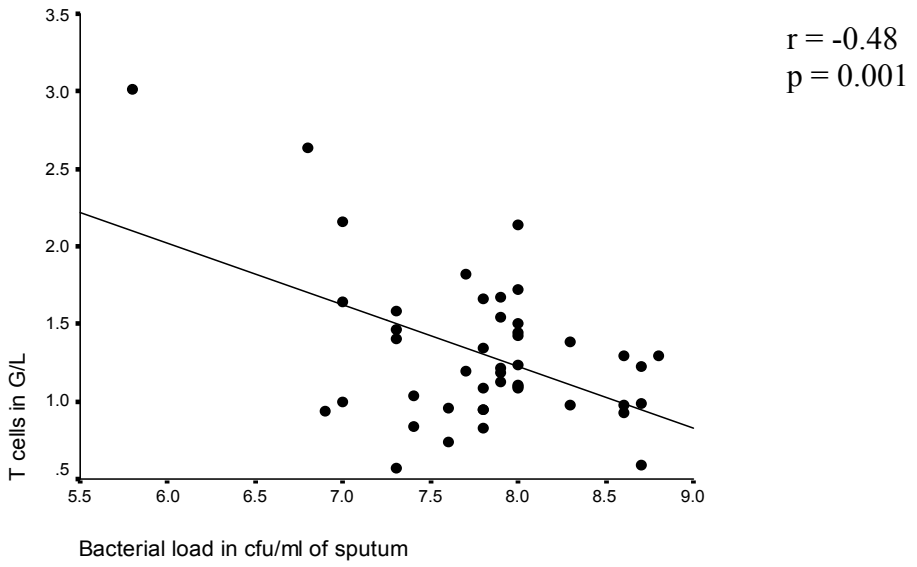
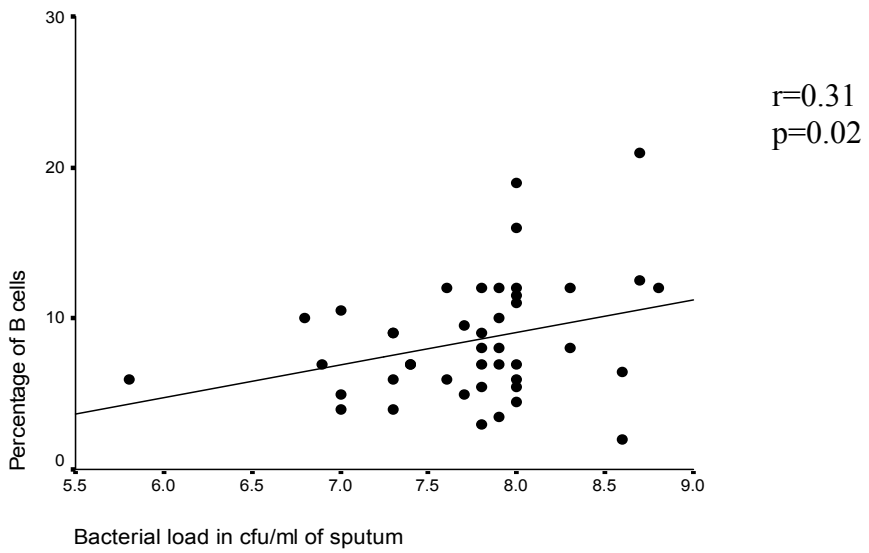


Figure 22: Relationship between circulating B lymphocytes (%) and sputum bacterial load (CFU/ml)



4.4.5 Changes in circulating leucocytes at exacerbation

Eighteen patients had an exacerbation during the study period and paired baseline-exacerbation samples were available for the following analysis. **Table 18** shows the baseline and exacerbation values of the leucocyte subsets and bacterial load for the 18 patients.

There was a small but non-significant fall in FEV_1 from 0.91(0.27) to 0.86(0.29) litres, p=0.29. There were no statistically significant changes in FVC and FEV1/FVC ratio.

Although the white cell count and neutrophil count remained within the normal range for our laboratory, there were significant changes at exacerbation. The total leucocyte count increased from 7.91 x 10^9/L to 10.18 x 10^9/L(p=0.04), neutrophil count increased from 5.23 x 10^9/L to 7.67 x 10^9/L (p=0.03) and monocyte count increased from 0.51 x 10^9/L to 0.69 x 10^9/L(p=0.016). Patients were sampled at exacerbation prior to any treatment with antibiotics and/or steroids.

At exacerbation there was a significant drop in the number of circulating lymphocytes from 1.81 x 10^9/L to 1.52 x 10^9/L (p=0.004). When the lymphocyte subsets were assessed we observed a drop in the total T lymphocytes (p=0.001); drop in CD4 lymphocytes (p=0.002) and a drop in CD8 cells (p=0.008). We did not observe any significant changes in eosinophils, basophils, B cells and NK cells numbers, CD4/CD8 ratio or bacterial load between baseline and exacerbation.

Table 18: Changes at exacerbation in 18 paired samples (paired *t*-test)

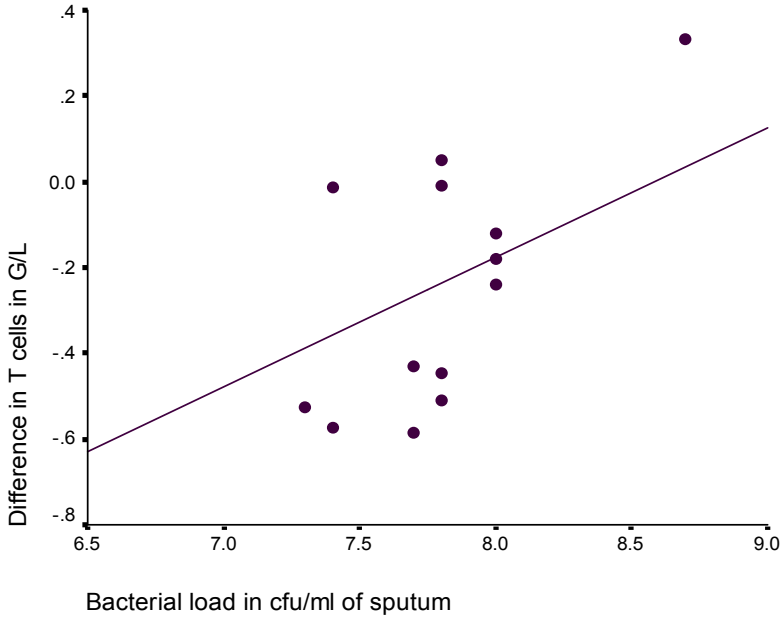
	Mean(SD) at baseline	Mean(SD) at exacerbation	p=
White cells x 10^9/mm^3	7.91(2.61)	10.18(4.00)	**p= 0.043**
Neutrophils x 10^9/L	5.23(2.16)	7.67(4.42)	**p=0.030**
Eosinophils x 10^9/L	0.26(0.22)	0.26(0.21)	p=1.00
Basophils x 10^9/L	0.08(0.07)	0.05(0.06)	p=0.170
Monocytes x 10^9/L	0.51(0.16)	0.69(0.32)	**p=0.016**
Lymphocytes x 10^9/L	1.81(0.61)	1.52(0.52)	**p=0.004**
B cells x 10^9/L	0.16(0.08)	0.13(0.10)	p=0.138
NK cells x 10^9/L	0.19(0.14)	0.22(0.14)	p=0.184
CD3 cells x 10^9/L	1.33(0.61)	1.07(0.49)	**p= 0.001**
CD 4 cells x 10^9/L	0.80(0.27)	0.63(0.26)	**p= 0.002**
CD 8 cells x 10^9/L	0.56(0.50)	0.46(0.39)	**p= 0.008**
CD4/CD8 ratio	2.11(1.270	2.01(1.30)	p=0.49
Bacterial load log cfu	7.60(0.63)	7.83(0.67)	p= 0.167

4.4.6 Relationship between baseline bacterial load and changes in leucocytes at exacerbation

The intensity of the change in the circulating total lymphocytes (r=0.63, p=0.016), T cells (r=0.64, p=0.013) and CD8 cells (r=0.61, p=0.019) at exacerbation was related

to the baseline bacterial load. The relationship between baseline bacterial load and the difference in T cells is illustrated in **Figure 23.** The changes in white cell count, neutrophil count, lymphocyte count (including lymphocyte subsets) between the stable state and exacerbation were not related to the baseline lung function.

Figure 23: Relationship between the change in circulating T cells at exacerbation and baseline bacterial load.



4.5 Discussion

This study was performed in a group of patients with well characterized COPD. When sampled in the stable state we demonstrate: 1) a relationship between the number of circulating neutrophils, B cells and the severity of COPD, 2) a relationship between the severity of the airway obstruction and the number of peripheral leucocytes, 3) a relationship between the airway bacterial load and the number of peripheral leucocytes. At exacerbation we demonstrate that the change in circulating lymphocytes and in-particular T lymphocytes is related to the baseline airway bacterial load.

Our data show that a higher neutrophil count is associated with a lower % FEV_1 and more severe airway obstruction. We also show that the severity of airway obstruction is inversely related to circulating leucocytes. These finding were previously described in epidemiological studies conducted in the general population (Weis et al 1995, James et al 1999). A recent observational study in a large cohort of COPD patients shows that a higher neutrophil count is related to more frequent exacerbations (Hurst et al 2010). The mechanism leading to an increased number of circulating leucocytes and neutrophils in COPD patients is unknown. One can speculate that the increased production of neutrophil chemo-attractants such as IL-8 and LTB-4 in the airways of COPD patients attracts the circulating neutrophils into the lungs and therefore stimulates the bone marrow to produce more cells to maintain the peripheral homeostasis. The increased T cells present in COPD airways can secrete GM-CSF which can in turn directly stimulate the bone marrow (Quint et al 2001). The presence of bacteria in the lower airway could also be the stimulus for a higher circulating leucocyte and neutrophil count.

Our finding of a lower circulating monocyte count in active smokers with COPD is in keeping with a previous study describing reduced levels of circulating monocytes in active smokers (Jensen et al 1998). Active smokers have higher numbers of airway macrophages; these secrete chemotactic agents stimulating the circulating monocytes to migrate into the lungs. In contrast to what is observed in the lungs, we found no significant relationship between the circulating lymphocytes subsets and the lung function indices.

To our knowledge this is the first study examining the relationship between circulating leucocytes and lower airway bacterial load in patients with COPD. Our data shows that the lower airway bacterial load is inversely related to the number of circulating leucocytes, neutrophils, lymphocytes and total T cells. These findings may reflect increased migration of these cells due to the presence of bacteria in the lungs of these patients. A study describing the morphological changes present in the small airways of COPD patients found that progression of disease is associated with a higher percentage of airways containing neutrophils, macrophages, CD4, CD8 cells and the apparition of lymphoid follicles with germinating centres (Hogg et al 2004). The authors speculated that bacterial colonization may lead to an adaptive immune response of the lungs and could explain the presence of the lymphoid follicles in patients with GOLD stage 3 and 4 disease. Our finding of a higher percentage of circulating B cells in patients with a higher airway bacterial load would also be in keeping with the above hypothesis.

T cells play a key role in orchestrating the immune response against bacteria and viruses. They are known to circulate between the airways, lung parenchyma, secondary lymphoid organs and the peripheral circulation as demonstrated in animal models (Lehmann et al 2001). The role of T cells in the COPD lung was known since the late nineties (O'Shaugnessy et al 1997, Saetta et al. 1998). The studies examining circulating T cells were summarized in Chapter 1. Studies comparing peripheral blood and BAL fluid showed higher numbers of T cells in the BAL fluid than in peripheral blood. Circulating T cells of smokers express $CXCR_3$ receptor more often than T cells of non-smokers and activation of $CXCR_3$ is known to promote migration of T cells into the lungs. Our data shows that a higher bacterial load is associated with a lower number of circulating lymphocytes and T cells. Again the lower airway bacteria could be the stimulus for the increased migration of T cells into the lungs.

The patients were prospectively followed until they experienced an exacerbation. At exacerbation we show a statistically significant rise in circulating white cells, neutrophils and monocytes. This may be related to decreased apoptosis of circulating neutrophils at exacerbation of COPD. In a study of patients hospitalized with COPD exacerbations reduced spontaneous apoptosis of the circulating neutrophils were observed when patients were compared with healthy controls. Furthermore when the

patient's admission sample neutrophil count was compared to their discharge sample neutrophil count, apoptosis was reduced only in the initial sample. (Peltz et al 2004). The other mechanism would be increased production of neutrophils by the bone marrow in response to the acute stress of the exacerbation.

We found a reduction in the circulating lymphocytes, T cells and CD8 cells at exacerbation. This could be due migration of these cells into the lungs or to trapping of lymphocytes in the secondary lymphoid organs. This drop is proportional to the baseline bacterial load. Patients with a higher number of bacteria in the lower airways have a smaller drop. It is possible that the stimulation level of T cells is already maximum in colonized patients and is not able to increase further. Or these patients may have a defect of the adaptive immune response that permits the lower airway colonization in COPD.

Our group and others have previously reported that the lower airway bacterial load is associated with greater airway inflammation (increased sputum IL-8 and IL6), faster decline in lung function and more frequent exacerbations. In the present study we demonstrate that the lower airway bacterial load is inversely related to the circulating leucocyte count, circulating neutrophil count and T cell count. The presence of bacteria in the lower airways may act as a stimulus for increased migration of inflammatory cells into the lung. The increased production of various cytokines by these inflammatory cells could lead to increased lung damage. This in turn could lead to a faster decline in lung function.

This study has several limitations. We did not have a group of smokers without COPD as controls. However, several of the studies presented in Chapter 1 did include control groups of smokers without COPD; they did not find any significant difference in the circulating T cell subsets between the two groups. We did not evaluate the airway leucocyte subsets in our patients. Finally we did multiple correlations and did not correct for them. Our data is hypothesis generating rather than a proof of concept.

4.6 Conclusion

In summary we demonstrate a new relationship between the number of circulating white cells, neutrophils and T cells and the number of bacteria present in the lower airways of patients with stable COPD. We also report the changes occurring to the different circulating white cells subsets at exacerbation.