The innate immune response of large and small airway epithelium to respiratory pathogens in Chronic Obstructive Pulmonary Disease

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Abstract

Chronic obstructive pulmonary disease (COPD) is a smoking-related inflammatory lung disease in which small airways remodelling contributes to loss of lung function. Bacterial colonisation in clinically stable COPD patients may play a role in persisting inflammation despite smoking cessation. I propose that the innate immune response from small airway epithelium is more potent than from large airway epithelium, an effect which may be dampened by cigarette smoke, favouring persistent bacterial colonisation in COPD.

Nineteen patients with COPD and eleven healthy volunteers were recruited for investigation. Bronchoalveolar lavage (BAL) differential cell counts and cytokine levels were analysed. Standard BAL culture and culture-independent bacterial DNA analysis were performed. Submerged cultures of epithelial cells from large (LAEC) and small (SAEC) airway were established. Pro-inflammatory cytokine release in response to cigarette smoke extract (CSE) and non-typeable *Haemophilus influenzae* whole cell lysate (HI) was investigated.

Increased neutrophil % and cytokine levels (IL-8, IL-6, IL-1β) were detected in COPD BAL samples compared to controls. Culture-independent microbiological assessment demonstrated bacterial DNA in BAL samples from both COPD patients and controls: the diversity of bacterial species identified was significantly less in COPD samples. Primary airway epithelial cell cultures were successfully established from 16 COPD patients and 10 healthy controls. LAEC and SAEC from COPD patients and controls demonstrated an increase in IL-8 release in response to combined CSE (5%) and HI. This effect was greater in SAEC, but no significant difference was observed between disease and control cell responses. The same pattern was observed for IL-6 release. Corticosteroid pre-treatment (1nM Dexamethasone or 17-Beclomethasone monopropionate) did not suppress the cellular responses observed.

Differences in large and small airway innate immune responses may be important in COPD pathogenesis. This is important to consider in modelling the disease *in-vitro* and in the development of new therapeutic targets.

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Table of Contents

Abstract i

Acknowledgements ii

Abbreviations x

List of Figures xii

List of Tables xv

Chapter 1. Introduction 1

1.1 Background 1

1.2 The pathophysiology of COPD 1

1.3 Effects of cigarette smoking 4

1.4 Infection in COPD 6

1.5 The lung microbiome 8

1.6 The role of infection in COPD pathogenesis 9

1.7 The airway epithelium 10

1.8 Airway epithelial cell cross-talk 11

1.9 Effects of cigarette smoke on the airway epithelium 13

1.9.1 Research using lung tissue and cells isolated from COPD patients 13

1.9.2 Research using *in-vitro* airway epithelial cell culture 14

1.10 Toll-like receptors 19

1.11 Toll-like receptors and the airway epithelial response to infection 21

1.11.1 TLR2 21

1.11.2 TLR3 21

1.11.3 TLR4 21

1.11.4 TLR5 23

1.11.5 Toll-like receptors and EGFR activation 23

1.12 Toll-like receptors in COPD 23

1.13 Effects of cigarette smoke on the immune response 25

1.14 *In-vitro* cell culture models of the airway epithelium 26

1.15 Current therapeutic strategies in COPD 28

1.16 Issues with *in-vitro* modelling of the COPD disease process 31

1.17 Unanswered questions 32
1.18 Hypothesis 34
1.19 Aims 34

Chapter 2. Study protocol, recruitment and sampling 35

2.1 Details from study protocol 35
2.2 Inclusion and exclusion criteria 37
2.3 Study procedure 37
2.3.1 Visit 0 37
2.3.2 Visit 1 38
2.3.3 Study samples 38
2.4 Sample processing and analysis 38
2.4.1 Urine samples 38
2.4.2 Blood samples 39
2.4.3 Sputum samples 39
2.4.4 Bronchoalveolar lavage (BAL) 39
2.4.5 Nasal, large and small airway epithelial cell culture 39
2.4.6 Endobronchial biopsies 39
2.5 Culture-independent microbiology analysis of BAL samples 41
2.6 Brushings sampling 43

Chapter 3. Materials and Methods 45

3.1 Cell types 45
3.1.1 Cell lines 45
3.1.2 Primary cells 45
3.2 Cell culture media 46
3.3 Antibodies and proteins 47
3.3.1 Immunocytochemistry 47
3.3.2 Western blot 48
3.4 PCR primers 48
3.5 Cell culture 49
3.5.1 Adherent cell culture (HBE, BEAS-2B) 49
3.5.2 Non-adherent cell culture (THP-1) 49

3.5.3 Primary cell culture 49
3.6 Cigarette smoke extract preparation 50
3.7 Bacterial whole cell lysate preparation 50
3.8 Cell treatments 51
3.8.1 Cell viability 51
3.8.2 Cell proliferation 51
3.8.3 Assessment of cytokine release 51
3.8.4 Corticosteroid treatments 52
3.8.5 Primary airway epithelial cell characterisation 53
3.9 FACS (fluorescence activated cell sorting) analysis of cell viability 54
3.10 Assessment of cell proliferation by BrdU incorporation 54
3.11 Haematoxylin and Eosin (H&E) staining 55
3.12 Immunocytochemistry (ICC) 55
3.13 RNA extraction 56
3.14 Reverse Transcription Polymerase Chain Reaction (RT-PCR) 56
3.15 Real time PCR 57
3.15.1 Primer assessment 57
3.15.2 Choice of housekeeping gene 59
3.16 Protein assay 60
3.17 Western blotting 60
3.18 Enzyme linked immunosorbent assay (ELISA) 62
3.19 Meso Scale Discovery assay cytokine analysis 63
3.20 Statistical analysis of results 64

Chapter 4. Assessment of airway and systemic inflammatory indices, and bacterial airway colonisation in samples from COPD and healthy control donors 65

4.1 Introduction 65
4.2 Study screening and recruitment outcomes 66
4.3 Study participant demographics 69
4.4 Complications and adverse events 74
4.5 Details of bronchoalveolar lavage processing 75

4.6 Review of bronchoalveolar lavage, full blood count and microbiology results 78
4.7 Bronchoalveolar lavage and serum cytokine analysis 81
4.7.1 Detection of pro-inflammatory cytokines in bronchoalveolar lavage 81
4.7.2 Serum results 86
4.8 Using BAL pilot data to determine sample size required for future research 89
4.9 Culture-independent microbiology analysis 91
4.9.1 Bacterial DNA identified in BAL samples from COPD and control donors 91
4.10 Comparison of results from standard culture and culture-independent microbiology analysis 97
4.11 The diversity of bacterial taxa identified and COPD and control BAL samples 99
4.12 Discussion 102
4.12.1 Summary of the data presented 102
4.12.2 Characteristics of COPD BAL and serum compared to control samples 103
4.12.3 Standard versus culture-independent microbiology analysis 103
4.12.4 Review of study recruitment and participant demographics 107
4.12.5 Further consideration of the research approach used 109
Chapter 5. Primary airway epithelial cell culture and characterisation 113
5.1 Introduction 113
5.2 Establishment of cell cultures 114
5.3 Primary airway epithelial cell culture and passage 117
5.4 Airway epithelial cell marker expression 122
5.5 Clara cell secretory protein (CCSP) expression 126
5.6 Primary airway epithelial cell Toll-like receptor expression 129
5.7 Discussion 131
5.7.1 Summary of data presented 131
5.7.2 Success of primary airway epithelial cell culture 131
5.7.3 Airway epithelial cell characterisation 132

5.7.4 Toll-like receptor expression in cultured airway epithelial cells 134

Chapter 6. Development of an *in-vitro* cell culture model to assess the effects of cigarette smoke and bacterial pathogens on innate immune responses in airway epithelial cells 137

6.1 Introduction 137

6.2 Approach to *in-vitro* cell culture work 138

6.3 Airway epithelial cell line culture model results 140

6.3.1 Effect of cigarette smoke extract and bacterial whole cell lysate on cell line viability and proliferation 140

6.3.2 Effect of cigarette smoke extract and bacterial whole cell lysate on pro-inflammatory cytokine release from airway epithelial cell lines 143

6.3.3 Investigation of the effect of corticosteroid treatment on TNF-α induced IL-8 release from BEAS-2B cells 150

6.4 Setting up the primary airway epithelial cell culture model 154

6.4.1 Effect of cigarette smoke extract and *Haemophilus influenzae* whole cell lysate on viability and proliferation in primary airway epithelial cells 155

6.4.2 Effect of *Haemophilus influenzae* whole cell lysate on IL-8 release from primary airway epithelial cells 157

6.4.3 Choice of cell culture conditions and media appropriate to cell type for further assessment of primary airway epithelial cell responses 161

6.5 Effect of cigarette smoke extract and *Haemophilus influenzae* whole cell lysate on IL-8 release from primary airway epithelial cells from healthy control and COPD donors 164

6.6 Effect of cigarette smoke extract and *Haemophilus influenzae* whole cell lysate on other pro-inflammatory cytokine release from primary large and small airway epithelial cells 171

6.6.1 Effect of cigarette smoke extract and *Haemophilus influenzae* whole cell lysate on LAEC and SAEC IL-6 172

6.6.2 Effect of cigarette smoke extract and *Haemophilus influenzae* whole cell lysate on LAEC and SAEC IL-1β 178

6.7 Review of the effect of cigarette smoke extract and *Haemophilus influenzae* whole cell lysate treatments on cytokine release from LAEC and SAEC 182
6.8 Baseline mediator levels in primary airway epithelial cells in culture 183
6.9 Effect of corticosteroid pre-treatment on primary airway epithelial cell responses 185
6.10 Discussion 194
6.10.1 Summary of results presented 194
6.10.2 Cell line findings 195
6.10.3 Primary cell findings 198
6.10.4 Reflection on results observed compared to the original study hypothesis 200
6.10.5 Review of corticosteroid treatment effects 202
6.10.6 Review of the *in-vitro* cell culture model used 203
Chapter 7. Final discussion 208
References 211

Abbreviations

ALI: Air-liquid interface

AM: Alveolar macrophage(s)

BAL: Bronchoalveolar lavage

BDP: Beclomethasone dipropionate

BEGM: Bronchial epithelial cell growth medium

17-BMP: Beclomethasone monopropionate

CAT: COPD assessment test

CCSP: Clara cell secretory protein

COPD: Chronic obstructive pulmonary disease

CSC: Cigarette smoke condensate

CSE: Cigarette smoke extract

CRP: C-reactive protein

DMSO: Dimethyl sulfoxide

$FEF_{25-75\%}$: Forced expiratory flow at 25-75% of forced vital capacity

FEV1: Forced expiratory volume in 1 second

FVC: Forced vital capacity

GOLD: Global Initiative for Lung Disease

HBE: 16HBE14o$^-$ human bronchial epithelial cell line

HDAC: Histone deacetylase

HI: Whole cell lysate preparation of clinical isolate of non-typeable *Haemophilus influenzae*

ICC: Immunocytochemistry

ICS: Inhaled corticosteroid

IHC: Immunohistochemistry

IL: Interleukin

LAEC: Large airway epithelial cell(s)

LLD: Lower limit of detection

LOS: Lipo-oligosaccharide

LPS: Lipopolysaccharide

MSD: Meso Scale Discovery

NAEC: Nasal airway epithelial cell(s)

OTU: Operational taxonomic unit(s)

PA: Whole cell lysate preparation of clinical isolate of *Pseudomonas aeruginosa*

PAMP: Pathogen associated molecular pattern

PBEC: Primary bronchial epithelial cell(s)

PPM: Potentially pathogenic micro-organism(s)

RV: Residual volume

SAEC: Small airway epithelial cell(s)

SAGM: Small airway epithelial cell growth medium

TGF-β: Transforming growth factor beta

TLC: Total lung capacity

TLCO: Transfer factor for carbon monoxide

TLR: Toll-like receptor

TNF-α: Tumour necrosis factor alpha

List of Figures

Figure 1 Pathological changes in the COPD airway 2
Figure 2 Toll-like receptor ligands and signalling pathways 20
Figure 3 Example of small airways brushing imaging performed during bronchoscopy 43
Figure 4 Primer optimisation 58
Figure 5 Choice of housekeeping gene for analysis of mRNA expression 59
Figure 6 Study screening and recruitment summary flow chart 67
Figure 7 Cytokine levels detected in BAL samples from COPD and control donors 83
Figure 8 Levels of pro-inflammatory mediators in BAL samples from COPD and control donors using ex- or never-smokers data only 84
Figure 9 Cytokine levels detected in serum samples from COPD and control donors 87
Figure 10 Serum IL-6 and CRP in samples from COPD donors 88
Figure 11 Detectable difference versus sample size for BAL indices 90
Figure 12 Details of bacterial DNA isolated from BAL samples from COPD donors 93
Figure 13 Details of bacterial DNA isolated from BAL samples from control donors 94
Figure 14 Details of proportions of most common bacterial DNA isolated from BAL samples from COPD and control donors 95
Figure 15 Principle co-ordinates analysis of bacterial OTU data 96
Figure 16 Analysis of bacterial DNA isolated from COPD and control BAL samples 100
Figure 17 Correlations between number of bacterial taxa present and disease severity/FEV1 101
Figure 18 Richness and Diversity by GOLD stage for COPD samples 101
Figure 19 Light microscopy of NAEC at P0 and P1 118
Figure 20 Light microscopy of LAEC at P3, with possible EMT changes 118
Figure 21 Light microscopy images of COPD cells in culture 119
Figure 22 Light microscopy images of healthy control cells in culture 120
Figure 23 H&E staining of COPD and healthy control cells fixed at P2 121
Figure 24 ICC analysis of epithelial cell marker expression in cells at P1 123
Figure 25 ICC analysis of non-epithelial cell marker expression 124
Figure 26 ICC analysis of cell marker expression in cells at P2 125

Figure 27 ICC analysis of CCSP expression in cells at P1 126
Figure 28 Western blot analysis of CCSP expression 127
Figure 29 CCSP mRNA expression in primary airway epithelial cells at P2 128
Figure 30 Baseline TLR expression in primary airway epithelial cells in culture 130
Figure 31 Cell proliferation following CSE exposure in airway epithelial cell lines 141
Figure 32 Effect of HI whole cell lysate preparations on airway epithelial cell line viability 142
Figure 33 HBE responses to CSE and bacterial whole cell lysate preparations 145
Figure 34 THP-1 responses to bacterial whole cell lysate preparations 146
Figure 35 BEAS-2B responses to CSE 148
Figure 36 BEAS-2B responses to HI bacterial whole cell lysates 149
Figure 37 Initial dose response experiments to assess the effect of *in-vitro* corticosteroid treatment on TNF-α induced IL-8 release from BEAS-2B cells 152
Figure 38 Extended dose response experiments to assess the effect of *in-vitro* corticosteroid treatment on TNF-α induced IL-8 release from BEAS-2B cells 153
Figure 39 Primary airway epithelial cell viability following CSE or HI treatment 155
Figure 40 Primary airway epithelial cell proliferation after CSE treatment 156
Figure 41 Healthy control cell responses to HI 159
Figure 42 COPD cell responses to HI 160
Figure 43 Review of LAEC cultured in different media types and IL-8 release in response to HI 162
Figure 44 Effect of culture media supplementation on observed cellular responses 163
Figure 45 Healthy control cell IL-8 release in response to CSE and HI 168
Figure 46 COPD cell IL-8 release in response to CSE and HI 169
Figure 47 Bland and Altman plot review of the effect of HI + 5% CSE on LAEC and SAEC IL-8 170
Figure 48 Healthy control cell IL-6 release in response to CSE and HI 174
Figure 49 Healthy control donor 4 LAEC and SAEC IL-6 raw data 175
Figure 50 COPD cell IL-6 release in response to CSE and HI 176
Figure 51 Bland and Altman plot review of the effect of HI and 5% CSE on LAEC and SAEC IL-6 177

Figure 52 Healthy control cell IL-1β release in response to CSE and HI 180
Figure 53 COPD cell IL-1β release in response to CSE and HI 181
Figure 54 The effect of combined CSE and HI on LAEC and SAEC IL-8 and IL-6 release 182
Figure 55 Baseline mediator release adjusted for cell counts 184
Figure 56 Healthy control cell corticosteroid pre-treatment results 187
Figure 57 Healthy control cell corticosteroid pre-treatment results - TNF-α controls 188
Figure 58 COPD cell corticosteroid pre-treatment results 189
Figure 59 COPD cell corticosteroid pre-treatment results - TNF-α controls 190
Figure 60 Dose response experiments to assess the effect of *in-vitro* corticosteroid treatment on TNF-α induced IL-8 release from healthy control LAEC and SAEC 191
Figure 61 Dose response experiments to assess the effect of *in-vitro* corticosteroid treatment on TNF-α induced IL-8 release from COPD LAEC and SAEC 192
Figure 62 Review of the effect of corticosteroid treatment on TNF-α induced IL-8 release from BEAS-2B cells 193

List of Tables

Table 1 Study inclusion and exclusion criteria 37
Table 2 Study samples to be collected at Visit 1 38
Table 3 Details of bronchoalveolar lavage processing 40
Table 4 Supplements added to SAGM and BEGM media 47
Table 5 Primary antibodies 47
Table 6 Isotype control antibodies 47
Table 7 Secondary antibodies 48
Table 8 Antibodies and proteins used for Western blot experiments 48
Table 9 PCR primers 48
Table 10 Acrylamide gel preparation (16%) 61
Table 11 Antibodies and reagents used for ELISA 62
Table 12 Reasons for potential participant ineligibility for study 68
Table 13 Study subject demographics and clinical characteristics - COPD patients 71
Table 14 Study subject demographics and clinical characteristics - Healthy volunteers 72
Table 15 GOLD criteria for assessment of severity of airflow obstruction 73
Table 16 Medical Research Council (MRC) dyspnoea scale 73
Table 17 BAL (and sputum) sample results - COPD patients 76
Table 18 BAL (and sputum) sample results - Healthy controls 77
Table 19 Details of BAL differential cell counts 79
Table 20 BAL Ex-smokers data only 79
Table 21 BAL COPD current versus ex-smokers data 79
Table 22 BAL COPD data for positive and negative microbiology samples 80
Table 23 BAL cytokine data (MSD ultrasensitive 7-plex assay results) 83
Table 24 BAL cytokine data: Ex- or never-smokers only 84
Table 25 BAL cytokine data - COPD current and ex-smokers 85
Table 26 BAL and serum cytokine data - COPD positive and negative microbiology 85
Table 27 Serum cytokine data (MSD ultrasensitive 7-plex assay results) 87
Table 28 BAL IL-8 sample size calculation results 90
Table 29 Comparison of standard and culture-independent microbiology results 98

Table 30 Details of primary airway epithelial cell cultures established from brushings from COPD subjects 115
Table 31 Details of primary airway epithelial cell cultures established from brushings from healthy control subjects 116
Table 32 Baseline healthy control cell IL-8 levels 159
Table 33 Baseline COPD cell IL-8 levels 160
Table 34 Baseline IL-8 levels in LAEC cultured in different media types 162
Table 35 Data for healthy control donors for *in-vitro* cell culture experiments 165
Table 36 Data for COPD donors for *in-vitro* cell culture experiments 165
Table 37 Values for lower limit of detection of MSD 4-plex Tissue Culture plates 171
Table 38 IL-1β levels detectable using MSD 4-plex assay in cell culture media samples 178
Table 39 Mean baseline mediator release adjusted for cell counts 184

Chapter 1. Introduction

1.1 Background

Chronic obstructive pulmonary disease (COPD) is an inflammatory lung disease which is most often related to cigarette smoking (American Thoracic Society, 1996). It occurs in 1 in 5 smokers, and is associated with loss of lung function which continues despite smoking cessation (Curtis *et al.*, 2007). COPD affects more than 3 million people in England and Wales (Department of Health, 2010), and causes more than 25,000 deaths per year (UK National Statistics). It is estimated that COPD will become the third leading cause of death worldwide by 2020 (Barnes, 2007).

Affected patients suffer from chest symptoms including cough and shortness of breath which have a significant impact on quality of life. Symptomatic disease exacerbations, often caused by viral or bacterial infection, further increase morbidity and mortality (Seemungal et al., 1998, Soler-Cataluna et al., 2005). Over time the disease process results in progressive loss of lung function, measured as decline in FEV1 (forced expiratory volume in 1 second) more accelerated than related to the normal ageing process (Curtis et al., 2007). Increasingly, bacterial colonisation is being recognised as an important driver of inflammation and exacerbations, and this may also contribute to disease progression (Hill et al., 2000, Donaldson et al., 2002).

In terms of disease management, smoking cessation is essential. However, loss of lung function continues despite this. Current therapies provide symptomatic relief but have little impact on disease progression. A translational gap exists in the development of new treatments for COPD; new targets need to be identified, validated and proof of concept demonstrated. Improved understanding of the disease process to allow development of novel treatments is urgently needed.

1.2 The pathophysiology of COPD

COPD affects the airways and lung parenchyma (see Figure 1). The airways become narrowed due to airway wall thickening in all components (Hogg et al., 2004). Squamous metaplasia and increase in goblet cell numbers contribute to epithelial thickening, and excessive mucus production results in mucus plugging. This causes the

"chronic bronchitis" phenotype with chronic productive cough. Peribronchial thickening as a result of collagen deposition and fibrosis is observed. Destruction of the lung parenchyma results in emphysema which is predominantly centri-lobular in distribution; there is loss of alveoli, the gas exchanging surface of the lung, and degradation of elastin fibres results in loss of tissue architecture. This contributes to air trapping due to airway collapse, which may be worse during expiration, and air trapping in bullae (Sturton et al., 2008). COPD is a heterogeneous disease and pathological changes observed vary both within and between affected individuals (Thurlbeck, 1990).

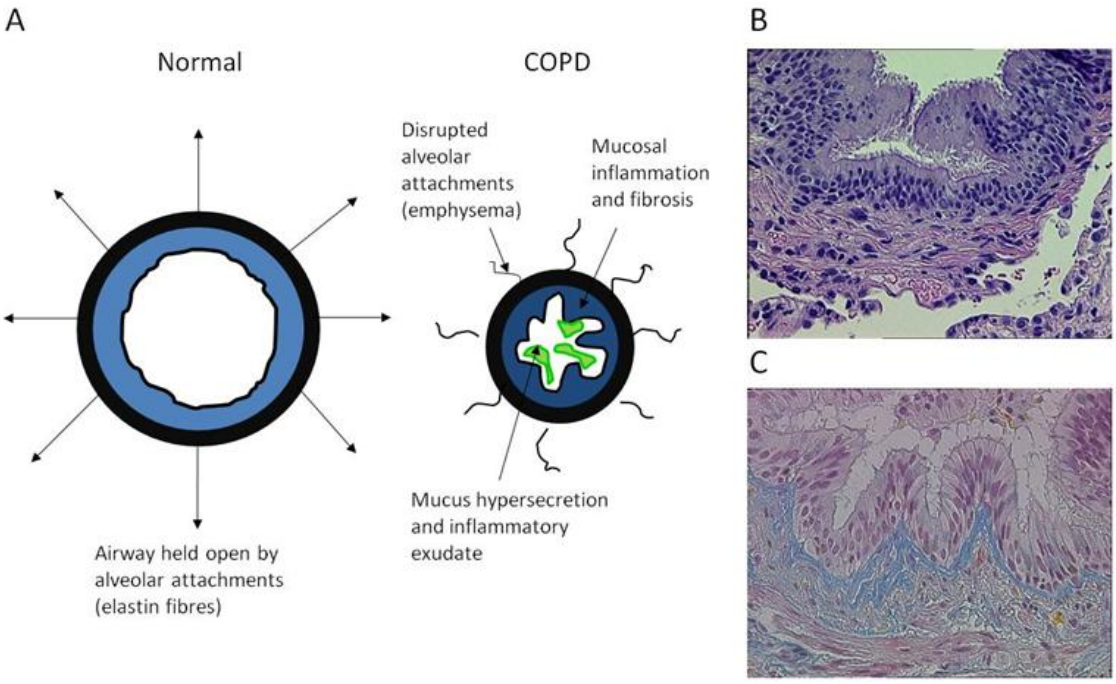


Figure 1 Pathological changes in the COPD airway

Figure (A) (adapted from (Figure 2, page 341, (Sturton et al., 2008)) is a schematic representation of changes in the COPD airway. Airway wall thickening results from epithelial hyperproliferation, collagen deposition in the lamina propria and an increase in smooth muscle in the submucosa. Mucus plugging contributes to airway occlusion. Loss of alveolar attachments contributes to airway collapse. Figure (B) shows an example of epithelial hyperproliferation in a COPD airway (Haematoxylin and Eosin stain). Figure (C) demonstrates new collagen deposition in the sub-epithelial layer in a COPD airway (Martius, Scarlet, Blue stain). Figures (B) and (C) provided by Dr Lee Borthwick (Fibrosis and Repair Group, Institute of Cellular Medicine, Newcastle University, unpublished data).

Although COPD affects the airways and lung parenchyma it is disease in the small airway (those less than 2mm in diameter) which is the main factor in causing airflow limitation (Hogg et al., 1968). In normal lungs the small airways contribute less than a quarter of total lung airflow resistance. However, in diseased lungs a fourfold increase in peripheral resistance has been demonstrated (Hogg et al., 1968). Importantly, in this study total lung resistance was only increased as a result of increased peripheral resistance in lungs showing moderate to severe disease; patients may have significant small airway disease long before becoming symptomatic with airflow limitation (Hogg et al., 1968). More recent work has also suggested that small airways disease may precede emphysematous changes in the lungs (Hogg et al., 2009). Based on histological analysis and micro-CT imaging of lung tissue in COPD it has been suggested that initial collagen deposition in damaged airways leads to bronchiolar narrowing, but as the disease progresses tissue loss and bronchiolar destruction ensues, with later emphysematous destruction (Hogg et al., 2009).

Stratification of patients based on a particular disease phenotype may allow more focused investigation of COPD disease mechanisms and development of more tailored treatment. Assessment of the relative contribution of airway disease, particularly the small airways, and emphysema to the disease process is important to consider in this respect (Stewart and Criner, 2013). The clinical diagnosis of COPD is made on the basis of symptoms, smoking history and evidence of airflow obstruction on spirometry. The extent of underlying emphysema may be reflected in reduction in gas transfer measurements on lung function due to loss of gas exchanging alveolar surfaces. Measurement of small airways function is difficult and there is no gold standard test for assessment of this (Burgel, 2011, Stewart and Criner, 2013).

Small airways disease is characterised by premature airway closure and air trapping; this can be reflected in an increase in residual volume (RV), and further expressed as relative to the total lung capacity (TLC) which is commonly increased in obstructive lung disease, as RV/TLC (Burgel, 2011). The forced expiratory flow at 25-75% of forced vital capacity (FVC) ($FEF_{25-75\%}$) is also used (Burgel, 2011). This measures the flow rate over the mid quartile range of FVC; this excludes the initial expiratory flow peak seen

in the flow-volume loop, and may capture the progressive increase in resistance due to small airways disease as the lung deflates and there is premature airway closure. The $FEF_{25-75\%}$ decreases more steeply than FEV1/FVC ratio with mild obstruction and therefore may be a more sensitive indicator of this. However, significant variability in measurement of this parameter, and previous research showing poor correlation with RV/TLC, raises issues with its use (Burgel, 2011).

"Lung structure-function relationships" have also previously been investigated using nitrogen breath wash out tests (Burgel, 2011). These involve measuring nitrogen concentration following a single inhalation of pure oxygen; multiple tests can also be used to quantify regional heterogeneity in small airways disease. However, use of these tests is currently limited to experienced research centres.

Imaging techniques can also be used to assess the extent of small airways disease and emphysema. Characteristic features of small airways disease on high resolution CT imaging include mosaic lung attenuation (on inspiratory images) and air trapping (on expiratory images) (Burgel, 2011). However, there is no standardised method in use to quantify the extent of the above changes. Development of computer software for quantification, and correlations with lung function parameters, has been proposed (Stewart and Criner, 2013).

1.3 Effects of cigarette smoking

Cigarette smoking is the main causative factor implicated in COPD, but only 1 in 5 smokers actually develop the disease (American Thoracic Society, 1996). This suggests that there may be underlying genetic or epigenetic factors which influence disease susceptibility. Variability in gene expression in small airway epithelial cells (SAEC) isolated from healthy individuals, smokers and patients with COPD has been reported (Ammous et al., 2008, Harvey et al., 2007). Particular genes of interest included anti-oxidant pathway related genes and those encoding cytokines. This heterogeneity in response may partly explain why COPD does not develop in all smokers and could also provide avenues of investigation into why the disease predominates in the small airways.

Cigarette smoke and associated reactive oxygen species production and epithelial damage triggers an influx of immune cells. Increased levels of neutrophils, macrophages, interleukin-8 (IL-8) and interleukin-1β (IL-1β) are found in bronchoalveolar lavage (BAL) from smokers compared to non-smokers in a dose-dependent manner (Kuschner et al., 1996). Increased IL-8 in BAL from smokers compared to non-smokers has been correlated with neutrophil cell count; IL-8 is a known potent neutrophil chemoattractant (Mio et al., 1997). These mediators are considered to be of potential importance in COPD pathogenesis as increased levels have been found in sputum and BAL in patients with COPD compared to smokers with normal lung function and non-smokers by many groups (Keatings et al., 1996, Riise et al., 1995).

Numbers of neutrophils, macrophages and lymphocytes in the airways increase with COPD disease severity (Hogg et al., 2004). In more advanced disease CD8+ cells and B cells predominate and formation of lymphoid follicles is observed. The degree of inflammation as assessed in sputum and BAL is associated with disease progression (Parr et al., 2006). The distribution of inflammatory cells may differ between large and small airways; increased numbers of neutrophils and mast cells have been observed in the lamina propria of small airways, compared to increased CD4+ cell numbers in large airways (Battaglia et al., 2006). This may affect the disease process at different airway levels. Airway remodelling with narrowing of the small airways may arise as a result of a vicious cycle of tissue damage and dysregulated tissue repair with persistent airway inflammation and resultant fibrosis.

The development of emphysema is thought to be related to an imbalance between proteases and anti-proteases produced by the airway epithelium and immune cells (Barnes et al., 2003). The link between a disease process resulting in tissue deposition with fibrosis alongside parenchymal destruction may also be explained by the action of these proteases. Matrix-metalloproteinases (MMP) produced by cells including neutrophils and macrophages cleave the latent form of Transforming growth factor-β (TGF-β), an important pro-fibrotic mediator (Barnes et al., 2003). This growth factor has also been implicated in airway remodelling and fibrosis in COPD in the absence of

inflammation. Increased expression of TGF-β has been demonstrated in the small airways of COPD patients (Takizawa et al., 2001). This correlated with smoking history (pack years) and indices of airway obstruction. In a mouse model, cigarette smoke exposure has been shown to cause an acute increase in growth factor and pro-collagen expression levels that occurred too early to be associated with a significant inflammatory response (Churg et al., 2006). With more prolonged exposure TGF-β expression actually decreased but there was ongoing evidence of downstream signalling. An oxidant-mediated activation of latent TGF-β was proposed.

1.4 Infection in COPD

The natural course of COPD, with a gradual decline in FEV1, is punctuated by exacerbations, many of which may be triggered by infection. Viral and bacterial infections increase airway inflammation in COPD resulting in acute deterioration in lung function and symptoms associated with this (Sapey and Stockley, 2006). Inflammation is increased in bacterial and viral infections compared to non-infective exacerbations (Sethi et al., 2008). Bacteria can be detected in more than half of acute exacerbations (30% bacterial infection, 25% combined bacterial/viral infection) (Papi et al., 2006). Common pathogens include non-typeable *Haemophilus influenzae* (NTHi), *Moraxella catarrhalis*, *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* (Sethi and Murphy, 2001). The frequency and severity of these exacerbations have a significant impact on morbidity (Seemungal et al., 1998). Hospital admissions due to COPD exacerbations generate a massive healthcare burden and the 2014 National COPD audit showed a median length of stay of 4 days and an inpatient mortality of 4.3% (The Royal College of Physicians and The British Thoracic Society, 2015). Mortality is directly related to the severity of the exacerbation (Soler-Cataluna et al., 2005). In addition to causing an acute decline in FEV1, patients who have more frequent exacerbations show accelerated loss of lung function over time compared to those with less frequent episodes (Donaldson et al., 2002). This may be related to the associated inflammation; patients with more frequent exacerbations have been shown to have increased levels of inflammatory cytokines including interleukin-6 (IL-6) at baseline, compared to less frequent exacerbators (Bhowmik et al., 2000).

It is now accepted that even out with these infective episodes, the airway is not a sterile environment, and bacteria may be present in health and disease (Zakharkina et al., 2013). Chronic bacterial colonisation is observed in COPD airways; common exacerbation-causing pathogens such as those listed above are isolated from patients in a stable disease state (Sethi and Murphy, 2001). Colonisation has been associated with current smoking status and severity of airflow obstruction (Zalacain et al., 1999), though other research has found colonisation in patients with less severe disease (Soler et al., 1999). Increasing rates of bacteria detected in patients with more severe COPD may be related to reduced mucociliary clearance in the more damaged airway (Sapey and Stockley, 2006).

The term "colonisation" suggests a merely bystander effect, but increasingly an active role of micro-organisms is supported (Sethi and Murphy, 2001). Potentially pathogenic micro-organisms may contribute to continued inflammation and effects on the innate immune system despite smoking cessation. Bacterial presence can impact on mucus hypersecretion and reduced ciliary beat frequency, and cause epithelial cell damage from adherence (Sapey and Stockley, 2006). The detection of bacteria by standard culture techniques during periods of stability has been associated with increased IL-8, IL-6 and tumour necrosis factor-α (TNF-α) levels in sputum (Zhang et al., 2010), and with neutrophil counts, IL-8 and TNF-α levels in BAL (Soler et al., 1999, Sethi et al., 2006). Chronic bacterial colonisation has been associated with increased inflammatory indices in sputum, related to bacterial strain and bacterial load (Hill et al., 2000). Colonised patients suffer from more frequent exacerbations (Patel et al., 2002). These may be due to symptomatic infection developing as a result of pathogens already present in the stable state. New strain infection has been shown to cause greater inflammation at the time of infection than pre-existing strain or non-infective exacerbations (Sethi et al., 2008). An increase in bacterial load and change in bacterial species present over time has also been associated with more rapid FEV1 decline (Wilkinson et al., 2003).

Patients with COPD report a high prevalence of nasal symptoms (Roberts et al., 2003), and nasal inflammation in addition to bronchial inflammation has been demonstrated

in biopsies from COPD patients (Vachier et al., 2004). Related to this, the relationship between nasal and lower airway colonisation and inflammation has been investigated; lower airway bacterial colonisation has been shown to be related to post-nasal drip and higher nasal bacterial load (Hurst et al., 2005). This suggests that nasal inflammation and colonisation in COPD may also impact on the lower airways and emphasises the importance of considering a united continuous airway, particularly with respect to cigarette smoke and pathogen exposure.

Overall this research suggests a role for chronic bacterial colonisation and associated inflammation in COPD disease progression. Furthermore, difficulty detecting pathogens in sputum and BAL samples may imply that this problem is a much greater issue than currently realised. Not all patients produce sputum and oropharyngeal contamination of both sputum and BAL specimens is problematic (Monso et al., 1995). In addition, the selective nature of culture-dependent techniques may not detect the full spectrum of bacterial diversity present (Rogers et al., 2009).

1.5 The lung microbiome

Culture-independent detection of bacterial presence is now being used to further characterise the microbiome of chronic respiratory diseases (Rogers et al., 2015). Amplification of the highly conserved 16S rRNA gene in the bacterial genome, followed by sequencing of the products can be utilised to allow detection of the diverse range of species present in airway samples. High throughput sequencing platforms are now being used for this.

Research to date has provided details of the microbiome present in never smokers, healthy smokers (with normal spirometry), and from subjects with COPD, through analysis of BAL samples (Erb-Downward et al., 2011). Erb-Downward *et al* found no overall significant differences in bacterial load and described a core microbiome with species including *Pseudomonas*, *Streptococcus*, *Prevotella*, Fusobacterium, *Haemophilus*, *Veillonella*, and *Porphyromonas*. In more severe COPD a reduction in bacterial diversity has been reported, and this has been associated with the presence of *Pseudomonas* (Erb-Downward et al., 2011, Zakharkina et al., 2013).

However a further study found an apparent increase in diversity in moderate and severe COPD patients, though this was shown to be influenced by age in the groups studied (Pragman et al., 2012). Separation of disease and control groups was found with clustering related to inhaled treatment but not disease severity. This group suggested that possibly only a minority of COPD patients exhibit low bacterial diversity which may skew results in studies with smaller sample sizes. Heterogeneity of findings has also been observed between samples from the same individual depending on source (upper versus lower airway *i.e.* sputum/BAL, or biopsy site in explanted lung) (Cabrera-Rubio et al., 2012, Erb-Downward et al., 2011). A greater understanding of the significance of the results from this work, in the context of disease activity and progression, and also management, particularly antibiotic treatment, is still required.

1.6 The role of infection in COPD pathogenesis

The exact contribution of infection to the disease process in COPD is yet to be fully elucidated. Dysregulation of the immune response initiated by exposure to cigarette smoke may lead to subsequent impairment of pathogen killing, and along with the structural abnormalities in COPD this may set up a niche for pathogen persistence. Certainly, smokers are known to be more at risk of upper and lower respiratory tract infections (Sopori, 2002). A "vicious cycle hypothesis" was previously proposed (Sethi and Murphy, 2001); in health the airway is "sterile"; smoking impairs mucociliary clearance and innate immune function, promoting pathogen invasion. Infection then contributes to further airway injury and disruption of mucociliary function, with excessive mucus production, and pathogen colonisation may result. On the other hand, an over-exuberant response may contribute to tissue damage and could promote dysregulated tissue repair creating an environment more favourable for pathogen invasion. Bacterial adherence may be increased during epithelial repair due to expression of different surface receptors or altered access to these in poorly differentiated cells (Puchelle et al., 2006).

1.7 The airway epithelium

The airway epithelium provides a continuous physical barrier from the nose and upper airway to the gas exchanging surface of the alveoli. The respiratory epithelium of the nasal and large airway mucosa consists of pseudostratified ciliated columnar epithelial cells with frequent mucus producing goblet cells, and basal cells (Jeffrey and Li, 1997). The mucociliary escalator provides important defence against inhaled noxious particles including pathogens. As the large airways branch, goblet cells are increasingly replaced by Clara cells, which are most predominant in the smaller airways. Clara cells express Clara cell secretory protein (CCSP), the exact function of which is not clear but it may have an anti-inflammatory role in regulating inflammation (Singh and Katyal, 1997).

The airway epithelium is not merely a simple barrier. It senses the lung microenvironment and responds to this, communicating with neighbouring cells and initiating changes in an autocrine and paracrine fashion (Bals and Hiemstra, 2004). Airway epithelial cells express Toll-like receptors (TLR) (Greene and McElvaney, 2005) (see Figure 2). TLRs are germ line encoded receptors which respond to conserved molecular signatures on invading pathogens, so called “pathogen associated molecular patterns” (PAMPs) (Medzhitov, 2001). These receptors may also be activated by “damage associated molecular patterns” (DAMPs) including substances which are normally intracellular and if found outside the cell indicate disruption of cellular integrity (*e.g.* heat shock proteins and high mobility group box protein 1). In response to signals from the microenvironment airway epithelial cells can release a number of different chemokines, such as IL-8, and anti-microbial peptides, such as human β defensin-2 (HBD2). Thus stimulation of epithelial cells as the first line in pulmonary innate immune defence can trigger an influx of immune cells and an appropriate inflammatory response if the epithelial barrier is breached.

1.8 Airway epithelial cell cross-talk

Communication with other cells in the lung microenvironment is essential in order to mount an effective immune response. This cross-talk and the other cells involved are therefore also important to consider in disease pathogenesis. Alveolar macrophages (AM) are key innate immune cells with an important role in pathogen recognition, phagocytosis and antigen presentation (Barnes, 2004). AM numbers are increased in the airways and BAL in COPD (Barnes, 2004), but their phagocytic ability may actually be reduced (Hodge et al., 2007). AM express TLRs but reduced TLR2 expression in AM from smokers and COPD patients has been reported (Droemann et al., 2005). These features may both contribute to susceptibility to infection. Cigarette smoke has also been shown to alter AM pro-inflammatory mediator release, but there is no overall consensus as to the effect on immune function (Smith et al., 2010).

Paracrine signals between the epithelium and AM are an important component of the pulmonary innate immune response to fight infection. A study using primary type II alveolar epithelial cells (ATII) and primary lung macrophages showed that ATII cells produced high levels of monocyte chemoattractant protein-1 (MCP-1), IL-8 and growth-related oncogene-α (GRO-α) in response to LPS, whereas macrophages produced greater levels of TNF-α and IL-1β (Thorley et al., 2007). These findings suggest that chemokines produced by the airway epithelium attract immune cells and epithelial activation is then further increased by pro-inflammatory mediators produced by these cells (Thorley et al., 2007).

In terms of the importance of small airway epithelial changes in COPD, interestingly Clara cells have specifically been shown to modulate AM responses in a mouse model *in-vivo* (Snyder et al., 2010). Increased TLR4 expression in AM from CCSP-/- mice and increased LPS-stimulated TNF-α production from these cells was observed, suggesting that Clara cells may normally attenuate these effects.

Fibroblasts are activated by pro-inflammatory mediators including IL-1 and TNF-α *in-vitro* (Elias et al., 1990), and therefore may also respond to production of these mediators by AM like the airway epithelium. Activated fibroblasts also produce

mediators including IL-6 (Elias et al., 1990). Fibroblasts also express TLRs and can respond to PAMPs and DAMPs; local pathogen invasion and tissue injury will activate fibroblasts to aid tissue repair (Wynn, 2008). However, this may become dysregulated in diseases such as COPD with evidence of peribronchial fibrosis. In addition to inflammatory mediators, growth factors including TGF-β activate fibroblasts. TGF-β stimulation of myofibroblasts can further increase collagen production, and this may continue once the growth factor stimulus is removed (Phan, 2008). These features of fibroblast activation are also important to consider in COPD, especially as epithelial cells can produce TGF-β and increased TGF-β expression has been found in the airway of COPD patients (Takizawa et al., 2001). Cigarette smoke may also directly modulate fibroblast function; studies of the effect on MMP production have however provided conflicting results with both increased (Ning et al., 2007), and decreased (La Rocca et al., 2007) production described.

Dendritic cells (DC) are present in the airway epithelium and submucosa throughout the respiratory tract (Stumbles et al., 2003). These cells are an important link between innate and adaptive immunity. They act as "sentinels", with the ability to take up and process inhaled antigens, followed by migration to draining lymph nodes for antigen presentation to naive T cells. Key to the interaction with T cells are associated DC signals which influence the outcome of the interaction in terms of tolerance to the antigen versus an immune response (Stumbles et al., 2003). Signals from the airway epithelium may influence DC activity; differential expression of immune surveillance genes has been reported in monocyte-derived DC (MDDC) co-cultured with airway epithelial cells (AEC); these "AEC-conditioned DC" were shown to upregulate Th1 chemokines with minimal change in Th2 chemokines, compared to MDDC cultured in isolation (Rate et al., 2012). Epithelial cells have also been shown to inhibit secretion of the pro-inflammatory cytokines TNF-α and IL-12 by monocytes, macrophages and DC in culture, by transfer of epithelial cell conditioned media (ECCM) from BEAS-2B cells (an airway epithelial cell line) (Mayer et al., 2008). In addition DC cultured in the presence of ECCM showed features of alternative activation with expression of IL-10 and arginase-1 (Mayer et al., 2008). These findings suggest that the airway epithelium

can modulate the activity of innate immune cells and this may help to maintain homeostasis in the local microenvironment.

1.9 Effects of cigarette smoke on the airway epithelium

As outlined above, in COPD a number of changes in the epithelium are observed which may be a direct response to chronic cigarette smoke exposure. The airway epithelium is thought to contribute a significant amount to the increased levels of inflammatory mediators present in sputum and BAL in smokers and in COPD patients.

1.9.1 Research using lung tissue and cells isolated from COPD patients

In-vitro, primary bronchial epithelial cells (PBEC) express and constitutively release baseline levels of IL-8, IL-6 and granulocyte-colony stimulating factor (GM-CSF) (Cromwell et al., 1992). Release of these mediators is further increased by TNF-α or IL-1β (Cromwell et al., 1992). PBEC isolated from patients with COPD have shown increased constitutive and TNF-α induced IL-8 expression which was not seen in cells from smokers with normal lung function and healthy controls (Schulz et al., 2003). Further work in this area later showed increased TNF-α induced IL-8 and GRO-α release from COPD PBEC compared to normal smokers, with no difference in TNF-α receptor subtype expression, suggesting an alternative mechanism of increased PBEC activation in COPD (Schulz et al., 2004). Further *in-vitro* culture work using differentiated air-liquid interface cultures has also reported increased constitutive IL-8 and IL-6 release from COPD PBECs compared to cells from smokers without evidence of airflow obstruction or healthy controls (Comer et al., 2013).

Analysis of peripheral lung tissue from COPD patients showed increased MCP-1 and IL-8 expression in bronchial epithelium compared to tissue from patients without airflow obstruction (de Boer et al., 2000). The increased MCP-1 expression correlated with increased numbers of intra-epithelial macrophages and mast cells, and CCR2 (the receptor for MCP-1) expression on these cells, suggesting an important role for MCP-1 in immune cell recruitment in COPD. Similar results have been found using laser micro-dissection techniques to isolate bronchiolar epithelial cells and macrophages from peripheral lung tissue (Fuke et al., 2004). Higher levels of IL-8, macrophage inflammatory protein-1α and MCP-1 were demonstrated in bronchiolar epithelial cells

from tissue of patients with mild COPD compared to levels in tissue from smoking and non-smoking controls. Increased CCR2 expression was also noted in bronchiolar epithelium, suggesting autocrine in addition to paracrine effects of the released mediators.

As discussed briefly above, microarray investigation has demonstrated differences in small airway epithelial gene expression profiles in smokers compared to non-smokers, and with greater variability observed in smokers with COPD compared to smokers without airflow obstruction (Ammous et al., 2008). This group described "smoking responsive genes" which were up- or down-regulated in smokers, in functional categories including anti-oxidant pathways. Increased IL-8 and intercellular adhesion molecule-1 (ICAM-1) expression and release has been demonstrated in SAEC isolated from smokers compared to non-smokers (Takizawa et al., 2000). No difference in expression was found when examining large airway epithelial cells between these groups in this study. This suggests that smoking may have differential effects on the epithelium depending on airway generation.

1.9.2 Research using in-vitro airway epithelial cell culture

Research has been undertaken to try to understand epithelial cell responses to cigarette smoke in elucidating underlying COPD disease mechanisms. One puff of cigarette smoke can contain up to $5x10^{14}$ free radicals (Pryor et al., 1983), a significant oxidant burden. More than 4700 chemical compounds have been identified in cigarette smoke including formaldehyde, acetaldehyde, acrolein, benzene and hydrogen cyanide (these are semi-volatile and vaporise easily), and polyaromatic hydrocarbons (in the particulate phase) (Thielen et al., 2008). In order to model exposure to cigarette smoke *in-vitro* a number of different methods can be employed (Clunes et al., 2008). Cigarette smoke extract (CSE) is produced by bubbling smoke through culture media using a vacuum pump system to achieve an aqueous solution of the water soluble gas and particulate phase matter produced. The use of CSE has been suggested to be relevant to *in-vivo* smoke exposure, as it is the soluble components of cigarette smoke which can pass through the aqueous mucus layer to contact the airway epithelium (Krimmer and Oliver, 2011). Cigarette smoke condensate (CSC) can be generated by machine-smoking cigarettes onto filter pads (Cambridge Filter pad,

which retains 99.9% of all particulate matter greater than 0.1 µm), followed by dimethyl sulfoxide (DMSO) extraction. These methods principally capture the water and lipid soluble particulate phase of whole cigarette smoke (Clunes et al., 2008); volatile and rapidly reactive components of smoke may be lost in these preparations (Rennard, 2004). The gaseous phase makes up 95% of whole cigarette smoke by weight (Clunes et al., 2008). Whole cigarette smoke can also be used in *in-vitro* model systems using a smoking machine and exposure chamber set up (Thorne and Adamson, 2013); this has been suggested as a method to allow consideration of both particulate and vapour phases combined. However, determination of the exact cellular exposure and measurement of "smoke dose" remains challenging.

The smoke exposure models described are usually reported to be standardised by use of the same reproducible method. Comer *et al* describe measurement of optical density (OD) of CSE at 450nm, which was found to be constant across CSE preparations using the same method (25ml of media and a single cigarette) (Comer et al., 2014). Laan *et al* also describe consistent OD results in CSE preparations, and use of "bioassays" to assess the activation of macrophage-like THP-1 cells (Laan et al., 2004). Whole smoke exposure "dosimetry" may be assessed using quantification of particulate deposition by spectrofluorometric analysis (*e.g.* high performance liquid chromatography and fluorescence detection) (Thorne and Adamson, 2013). Clunes *et al* describe measurement of nicotine and its breakdown product cotinine by mass spectrometry, in airway surface liquid collected from lavage of *in-vitro* cell cultures treated with whole cigarette smoke (Clunes et al., 2008).

In-vitro studies have investigated the effect of cigarette smoke on various epithelial cell properties and functions using the models described above. Both cell lines and primary cells, and submerged and air liquid interface (ALI) cultures have been investigated. In submerged culture models, epithelial monolayers are grown to confluence under a layer of culture medium which provides the necessary nutrients. The use of ALI cultures attempts to model the *in-vivo* cell differentiation in the airway epithelium; cells are seeded into transwell inserts, and cultured with media at the basal surface only once attached, with the apical cell surface exposed to air (Karp et al.,

2002). The resultant cell cultures become differentiated with the presence of ciliated cells, basal cells and mucus-producing goblet cells. ALI cultures are polarised with apical and basolateral membranes, and form an epithelial barrier with formation of tight junctions. These models can be used to evaluate trans-epithelial transport and drug delivery, in addition to investigation of interaction with environmental agents including cigarette smoke to create models of disease pathogenesis.

To date research on the effect of cigarette smoke in *in-vitro* airway epithelial cell models have generated conflicting results. CSE has been shown to increase basal IL-8 release from a human bronchial epithelial cell line (16HBE-14o)(Mortaz et al., 2011), normal PBEC (Mio et al., 1997), and SAEC (Kode et al., 2006, Moretto et al., 2009). In contrast, in both A549 cells (a type II alveolar epithelial cell line) and primary type II alveolar epithelial cells, CSE has been shown to inhibit IL-8 expression and chemokine release (Moodie et al., 2004, Witherden et al., 2004). However, a further group showed an increased in mRNA expression of IL-8, IL1-β, GM-CSF, and sICAM-1 following CSC exposure in both A549 cells and normal PBECs (Hellermann et al., 2002).

The different effects of cigarette smoke reported may be related to the method and duration of cigarette smoke exposure used, or a threshold for oxidant effects of cigarette smoke with differing susceptibility depending on cell type and airway level. The underlying mechanisms proposed for effect of cytokine release often relates to the oxidant effect of cigarette smoke on cell signalling pathways involved in pro-inflammatory gene expression. CSE has been shown to have both positive and negative effects on activation of the redox sensitive transcription factor nuclear factor κ B (NFκB) (Kode et al., 2006, Moretto et al., 2009). Other pathways involved in IL-8 expression include the JUN-N terminal kinase (JNK), extracellular protein kinase (ERK) and p38 mitogen activated protein kinase (MAPK) signalling cascades which activate the activating protein 1 (AP-1) transcription factor (Hoffmann et al., 2002). The p38 MAPK pathway is also important post-transcriptional regulation of IL-8 with a role in mRNA stabilisation. These pathways have also been implicated in contributing to the effects of CSE on IL-8 (Moretto et al., 2009).

Pre-treatment of cells with antioxidants such as glutathione (GSH), has been shown to protect against some of the effects of CSE observed (Witherden et al., 2004). In bronchial epithelial cells isolated from explanted lung tissue, baseline GSH levels were higher in cells isolated from tissue of patients with COPD and smokers with normal lung function, compared to controls (Rusznak et al., 2000). Short (20 minute) cigarette smoke exposure depleted GSH levels, an effect that was much more pronounced in cells from COPD tissue than those from smokers or controls.

CSE may increase IL-8 release in PBEC via an epidermal growth factor receptor (EGFR) dependent mechanism (Richter et al., 2002). CSE has been shown to cause rapid ligand independent phosphorylation of the EGFR, but additionally in this study CSE induced expression and release of EGFR ligands (Richter et al., 2002). IL-8 expression and release in response to CSE was found to be dependent on EGFR activation by these ligands. This may be important in a more sustained inflammatory response once acute oxidative mechanisms are no longer active (Richter et al., 2002). Of note, low CSE concentrations promoted cell survival, potentially via increasing EGFR ligand levels and hence EGFR activation. Increased EGFR expression has been reported in smokers and patients with COPD (Burgel and Nadel, 2008). EGFR activation is important for mucin production and epithelial maintenance and repair processes, both of which are dysregulated in COPD.

The effects of cigarette smoke on epithelial integrity and repair has also been investigated. CSE has been shown to inhibit epithelial repair processes including chemotaxis, proliferation and contraction of 3D gels as a model of extracellular matrix remodelling (Wang et al., 2001). Reduced fibronectin and TGF-β release were observed; addition of endogenous TGF-β but not fibronectin partially reversed effects on collagen gel contraction, suggesting this factor has a more important role in the effect on remodelling. The findings are in contrast to increased TGF-β expression noted on acute cigarette exposure in mice (Churg et al., 2006) and in the small airways of smokers and patients with COPD (Takizawa et al., 2001). However, these other studies focussed on TGF-β expression and not protein release, and this could also reflect different mechanisms of acute and chronic smoke exposure.

In addition to the above research, comparison of normal PBECs with those isolated from patients with COPD has been performed. Comer *et al* report an increase in IL-8 in response to CSE in normal PBECs grown at ALI (Comer et al., 2013). Nadigel *et al* report a similar increase in IL-8 in response to CSC in normal PBEC in submerged culture (Nadigel et al., 2013). However, in both models this effect was not observed in cells isolated from COPD patients. In one study an immunosuppressive effect of CSE was observed in COPD cells, with additional findings of reduced TLR4 expression, and reduction of MAPK and NFκB activation compared to normal PBECs (Comer et al., 2013). Use of inhibitors showed that the response to CSC in normal PBECs was TLR4 and ERK-1/2 dependent, with no change inTLR4 expression observed (Nadigel et al., 2013). Overall, this work suggests that cigarette smoke may exert effects through TLRs and associated signalling pathways, which are also important to consider further in terms of the epithelial response to infection.

1.10 Toll-like receptors

Toll-like receptors (TLR) were originally identified in Drosophila species with finding of the dToll protein, first noted to be of importance for dorsal-ventral axis development and later found to be essential for an immune response to fungal infection (Medzhitov, 2001). Following this discovery homologous proteins were found in other species, and to date 11 TLRs have been identified in humans (Parker and Prince, 2011). These are expressed by a wide variety of cells, in particular those of the innate and adaptive immune systems. Various studies have shown that TLRs 1-10 are all expressed by airway epithelial cells, though at differing levels in the resting state and in response to stimulation (Greene and McElvaney, 2005). The highest expression levels have been reported for TLRs 2-6, with expression of TLRs 7-10 reported depending on the cell type studied (Parker and Prince, 2011). TLRs 1, 2, 4, 5 and 6 are present at the plasma membrane, whereas TLRs 3, 7, 8 and 9 are found at the endoplasmic reticulum from where they are chaperoned to endolysosomes (Parker and Prince, 2011). More recent work has also demonstrated the presence of TLR4 in cytosolic compartments in airway epithelial cells as further discussed below (Guillot et al., 2004). TLRs differ in their ability to respond to different ligands as shown in Figure 2. Due to the different potential ligands which can be recognised by one or more TLRs and the complex signalling cascades which can result, a cell may respond in a number of different ways to a pathogen depending on differential TLR activation. This innate response can then signal to initiate an appropriate adaptive immune response.

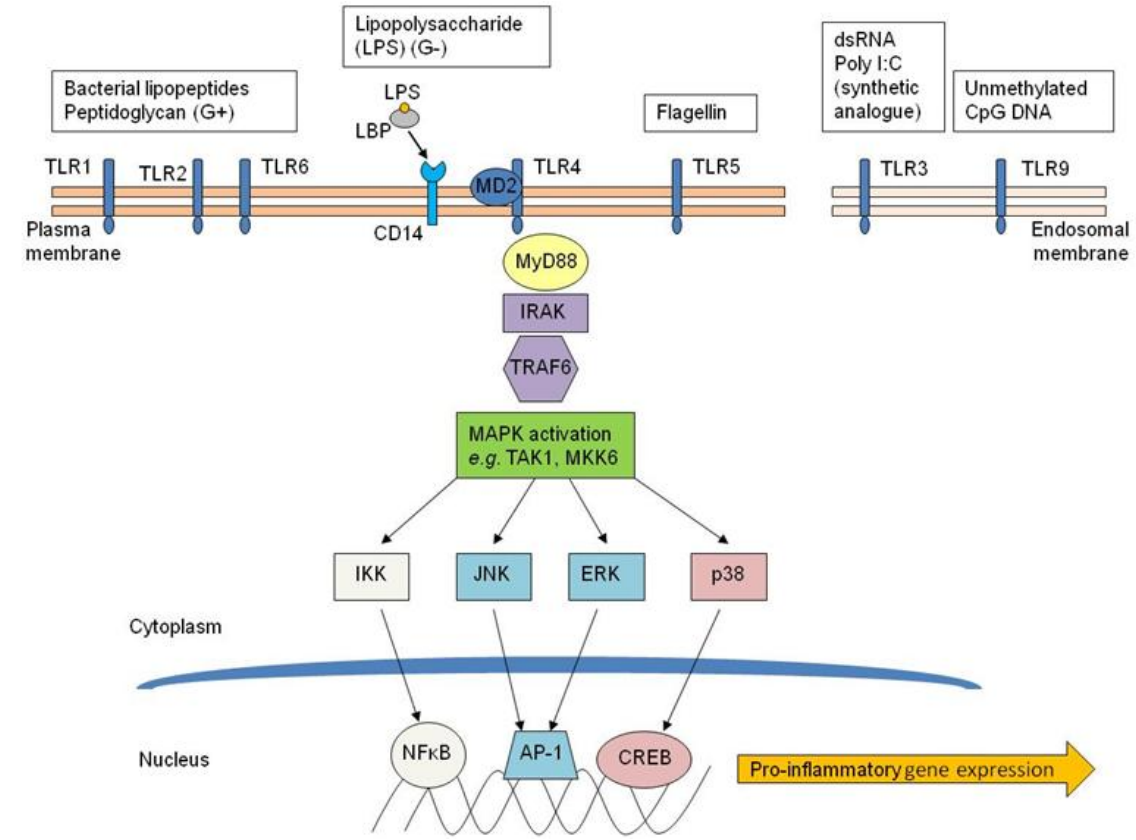


Figure 2 Toll-like receptor ligands and signalling pathways

Figure adapted from figures 3 and 4 (Medzhitov, 2001), and figure 1 (Gay et al., 2014). Toll-like receptors (TLR) are transmembrane receptors with an extracellular ligand binding domain (with leucine rich repeats), and a highly conserved cytoplasmic Toll/IL-1R domain (TIR), similar to the IL-1 receptor (IL-1R). TLRs can be divided into those expressed at the cell surface, which recognise lipid and protein ligands; and those which are expressed on the membranes of endosomal compartments, and respond to non-self nucleic acids. TLR2 forms heterodimers with TLR1 and TLR6 and as a result can recognise a wide range of ligands. TLR4 requires accessory proteins CD14 and MD2 for activation by LPS (associated with LPS binding protein).

TLR activation triggers an intracellular signalling cascade with the end result of gene expression of inflammatory mediators to modulate local cellular immune responses. Ligand-binding induces dimerisation of TIR domains, which forms a scaffold for association of adapter proteins including MyD88 and TRIF. One of the most well understood signalling pathway involves MyD88 adaptor protein and activation of NFκB. However, other pathways also lead to activation of AP-1 and CREB via MAP kinases of the JNK, ERK and p38 families.

(MyD88: Myeloid differentiation response protein 88, TRIF: TIR domain-containing adaptor protein inducing IFN-β, IRAK: IL-1R-associated kinase, TRAF6: TNF-receptor associated factor 6, TAK1: TGF-β activated kinase, MKK6: MAP kinase kinase 6, IKK: IκB kinase, CREB: cAMP-responsive element-binding protein).

1.11 Toll-like receptors and the airway epithelial response to infection

1.11.1 TLR2

TLR2 is important for the detection of both Gram-positive and Gram-negative bacteria, through formation of heterodimer complexes with TLR1 and TLR6. Bacterial lipopeptide-mediated activation of TLR2 in primary tracheobronchial epithelial cells has been shown to induce increased expression and release of HBD2 (Hertz et al., 2003). TLR2 expression is upregulated by NTHi in *in-vitro* PBEC cultures, with increased NFκB pathway activation (Shuto et al., 2002). Of interest the addition of glucocorticoids, whilst reducing cytokine release in response to NTHi through inhibitory actions on NFκB, has been shown to further increase this effect on TLR2 expression (Shuto et al., 2002, von Scheele et al., 2010). This may be due to effects at the transcriptional level at the TLR2 promoter, or an inhibitory effect on the p38 MAP kinase pathway which normally negatively regulates TLR2 expression (Shuto et al., 2002).

1.11.2 TLR3

TLR3 is important in the response to viral infection through recognition of dsRNA. Investigation of the BEAS-2B bronchial epithelial cell line, and primary SAEC has shown that stimulation of TLR3 with a synthetic analogue, Poly I:C elicits the most profound pro-inflammatory response compared to stimulation of other TLRs with their own ligands (Ritter et al., 2005, Sha et al., 2004). In addition to viral responses, TLR3 has been shown to be important in PBEC responses to NTHi, with production of the chemokines CCL-5 and CXCL-10 (Teng et al., 2010). This may be important in influx of macrophages and T lymphocytes which express the receptors for these chemokines, CXCR3 and CCR5 respectively. NTHi has also been shown to increase TLR3 and ICAM-1 expression, thereby potentiating epithelial cell responses to subsequent infection with rhinovirus (Sajjan et al., 2006).

1.11.3 TLR4

TLR4 is important in the recognition of lipopolysaccharide (LPS) on the cell surface of Gram-negative bacteria. TLR4 activation requires adapter proteins which facilitate the transfer of LPS to the receptor, and receptor activation. These include LPS-binding protein (LBP), CD14 and MD2. LBP aids the transfer of LPS to membrane bound or

soluble CD14, a high affinity LPS receptor (Medzhitov, 2001). The exact role of MD2 is still not clear but it is necessary for recognition of LPS by TLR4 (Shimazu et al., 1999).

Primary human tracheobronchial epithelial cells differentiated in culture have shown poor responsiveness to endotoxin as a result of low MD2 expression; this was corrected by the addition of soluble MD2 or MD2:endotoxin complexes (Jia et al., 2004). This group used Lipo-oligosaccharide (LOS) isolated from NTHi as a relevant PAMP to model respiratory tract infection. Exposure of cells to heat inactivated NTHi upregulated MD2 mRNA expression and increased cellular responses to subsequent LOS stimulation. Other groups have shown that airway epithelial cells can respond to LPS; LPS-induced production of HBD2 from tracheobronchial epithelial cells has been demonstrated (Becker et al., 2000). However, much higher doses were required (100ng/ml to 1µg/ml) compared to those which could stimulate monocyte-derived cells (10ng/ml).

In addition to a requirement for accessory proteins, the cellular location of the TLR4 receptor complex may also impact on exposure to and activation by LPS (Guillot et al., 2004). TLR4 was shown in intracellular compartments in airway epithelial cell lines (BEAS-2B, A549) and also in primary differentiated polarised bronchial epithelial cells, where it was located in an intracellular, subapical pattern (Guillot et al., 2004). This is in contrast to both surface and intracellular expression demonstrated on monocytic cells. Doses of 1µg/ml LPS were required to stimulate IL-8 release from BEAS-2B cells in this study. Stimulation did not alter TLR expression levels or induce surface expression. Instead, transfer of LPS across the plasma membrane was thought to be responsible for TLR4 activation (Guillot et al., 2004). Regulation of expression of necessary adaptor proteins and cellular localisation of TLR4 may both play an important role in the prevention of undue TLR4 activation at airway epithelial surfaces with environmental exposure to LPS in inhaled air.

“Endotoxin tolerance” is described in monocytes and macrophages in response to LPS (Dobrovolskaia and Vogel, 2002). Repeated exposure to inhaled LPS has been shown to result in changes in the inflammatory response to subsequent exposure in a mouse

model (Natarajan et al., 2010). In an *in-vitro* cell culture model, primary human tracheobronchial epithelial cells treated with *Pseudomonas aeruginosa* culture filtrate demonstrated an initial pro-inflammatory response with activation of the NFκB and AP-1 signalling pathways, which was significantly attenuated on repeat exposure (Wu et al., 2005). Tolerance may also be directed by interactions between the airway epithelium and dendritic cells as described in section 1.8.

1.11.4 TLR5

TLR5 is involved in the immune response to flagellin, the protein component of bacterial flagella which provides motility and chemotaxis towards bacterial substrates (Zhang et al., 2005). TLR5 is expressed at the apical surface of airway epithelial cells and is involved in the innate immune response to *Pseudomonas aeruginosa* (PA). This Gram-negative pathogen which is a common opportunistic pathogen in chronic lung diseases including COPD, expresses flagellin. It has been shown that the response to PA is predominantly induced through TLR5; transfection of a dominant negative TLR5 gene (with reporter) into human airway epithelial cells, followed by heat killed PA treatment, resulted in loss of NFκB activation (Zhang et al., 2005). PA may also activate TLR2 via lipoteichoic acid, but use of a dominant negative TLR2 model showed only minimal reduction in response compared to transfection of a non-dominant negative TLR2.

1.11.5 Toll-like receptors and EGFR activation

In addition to the production of pro-inflammatory mediators, TLR activation can also modulate epithelial cell repair after injury through effects on EGFR activation via signalling from TLR pathways (Koff et al., 2008). Activation of TLR2 and TLR5 by bacterial products of *Staphylococcus aureus* stimulated EGFR-mediated increased epithelial cell survival and proliferation, and epithelial repair after injury (Shaykhiev et al., 2008). Rates of repair were shown to increase in the presence of bacterial products, mediated via EGFR as demonstrated by loss of this effect on EGFR inhibition. This effect was independent from mediator release observed including IL-8 and TNF-α.

1.12 Toll-like receptors in COPD

TLR activation and signalling have been of particular interest in COPD with respect to frequent bacterial and viral infections, and bacterial colonisation. TLR4 expression has

been shown to be increased in tracheobronchial epithelial cells from patients with mild-moderate COPD compared to smoking controls; this was in contrast to reduced in expression in severe disease (MacRedmond et al., 2007). Tracheobronchial expression levels were found to correlate with nasal mucosal expression in a subgroup of COPD patients. Levels of HBD2 expression were also shown to parallel these differences in TLR4 expression. The differences in TLR expression found at different stages of the disease could be related to adaptation to the inflammatory environment and increasing bacterial load, but may also have implications for disease pathogenesis. Investigation of TLR expression in primary SAEC has shown reduced TLR5 expression in cells isolated from smokers without airflow limitation, and smokers with COPD, compared to healthy controls (Wang et al., 2012). This suggests the possibility of smoking-related down-regulation of TLR5 expression; this effect was subsequently investigated *in-vitro* using PBEC exposed to CSE and reduced TLR5 expression was observed.

Levels of LBP and soluble CD14 (sCD14) detected in BAL are increased in smokers and COPD patients compared to healthy controls (Regueiro et al., 2009). *In-vitro* work has demonstrated LBP and CD14 release from PBEC in response to IL-6 and IL-1β (Regueiro et al., 2009). Levels of both LBP and CD14 also increased following exposure to CSC, with an associated increase in mRNA expression. These results may explain the findings of increased LBP and CD14 levels observed *in-vivo*. Interestingly, the presence of LBP and sCD14 reduced the inflammatory response of PBEC to NTHi, but adhesion and internalisation of this pathogen were increased by their combined presence (Regueiro et al., 2009). This may reflect an immunomodulatory reaction to bacterial presence to prevent an overwhelming inflammatory response, and could also be important in COPD where chronic colonisation is frequently found.

Single nucleotide polymorphisms (SNP) in the genes encoding TLRs may alter receptor function; two common TLR4 SNP, Asp299Gly and Thr399Ile, affect the extracellular domain of the receptor, and have been shown to reduce primary airway epithelial cell and macrophage LPS responsiveness *in-vitro* (Arbour et al., 2000). These SNP have therefore been investigated in COPD. The Asp299Gly SNP was not associated with

disease severity in a COPD population of 289 subjects (Sabroe et al., 2004). In contrast, a 2.4 fold increased risk of COPD development related to the Thr399Ile polymorphism was suggested in a study group of 240 smokers (136 with COPD) (Speletas et al., 2009). These studies have inherent difficulties as screening of a very large population may be required to detect a significant number of SNP and any associated disease features (Sabroe et al., 2004). Investigation of SNPs in TLR2 and TLR4, in 110 patients with moderate-severe COPD, identified SNPs which were associated with more accelerated FEV1 decline and higher numbers of sputum inflammatory cells (Budulac et al., 2012). Further investigation is required to validate these findings in a larger population, and to consider the effects of the SNPs investigated on TLR expression and function in cells in the COPD lung.

1.13 Effects of cigarette smoke on the immune response

Cigarette smoke may influence airway epithelial cell responses to infection, including through effects on TLR. CSE has been shown to reduce TLR4 expression at both the mRNA and protein level in A549 cells (MacRedmond et al., 2007). Related to this CSE attenuated IL-8 release in response to LPS. Corticosteroids induced the same effects on both TLR expression and LPS response. In contrast salmeterol (a long acting β-agonist used to relieve bronchoconstriction) increased TLR4 surface expression and could reverse the inhibitory effect of corticosteroids on the response to LPS, but not in the presence of CSE. This is interesting in the context of COPD treatment with a combined corticosteroid/long acting β-agonist preparation and the improvement in exacerbation rates seen with this treatment in the TORCH trial (Towards a Revolution in COPD Health) (MacRedmond et al., 2007).

Further work using A549 cells has shown that CSC reduced (heat-killed) NTHi and *Staphylococcus aureus* induced IL-8 release. This was found to be related to NFκB inhibition and reduced IL-8 mRNA production, effects which were reversed by pre-treatment with the anti-oxidant N-acetylcisteine (Kulkarni et al., 2010). Suppression of mediator release in response to LPS has also been demonstrated BEAS-2B cells (Laan et al., 2004). However, the CSE effects were shown to be mediated via inhibition of LPS-induced AP-1 activity.

In contrast other research has shown that CSE may accentuate the response to pathogens. CSE has been shown to increase total TLR4 protein and surface expression in 16-HBE cells, with associated increased LPS binding (Pace et al., 2008). There was no effect on TLR4 mRNA pointing to a post-translational effect. CSE increased IL-8 release from BEAS-2B cells and PBEC in response to rhinovirus (Hudy et al., 2010). CSE inhibited rhinovirus induced IL-8 promoter activation, but increased IL-8 levels were found to be due to increased mRNA stability related to increased p38 MAPK phosphorylation. In a primary cell culture ALI model, CSE has been shown to increase IL-8 and IL-6 release in response to *Pseudomonas aeruginosa* LPS in PBECs from healthy controls and smokers without evidence of airflow obstruction (Comer et al., 2013). However, in PBECs isolated from COPD patients the LPS response was reduced by CSE pre-treatment, with reduced TLR expression, and reduced MAPK and NFκB activation demonstrated following CSE in this cell type, but not in control cells.

1.14 *In-vitro* cell culture models of the airway epithelium

The models described above have been used to investigate specific airway epithelial cell responses. Single culture models have the advantage of analysing the output from a particular cell type, *e.g.* investigating changes in gene expression or mediator release. However, in assessing the relevance of results to the COPD disease process, these models are limited by the lack of other important cell types which communicate with the airway epithelium *in-vivo*, such as those as described in section 1.8. "Conditioned" media from cells treated separately can be used to investigate potential effects of mediators released from other cell types; Thorley *et al* analysed the cytokine and chemokine content of media collected from LPS-stimulated alveolar epithelial cells and macrophages. The media was subsequently used in neutrophil migration assays, with anti-chemokine antibody blockade used to delineate which chemokines were involved (Thorley et al., 2007).

Co-culture models can also be utilised; these may be more technically challenging due to the specific culture requirements of different cell types (Adamson et al., 2011). Treatment of more than one cell type in the same model may also require more in depth analysis to determine the source of any detected signal *e.g.* change in cytokine level. This could be investigated by use of concurrent single and co-culture models.

The importance of interactions between airway epithelium and monocytes in the response to rhinovirus infection has been investigated using mono- and co-culture models with primary bronchial epithelial cells and monocytes isolated from peripheral blood samples; a synergistic effect on IP-10 and MCP-1 release was reported in the co-culture set up (Korpi-Steiner et al., 2010). The use of inserts to culture cells in different compartments but allow communication through soluble factors in the media can also help to work around this issue. Models have been developed incorporating epithelial cells, macrophages and dendritic cells, to investigate particle uptake and translocation in the airway, with addition of macrophages above the epithelial layer and dendritic cells below (Rothen-Rutishauser et al., 2005). Liu *et al* co-cultured A549 cells and fibroblasts in transwell plates followed by exposure to CSE; augmentation of TGF-β1 and induction of EMT was observed in co-cultured A549 cells, but not in those in mono-culture (Liu et al., 2010).

As an extension of ALI models, "organotypic" cultures have been developed incorporating a differentiated, ciliated epithelium on a layer of fibroblasts within type I collagen (Choe et al., 2006). A tri-layer model incorporating epithelial cells, fibroblasts and dendritic cells has also been developed (Harrington et al., 2014). These more complex models may allow a much more accurate representation of the airway epithelium *in-vivo* and can be maintained in culture for 4 weeks and beyond, with further differentiation of the extracellular matrix, including deposition of types III and IV collagen and fibronectin, during this time (Choe et al., 2006). The longer lifespan of these more complex cultures may allow exposure to stimuli of interest over a longer duration, including cigarette smoke, which is more relevant than an acute exposure in understanding the disease process.

In addition, the influence of other local factors including the microbiome, and potential inflammatory milieu, is not addressed by the culture models described above. The use of individual TLR ligands or exposure to individual pathogens does not reflect the reality *in-vivo*, where there are complex interactions between the host and the lung microbiome. Modelling this is clearly much more complicated. Use of mono- and mixed cultures of commensal and potentially pathogenic bacteria in culture with

human gingival keratinocytes has shown that responses to commensal flora may alter the reaction to pathogenic species, using microarray analysis of gene expression (Mans et al., 2009). Consideration of use of both individual and multiple bacterial species, known to be present in the lung microbiota may further insights into the role of the microbiome in COPD. It may be possible to utilise clinically relevant biological samples *e.g.* BAL, as a means of investigating the impact of both the microbiome and mediators which would be present in the airway *in-vivo*.

1.15 Current therapeutic strategies in COPD

Inhaled corticosteroids (ICS) are used as part of the current management strategy in COPD (National Institute for Health and Clinical Excellence (NICE), 2010). However, in comparison to other inflammatory lung diseases such as asthma, COPD is known to be inherently more resistant to this therapy, and in recent years the reasons behind this apparent steroid resistance have become much more fully understood (Barnes, 2013).

Corticosteroids exert their effect through modulating gene expression, of both anti- and pro-inflammatory genes, and also through post-transcriptional mechanisms (Barnes, 2013). Corticosteroids bind to the glucocorticoid receptor (GR) in the cell cytoplasm, and this complex translocates to the nucleus. Here it binds to glucocorticoid response elements (GRE) at the promoter of various genes. This can switch on expression of anti-inflammatory genes through recruitment of transcription co-activator molecules, *e.g.* CREB. CREB has intrinsic histone acetyltransferase activity, resulting in chromatin remodelling, and recruitment of RNA polymerase II. The main corticosteroid action is however, to turn off expression of pro-inflammatory genes, and this occurs through interaction with co-repressor molecules. In particular this involves recruitment of histone deacetylase (HDAC) 2, with deacetylation of core histones in the chromatin structure, and resultant repression of gene transcription. There are at least two forms of GR: GRα which binds to corticosteroids and to DNA, and GRβ which can only bind to DNA. GRβ can therefore compete with GRα for DNA binding sites to inhibit steroid action. The main mechanism of steroid resistance in COPD is however thought to be related to reduced HDAC 2 activity.

Reduced HDAC 2 expression was initially found in AM from healthy smokers compared to non-smokers, and was associated with reduced Dexamethasone suppression (10^{-6}M) of IL-1β stimulated TNF-α release in these cells (Ito et al., 2001). AM from COPD patients have also been shown to exhibit steroid resistance compared to cells from healthy smoking controls (Culpitt et al., 2003). HDAC 2 expression is reduced in peripheral lung tissue and AM from COPD patients, with more marked reduction noted with increased disease severity (Ito et al., 2005). HDAC 2 expression is significantly downregulated in small airways compared to large airways in smokers with COPD (Isajevs et al., 2011).

This effect on HDAC 2 is thought to be mediated by cigarette smoke and associated oxidant stress, which may continue in the inflamed airways even once smoking has ceased (Barnes, 2013). Oxidative stress results in the formation of peroxynitrite; this can alter tyrosine residues on HDAC 2, rendering it inactive and resulting in ubiquitination, which targets the protein for degradation by the proteosome. In addition, oxidative stress can activate the phosphoinositide-3-kinase (PI3K) pathway, in particular PI3K-δ, leading to the downstream phosphorylation and inactivation of HDAC 2. Therapeutic strategies to reverse these effects on HDAC 2 are under assessment. This includes a study using low dose Theophylline, which appears to impact on the PI3 kinase pathway.

The studies above have focused on AM and analysis of HDAC 2 expression in lung tissue. The effect of oxidative stress on airway epithelial cell responses to steroid treatment has been shown using *in-vitro* cell culture models. Heijink *et al* used hydrogen peroxide and CSE to induce oxidative stress, and assessed the effect on PBECs from patients with asthma, COPD, and smoking and non-smoking controls (Heijink et al., 2014). Budesonide reduced TNF-α induced IL-8 and GM-CSF release in cells from all groups (1-100nM), but this effect was much less marked in the GM-CSF release from asthma and COPD cells. Hydrogen peroxide reduced the inhibitory effect of Budesonide (100nM) on GM-CSF but not IL-8 release from non-smoking control PBECs. Epithelial barrier resistance was reduced by treatment with CSE (5%); Budesonide pre-treatment partially protected against this effect in normal and

smoking control PBECs, but not in PBECs from COPD patients. In PBECs from patients with severe COPD the protective effect of Budesonide was completely absent. The normal airway epithelium may have more capacity to compensate for oxidative stress, which is lost in asthma and COPD (Heijink et al., 2014).

A further study of primary cells from asthma patients and healthy controls, showed that stimulated cells (using IL-1β ± IFN-γ, or Poly I:C) were broadly unresponsive to pre-treatment with Prednisolone (up to 10μM) (Woodman et al., 2013). An IκB kinase (IKK) 2 inhibitor was however, shown to have an effect, suggesting that the corticosteroid unresponsiveness observed may be related to features of the NFκB pathway in airway epithelial cells, rather than any specific resistance mechanism in asthmatic epithelium.

In addition to consideration of steroid resistance in COPD, there may also be issues with appropriate direction of therapy; poor efficacy of inhaled corticosteroid treatment may also related to deposition in larger rather than small airways where the disease process is active (van den Berge et al., 2011). The pattern of lung deposition of inhaled treatment is related to particle size as well as other factors including patient inhaler technique (Leach et al., 2002). A small study of 18 patients considered the effect of Beclomethasone/Formoterol (BDP/F, with extra-fine particle Beclomethasone) compared to Fluticasone/Salmeterol as a control (Tzani et al., 2011). This showed a reduction in air trapping (based on residual volume) and breathlessness in the BDP/F group. A retrospective observational study has compared extra-fine Beclomethasone (small particle ICS), and larger particle size Fluticasone in a primary care population (Postma et al., 2015). Small particle ICS was used at lower dose, and was associated with greater odds of treatment stability, and lower odds of treatment change in the 2 year follow-up period studied. This could be related to greater lung deposition, particularly in the small airways. The responsiveness of the small airway epithelium to corticosteroid treatment, compared to large airway epithelium, has however not been investigated.

1.16 Issues with *in-vitro* modelling of the COPD disease process

The research discussed so far highlights the importance of consideration of which cells are used in trying to model the disease process *in-vitro*, as this may significantly impact on the results observed. This includes cells isolated from healthy and smoking controls without evidence of airflow limitation, and cells isolated from patients with COPD. Primary cells may behave differently to cell lines and are usually considered to be the optimal cell type to use where possible, though this relies on an appropriate source and multiple passages are usually not possible, with a limited duration of cell culture. As cells lining a united airway, it has been suggested that nasal airway epithelial cells could be used to model the lower airway in research and drug development studies (Hurst, 2010). This would be an attractive model to use due to ease of access for cell sampling which would potentially be acceptable to a wider population. In addition, this would allow sampling of cells from COPD patients with more severe disease, which may not be possible from bronchoscopic sampling in terms of safety considerations and ethical issues.

One study has investigated cellular responses from paired nasal and bronchial airway epithelial cells sampled by brushing from 35 subjects undergoing bronchoscopy for a number of different potential lung pathologies (McDougall et al., 2008). Resting and stimulated cytokine release, in response to IL-1β or TNF-α, were assessed. Significantly higher constitutive mediator release was observed from nasal cells compared to bronchial epithelial cells in submerged culture. However, the responses to stimulation were similar, with correlations between nasal and bronchial epithelial cell mediator release (including IL-6, MCP-1 and MMP-9 release). However, other studies have shown significant differences in cellular responses *in-vitro* depending on the location of sampling (Comer et al., 2012, Woodman et al., 2013). Comer *et al* showed differences in innate immune responses and TLR expression between nasal and large airway epithelial cells cultured from COPD patients (Comer et al., 2012). Woodman *et al* also showed differences in nasal and bronchial airway epithelial cell responses in cells isolated from asthma patients and healthy controls (Woodman et al., 2013). Intrinsic differences in responses to cigarette smoke and pathogens at different airway levels may play a significant role in COPD. Small airway epithelial cell cultures have more

recently been established from guided brushings sampling to allow research into this important area (Banerjee et al., 2009). However, there has been no direct comparison of the response of cells from the nasal, large and small airway epithelium.

1.17 Unanswered questions

Throughout the above review of the literature it is clear that that small airways disease is of key importance in COPD. Differences in gene expression profiles of small airway epithelial cells from smokers and patients with COPD compared to non smoking controls are reported (Ammous et al., 2008, Takizawa et al., 2001, Wang et al., 2012). Differences in inflammatory cell infiltrate between large and small airway epithelium are described (Battaglia et al., 2006, Isajevs et al., 2011). The findings discussed may reflect a different reaction to cigarette smoke in small compared to larger airways. In *in-vitro* studies, just as results from large airway epithelial cell culture may not be representative of nasal airway cell responses, the same may be true for the small airway epithelium compared to larger airways. Further investigation will therefore require direct comparison between cells from different airway levels. Understanding potential different responses in the small airways is important in understanding the disease process in this area and developing novel targeted treatments.

Bacterial presence in the airway is now recognised in health and disease. The effect of the recently discovered microbiome on airway and systemic inflammatory parameters, which may be of relevance to the disease process in COPD has however not yet been investigated. In maintaining homeostasis and avoiding unnecessary immune cell recruitment it is important that the airway epithelium responds to appropriate stimuli but not frequent non-noxious environmental exposures from inhaled air, or indeed the "healthy" microbiome. Mechanisms of tolerance have been discussed above, including modulation of epithelial cell TLR expression and interactions with innate immune cells such as AM and DC. Proximal airways may be exposed to greater potential antigen load compared to the more distal small airways. The less exposed small airway epithelium may be less "tolerant" to such environmental stimuli, and could have a lower threshold for orchestration of an immunogenic response than the large or nasal airway epithelium; repeated exposure to the same insults in the upper airway may reduce the effect seen here in comparison. On this basis, the small airway epithelium

could demonstrate a more potent innate immune response to pathogen exposure, compared to large or nasal airway epithelium. Cigarette smoke has been shown to reduce the inflammatory response to pathogen exposure in some *in-vitro* models (Laan et al., 2004, Kulkarni et al., 2010). Smokers are also more at risk of infection, suggesting that this has a negative effect on immune system (Sopori, 2002). If cigarette smoke negatively impacts on the normally more robust response from the small airway epithelium, this could affect pathogen clearance and local inflammation. These considerations lead onto the hypothesis which was generated for investigation in this PhD study.

1.18 Hypothesis

In health, respiratory pathogens induce a more potent innate immune response from small airway epithelium compared to large airway or nasal epithelium. However, this response is blunted by cigarette smoke, and in established COPD this dampened innate response causes persistent airway colonisation and low grade inflammation, and creates an environment favouring small airway fibrosis.

1.19 Aims

1. To evaluate associations between airway bacterial colonisation and inflammation in a cohort of well characterised COPD patients compared to healthy controls.
2. To establish primary airway epithelial cell cultures of nasal, large and small airway epithelial cells from COPD patients and healthy controls, with characterisation of cell markers and Toll-like receptor expression.
3. To establish an *in-vitro* cell culture model to assess airway epithelial cell innate immune responses to pathogen associated molecular patterns and cigarette smoke extract.

Chapter 2. Study protocol, recruitment and sampling

In order to investigate the hypothesis proposed, a study protocol was developed for recruitment of healthy volunteers and patients with COPD for investigation. The following sections are details from the study protocol and describe the original plan for recruitment, sampling and analysis.

I developed the study protocol with my supervisors, and with input from the Respiratory and Inflammation Group at Astrazeneca (previously based at Loughborough, UK). I completed the necessary ethics and R&D applications supported by my supervisors, staff from the Sir William Leech Research Centre, and with input from Dr L Mitchell (Consultant Radiologist, Freeman Hospital) and Dr J Kotre (Consultant Clinical Scientist, Medical Physics, Freeman Hospital). Screening and recruitment was performed by myself, Dr De Soyza and Professor Fisher, with help from the Sir William Leech Research Centre team. The bronchoscopy procedures were performed in the Cardiothoracic screening room at the Freeman hospital by myself (supervised by Dr De Soyza or Professor Fisher). Samples were sent for analysis to the Freeman laboratories as detailed. BAL and serum samples were processed by myself and the Sir William Leech Research Centre laboratory team including Gail Johnson and Kasim Jiwa, as described below.

2.1 Details from study protocol

We plan to recruit 40 stable COPD patients and 15 healthy volunteers for investigation. This research study was developed as a pilot study. This should generate data about important parameters in these groups, and also address the feasibility and practicality of study design. The data generated, including standard deviations, may be used to allow calculation of sample sizes for subsequent research in this area.

As a pilot study, for exploratory analyses as described, no formal power calculation was performed. The recruitment targets chosen are based on numbers which may be required to demonstrate significant differences in inflammatory indices in BAL, and also with consideration of numbers required for cell culture work. Previous studies have shown adequate power to detect significant results in comparison of sputum and

BAL cytokine levels and bacterial load in COPD patients with samples of this size (Patel et al., 2002, Sethi et al., 2006, Tumkaya et al., 2007). Sufficient sample size is also required to account for potential cell culture failure rates. As described in previous studies, the success rate of establishing primary airway epithelial cell culture from bronchial brushings can be influenced by patient derived infection and underlying airways disease (Forrest et al., 2005, Banerjee et al., 2009). In our laboratory, we have a history of successful cell culture from approximately 80-90% of brushings in the winter period, but in the summer this can fall to only 20-30% (due to increased infection rates), with an overall success rate of approximately 50-60%.

An external statistical review was provided in consideration of the sample size required for this study (Dr Andrew Lloyd, Principal Statistician, AstraZeneca, Respiratory Disease and Inflammation Clinical Discovery Team, Alderley Park, UK). The following is a summary of this review which was provided and written by Dr Lloyd:

"There is no single primary endpoint to be investigated in this study, but a number of exploratory hypotheses are of interest. The sample size has been determined therefore, based upon a number of similar studies and feasibility to recruit sufficient subjects to provide enough evaluable subjects to test hypotheses of interest. For an exploratory study, the recommendation is a minimum sample size of 12 per group. The justifications for this sample size are based on rationale about feasibility, and precision about the mean and variance (Julious, 2005). The contexts of the justifications are that future studies will use the information from this exploratory study in their design, and the number is considered sufficient for estimating variability, and estimating a clinically meaningful difference between the subject groups of interest".

Ethical approval has been obtained for the study from the Newcastle and North Tyneside 1 REC (Reference no: 10/H0906/44). Recruitment will take place from the Sir William Leech Research Centre, Freeman hospital, from patients referred from outpatient clinics and GP practices within the Newcastle upon Tyne Hospitals NHS

Trust. Healthy volunteers will be recruited through advertising within Newcastle upon Tyne Hospitals NHS Trust and Newcastle University.

2.2 Inclusion and exclusion criteria

Inclusion criteria
Confirmed diagnosis of COPD (as defined by GOLD criteria) or Healthy volunteer
Exclusion criteria
FEV1 <30% predicted
SpO_2 <92% on room air
Long term oxygen treatment
Evidence of other co-morbid lung disease
Significant cardiac, liver or renal disease
Other significant co-morbidity felt by the investigating team to preclude bronchoscopy for research purposes, *e.g.* Malignancy
Prior history of intolerance to bronchoscopy or bronchoscopy associated medications
Recent lower respiratory tract infection or systemic antibiotics within the last 30 days
Any evidence of systemic immunodeficiency or immunosuppressant medication
Patients on warfarin, clopidogrel or dual antiplatelet therapy
Age <18 or >80 years

Table 1 Study inclusion and exclusion criteria

2.3 Study procedure

2.3.1 Visit 0

Following written informed consent, baseline clinical information will be recorded for each participant. This will instruct whether a participant can be involved in the study (in particular safety for investigations including bronchoscopy), and outline a clinical phenotype. Information including participant demographics and medical history will be collected. Health status questionnaires will be completed. Clinical investigations including baseline routine chemistry and haematology, ECG and sputum culture will be performed. Full lung function tests including spirometry (pre- and post-bronchodilator), lung volumes and gas transfer measurement will be undertaken in all

COPD patients (to be performed by the Lung Function Department, Freeman Hospital). In healthy volunteers pre- and post-bronchodilator spirometry will be completed.

2.3.2 Visit 1

Following review of clinical information participants will proceed to study sampling. Each subject will have a single set of investigations for cross-sectional study.

2.3.3 Study samples

Sample	Details
Urine	For cotinine analysis
Blood	30 ml for whole blood and serum analysis
Sputum	To be collected when possible for standard microbiological culture
Bronchoscopy	Procedure for sampling to include bronchoalveolar lavage, brushings and endobronchial biopsies
Bronchoalveolar lavage	To instil 3x60mls 0.9% Saline (total 180ml) into right middle or lower lobe, with collection on low suction to minimise airway collapse and improve return
Bronchial brushings	2 samples from large airways to be taken from 2^{nd}-3^{rd} generation airways. 2 samples from small airways to be performed under x-ray screening (fluoroscopy).
Endobronchial biopsies	4-6 biopsies from large airways at 2^{nd}-3^{rd} generation
Nasal brushings	2 brushings to be taken from nasal airway epithelium

Table 2 Study samples to be collected at Visit 1

2.4 Sample processing and analysis

N.B. Urine cotinine analysis, full blood count, CRP and standard sputum and BAL cultures all to be performed as arranged by Freeman laboratories.

2.4.1 Urine samples

Samples will be stored frozen at -20^{o}C until assayed as a batch for cotinine levels by ELISA. The results of this analysis will be correlated with recorded smoking history.

2.4.2 Blood samples

A full blood count will be performed to provide a differential white cell count. CRP will be measured. The serum fraction will be separated and frozen at -80^{o}C in 0.5ml aliquots for examination of a panel of inflammatory cytokines using a multiplex ELISA.

2.4.3 Sputum samples

Spontaneously produced sputum (when available) will be collected and processed for standard microbiological culture.

2.4.4 Bronchoalveolar lavage (BAL)

A proportion of the BAL will be sent for standard culture. Where a sufficient volume of BAL is available, an unprocessed aliquot will of this will be stored at -80^{o}C to be sent for later molecular microbiology analysis (as detailed below). The remaining BAL will be processed to separate the cellular component from the supernatant (see Table 3). A total and differential cell count will be performed using prepared cytospins. Depending on BAL volume and cell count, the alveolar macrophage component will be separated from the other cells via selective adherence. The level of pro-inflammatory cytokines will be measured in BAL using the same assays as for serum.

N.B. Initial BAL and serum processing, and BAL total and differential cell counts performed by myself, Gail Johnson (Senior technician) and Kasim Jiwa (Laboratory technician) in the Sir William Leech Research Centre laboratory. Subsequent analysis of pro-inflammatory cytokine levels performed by myself using methods as described in Chapter 3.

2.4.5 Nasal, large and small airway epithelial cell culture

Primary cultures of airway epithelial cells from the nose, large and small airways of each study participant will be established from seeding of brushed cells.

2.4.6 Endobronchial biopsies

Endobronchial biopsies will be fixed in 4% PFA and embedded in paraffin wax for sectioning and later analysis including Haematoxylin and Eosin staining, and immunohistochemistry. *N.B. These samples were collected as part of the study protocol but were not used in the current PhD project.*

Initial BAL processing
BAL filtered through sterile gauze and volume documented Centrifuged at 1250 rpm (183 g) for 6 minutes at 4°C (MSE Mistral 3000i centrifuge)
Supernatant
Supernatant decanted and centrifuged at 2500 rpm (734 g) for 6 minutes at 4°C Supernatant divided into 600µl aliquots and frozen at -80°C
Cytospins and cell count
Cell pellet resuspended in appropriate volume of 1xPBS (Sigma: D8537) to give an opaque suspension Total cell count calculated using Neubauer haemocytometer (cells in 4 large outer squares counted) Cytospins prepared using 100µl of re-suspended cells centrifuged at 300 rpm (9 g) for 3 minutes (Shandon Cytospin 3) 1xcytospin fixed in acetone at room temperature for 10 minutes and air dried Remaining cytospins air dried overnight, wrapped in cling film and stored at -80°C for later analysis Acetone fixed cytospin stained with Giemsa and differential cell count performed by counting 500 cells and calculating percentages of cells present
Cell pellets
Once cytospin preparation is complete, the cell suspension is recentrifuged as above Supernatant discarded and cells resuspended in 1xPBS (to give concentration of 2-3 million cells/ml) 1ml aliquots centrifuged at 300 rpm (352 g) for 4 minutes Supernatant discarded and pellets stored at -80°C
Alveolar macrophage (AM) isolation
BAL filtered as above and centrifuged at 300 g for 4 minutes Cell pellet resuspended in RMPI media (Sigma: R0883) Cells seeded at an appropriate cell density and incubated at 37°C for 1 hour to allow AM to adhere Wells washed with sterile 1xPBS and fresh RPMI media added AM attachment reviewed and cells fixed in 4% PFA within 24 hours

Table 3 Details of bronchoalveolar lavage processing

2.5 Culture-independent microbiology analysis of BAL samples

As described, where sufficient BAL was available, an unprocessed aliquot was stored to be sent for culture-independent microbiological assessment. This work was performed by Professor Stephen Cummings group, Department of Applied Sciences, Faculty of Health and Life Sciences, University of Northumbria, using the NU-OMICS sequencing facility. The Illumina MiSeq platform was used following the protocol described by Kozich *et al* (Kozich et al., 2013). In particular Hazel Ingram (DNA extraction), Dr Darren Smith (Illumina MiSeq processing), and Dr Andrew Nelson (Illumina MiSeq processing and data analysis) were involved in this work.

Briefly, the technique used involves extraction of bacterial DNA from the BAL samples using the PowerLyser® PowerSoil® DNA isolation kit (MoBio) which uses both chemical and mechanical lysis, followed by patented PCR inhibitor removal technology to obtain genomic DNA of high yield and purity. Universal PCR primers are then used to amplify the V4 variable regions of the 16S rRNA gene in the bacterial genomes, alongside negative controls. Further limited cycle PCR is performed to add sequencing adaptors and barcodes to each target to create an amplicon library (Kozich et al., 2013). The fragment size is confirmed using the Agilent Bioanalyzer and DNA concentration confirmed by quantitative PCR. Paired-end sequencing is then performed using the Illumina MiSeq platform. The Mothur MiSeq SOP is used to assemble paired-end reads, and sequences with a length >270bp and containing any ambiguous bases are removed. The contigs are aligned to the SILVA database and chimeric sequences are removed using UCHIME (Edgar et al., 2011). Sequences present in the negative control are removed from the downstream analysis. Furthermore, potential contaminating bacterial species are excluded; there have been concerns regarding contamination of the kits used in the above process with environmental species (Salter et al., 2014). The sequencing data is analysed to provide details of operational taxonomic units (OTU) present in each sample processed, and also to normalise the data (making the reads for each OTU a proportion of the sample: normalised value=sum of all reads for the sample/number of reads for each OTU in the sample).

Following the above I was provided with an OTU table with details of bacterial DNA present in each sample (Phylum, Class, Order, Family, Genus). Further analysis of this was performed with help from Dr Nelson using PAST software (http://folk.uio.no/ohammer/past/) for assessment of diversity and principle co-ordinates analysis.

2.6 Brushings sampling

Brushings were collected using a single use protected cytology specimen brush. For each site, 2 brushings were collected and dispersed into 5ml of RPMI culture media (further details in methods section 3.5.3). Small airway sampling was performed under fluoroscopic guidance (see Figure 3) to allow safer and more accurate sampling of these airways which cannot be sampled under direct vision as for large airway brushings; the unsheathed brush tip was directed 2-3 cm from the pleural surface for sampling.

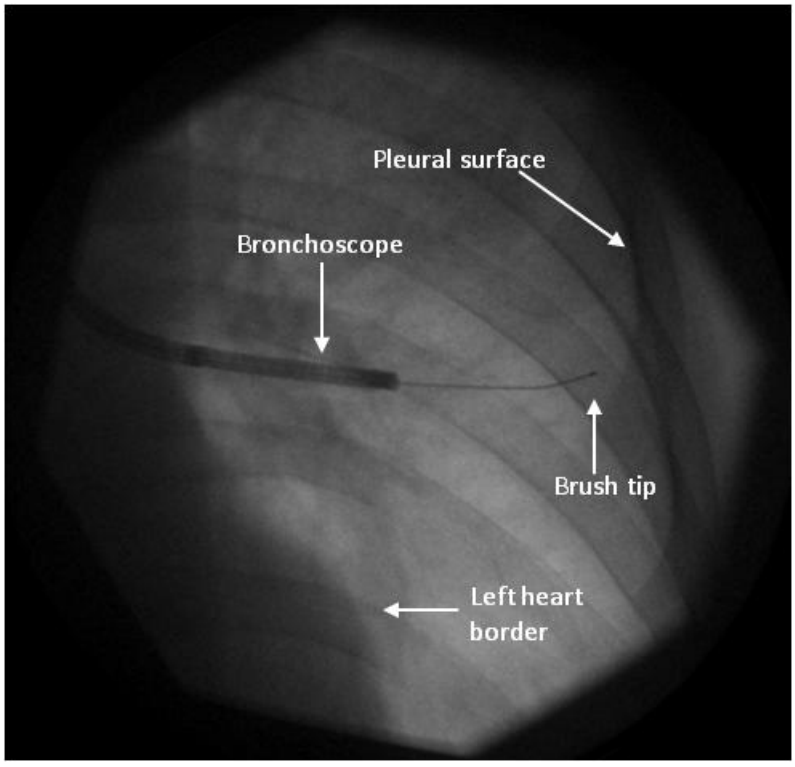


Figure 3 Example of small airways brushing imaging performed during bronchoscopy

Nasal and large airway brushings were all performed using a Cook cytology brush (Cook UK LTD, Hitchin, Hertfordshire: BCB-5-120-3-S). Although the diameter of the unsheathed brush is <2mm (5 French = 1.67mm), once open the whole brush diameter including bristles is 3mm. This specimen brush was used for small airway sample collection in the first 5 COPD donors only. On reflection at this point in the study it was felt that although this brush should be able to sample the small airways with an unsheathed diameter of <2mm to access this level of the bronchial tree, that sampling when the brush was deployed at 3mm could potentially cause local trauma and may reduce the yield of cells collected. Therefore, an Olympus cytology brush with a

diameter of 1.2mm including bristles of 0.064mm (Olympus UK, Southend-on-Sea: BC-202D-1210) was used for small airway brushings in the remaining donors. SAEC collected from the first 5 COPD donors were used for early characterisation work only, which was also repeated in samples from later donors, and these cells were not used for any of the culture experiments described. Therefore this change is not considered to have impacted on the results reported.

Chapter 3. Materials and Methods

3.1 Cell types

3.1.1 Cell lines

• 16-HBE14o- (HBE) cells are a human bronchial epithelial cell line immortalised by transformation with Simian virus 40 (SV40) large T-antigen.

• BEAS-2B cells are a human bronchial epithelial cell line derived from normal bronchial epithelium immortalised by infection with a hybrid virus of adenovirus 12 and SV40.

• THP-1 cells are a monocytic cell line derived from acute monocytic leukaemia cells in peripheral blood. Differentiation to a macrophage-like phenotype can be induced by the phorbol ester PMA (phorbol-*12*-myristate-*13*-*acetate).*

3.1.2 Primary cells

• Primary nasal, large and small airway epithelial cells were obtained from brushings from COPD patients and healthy volunteers recruited for the study.

The following cell types were used as positive controls for the immunocytochemistry experiments only:

• Primary alveolar macrophages (AM) were isolated by selective adherence from BAL (see BAL processing details in study protocol section 2.4.4).

• Primary endothelial cells were kindly donated by Dr Laura Mackay (previously Institute of Cellular Medicine, Newcastle University). Cells were isolated from normal peripheral lung tissue from patients undergoing lobectomy/pneumonectomy, County Durham and Tees Valley 2 Research Ethics Committee, reference 09/H0908/35 (Mackay et al., 2013).

• Primary human lung fibroblasts were kindly donated by Dr Monika Suwara (previously Institute of Cellular Medicine, Newcastle University). Cells were isolated from donor lung tissue not used for transplantation and allocated for research, Newcastle and North Tyneside Local Research Ethics Committee, reference 2001/179 (Suwara et al., 2014).

3.2 Cell culture media

• MEM/EBSS, HyClone Classical Liquid Media: Minimum Essential Medium with Earle's Balanced Salts (Fisher Scientific, Loughborough, UK: SH30244.FS), supplemented with 10% fetal bovine serum (Sigma, Gillingham, UK: 9665), 1% Penicillin/Streptomycin (100units/ml, 0.1mg/ml, Sigma: P0781) and 1% L-glutamine (2mM, Sigma: G7513). **For HBE culture.**

• RPMI-1640 medium (Sigma: R0883), supplemented with 10% FBS, 1% Penicillin/Streptomycin and 1% L-glutamine. **For THP-1 and AM culture.**

• BEBM, Bronchial Epithelial cell Basal Medium (Lonza, Castleford, UK: CC-3171) with Bronchial Epithelial Cell Growth Medium SingleQuot Kit (Lonza: CC4175) and 1% Penicillin/Streptomycin. **For BEAS-2B and primary airway epithelial cell culture.**

• SABM, Small Airway cell Basal Medium (Lonza: CC-3119) with Small Airway cell Growth Medium SingleQuot kit (Lonza: CC4124) and 1% Penicillin/Streptomycin. **For primary airway epithelial cell culture.**

Details of the supplements and growth factors added to SABM and BEBM media in the SingleQuot Kits are provided in Table 4. "Complete media" was prepared with the addition of SingleQuot kits and Penicillin/Streptomycin, and designated BEGM and SAGM. "Incomplete media" also used for selected experiments with addition of Insulin, Transferrin and Gentamicin/Amphotericin SingleQuots only, and Penicillin/Streptomycin, as detailed in the relevant results section.

List of supplements and growth factors (*only in SAGM)
Bovine pituitary extract (BPE)
Hydrocortisone
Epinephrine
Insulin
Triiodothyronine
Transferrin
Gentamicin/Amphotericin-B
Retinoic acid
*Bovine serum albumin (fatty acid free)

Table 4 Supplements added to SAGM and BEGM media

3.3 Antibodies and proteins

3.3.1 Immunocytochemistry

Antibody	**Supplier (Cat. No.)**	**Description**	**Concentration**	**Dilution for ICC**
CD31/PECAM	Dako (M0823)	Mouse IgG1	0.2mg/ml	1/20
CD45	Abcam (ab10559)	Rabbit polyclonal	0.5mg/ml	1/25
CD68	Abcam (ab955)	Mouse IgG1	Not determined	1/100
Cytokeratin 19	Abcam (ab7754)	Mouse IgG2a	1mg/ml	1/100
Cytokeratin 17	Abcam (ab53707)	Rabbit polyclonal	1mg/ml	1/200
E-cadherin	BD Biosciences (610181)	Mouse IgG2a	0.25mg/ml	1/25
Fibronectin	Sigma (F3648)	Rabbit polyclonal	0.5mg/ml	1/100
Uteroglobin (CCSP)	Abcam (ab40873)	Rabbit polyclonal	Not determined	1/50

Table 5 Primary antibodies
(Dako, Glostrup, Denmark; Abcam, Cambridge, UK; BD Biosciences, Oxford, UK)

Antibody	**Supplier (Cat. No.)**	**Description**	**Concentration**
Mouse IgG1	Abcam (ab18447)	Isotype control	1mg/ml
Mouse IgG2A	R&D (MAB003)	Isotype control	0.5mg/ml
Rabbit IgG	R&D (AB-105-C)	Isotype control	1mg/ml

Table 6 Isotype control antibodies
(R&D, Abingdon, UK)
NB Dilutions used to match concentration of primary antibody.

Antibody	Supplier (Cat. No.)	Conjugate	Raised in	Dilution for ICC
Anti-Mouse IgG	Sigma (F2012)	FITC	Goat	1/100
Anti-Rabbit IgG	Sigma (T6778)	TRITC	Goat	1/100

Table 7 Secondary antibodies
FITC - Fluorescein isothiocyanate, TRITC - tetramethylrhodamine isothiocyanate.

3.3.2 Western blot

Antibody/protein	Supplier (Cat. No.)	Description	Concentration	Dilution for WB
Uteroglobin (CCSP)	Abcam (ab40873)	Rabbit polyclonal	Not determined	1/4000
Recombinant human uteroglobin	R&D (4218-UT)	Recombinant protein	100µg/ml	NA
β-actin	Sigma (A2228)	Mouse IgG2A	2mg/ml	1/5000

Table 8 Antibodies and proteins used for Western blot experiments

3.4 PCR primers

Gene	Forward primer sequence (5' - 3')	Reverse primer sequence (5' - 3')
Gapdh	GAGTCAACGGATTTGGTCGT	GACAAGCTTCCCGTTCTCAG
18S	TAACCCGTTGAACCCCATTC	TCCAATCGGTAGTAGCGACG
CCSP	CTTTCAGCGTGTCATCGAAA	TGATGCTTTCTCTGGGCTTT
TLR2	TTGTGGATGGTGTGGGTCTT	AGGTCACTGTTGCTAATGTA
TLR3	AGATTACCAGCCGCCAACTT	TTTCTGGATTGAGTTGGACA
TLR4	TTTTTCTAATCTGACCAATC	TCATAGGGTTCAGGGACAGG
TLR5	TACCCCCTTGACTATTGACA	ATAACCATCTTTCAATACAG
TLR9	TACCTTGCCTGCCTTCCTAC	CACTTGAGGTTGAGATGCCG

Table 9 PCR primers
(All purchased from Sigma)

3.5 Cell culture

All cell culture was performed under aseptic conditions in a class II safety cabinet, in line with Newcastle University Health and Safety guidelines. Cells were grown at 37^{o}C, 5% CO_2/95% air in a Sanyo humidified incubator. The culture work and experiments described below were performed by myself, with help from Andrew Walker (Laboratory technician) during periods of high culture numbers only.

3.5.1 Adherent cell culture (HBE, BEAS-2B)

Cells were grown in T75cm^2 flasks and were passaged when approximately 90% confluent. Cells were washed with 1xPBS and incubated with trypsin-EDTA (Sigma: T3924) to detach the cells. Once detached an equal volume of media was added to neutralise the trypsin and the cells pelleted at 300 g for 4 minutes. Cells were resuspended in fresh media at the required cell density and seeded into T75cm^2 flasks for culture. Media was changed every 48-72 hours until cells reached 90% confluence.

3.5.2 Non-adherent cell culture (THP-1)

Cells were cultured in T75cm^2 flasks at a density of 0.5x10^{6}cells/ml. Cell counts and media change were performed every 48-72 hours. For selected experiments, THP-1 cells were differentiated with PMA (5ng/ml) for 24 hours. Differentiation was confirmed by adherence to cell culture plastic. Non-adherent cells were removed by washing with 1xPBS before treatments.

3.5.3 Primary cell culture

Brushings (as detailed in the study protocol section 2.3.3 and 2.6) were collected using a single use protected cytology specimen brush. Following collection brushed cells were dispersed into RPMI media and centrifuged at 1000 g for 4 minutes. The cell pellets were resuspended in media appropriate to cell type and seeded into collagen coated (Purecol 0.5%, Nutacon: 5409) T25cm^2 flasks. Primary small airway epithelial cells were all culture in SAGM. Large and nasal cells were cultured in both SAGM and BEGM as detailed in the results sections.

Cells were passaged when approximately 90% confluent. Cells were washed with 1xPBS prior to detachment using trypsin-EDTA (Lonza: CC-5012). Following detachment, trypsin neutralising solution (Lonza: CC-5002) was added and cells

pelleted at 300 g for 4 minutes. Cells were resuspended in fresh media and seeded into collagen coated (Purecol 0.5%) T25cm^2 flasks at the required cell density.

3.6 Cigarette smoke extract preparation

All preparation was performed in a fume hood in keeping with Newcastle University Health and Safety Regulations. The smoke produced from one research grade cigarette (4A1, University of Kentucky, provided by AstraZeneca, Respiratory and Inflammation Group, Loughborough, UK) was drawn through 25ml of media over 2-3 minutes using a vacuum pump. Media appropriate to cell type was used. The medium was sterile filtered (0.2µm pore Minisart filter, Sartorius) and designated 100% CSE. CSE was diluted to concentrations as required for stimulation experiments, with media appropriate to the cell type treated. Once prepared, CSE was used for cell treatments within 30 minutes.

3.7 Bacterial whole cell lysate preparation

Whole cell lysates of non-typeable *Haemophilus influenzae* (HI) and *Pseudomonas aeruginosa* (PA) were prepared from clinical isolates obtained from COPD patients, provided by Professor Perry at the Freeman Hospital, Newcastle upon Tyne. Three isolates of HI (803222P, 794818, 800891), and three isolates of PA (80788M, 807173, 807173B) were processed. This work was performed with the help of Dr Sonya Carnell.

As per protocol (De Soyza *et al.*, 2004), strains were grown on appropriate growth medium for 24 hours, harvested into 1xPBS and standardised to 0.2 at an optical density of 600nm. Bacterial suspensions were lysed by sonication (Branson sonifier 150) and treated with deoxyribonuclease II (200µg/ml, Sigma: D8764) at 37^{o}C for one hour (to break down bacterial DNA). Proteinase K (2mg/ml, Sigma: P6556) was added and lysates were incubated at 60^{o}C for 2 hours, boiled for 20 minutes (to inactivate Proteinase K) and stored at -80^{o}C prior to use.

3.8 Cell treatments

3.8.1 Cell viability

Cells were seeded into 12 well plates (collagen coated for primary cells) and grown to 90% confluence prior to treatment with CSE (0, 5, 10%) or HI (0, 3.125, 12.5, 25µl/ml) for 24 hours. Cell viability was assessed by flow cytometry.

3.8.2 Cell proliferation

HBE, BEAS-2B (seeded at $2x10^4$ cells/ml in 96 well plates for 24 hours), and primary cells (seeded at $2x10^4$ cells/ml in 96 well plate collagen coated wells, for 72 hours) were treated with CSE (0, 2.5, 5, 7.5, 10%) for 24 hours, and effects on proliferation analysed using the Cell Proliferation ELISA, BrdU (colorimetric) (Roche, Sussex, UK: 11 647 229 001).

BEAS-2B cells were seeded into 12 well plates (200,000 cells/well for 24 hours) and treated with CSE (0, 2.5, 5, 7.5, 10%) for a total of 48 hours (to allow for BrdU labelling of concurrent proliferation experiment as described below). Cells were harvested for cell counts to be performed: cells were washed with 1xPBS and incubated with trypsin-EDTA (Sigma: T3924) to detach the cells. Once detached an equal volume of media was added to neutralise the trypsin and the cells pelleted at 300 g for 4 minutes. Cells were resuspended in 1ml 1xPBS and 10µl of the suspension counted on a Neubauer haemocytometer.

3.8.3 Assessment of cytokine release

Cell line or primary airway epithelial cells were seeded into 6 or 12 well plates and grown to 90% confluence prior to treatment with CSE (0, 1, 2.5, 5, 10%), HI (0, 3.125, 12.5, 25, 50µl/ml) or PA (0, 3.125, 12.5, 25, 50µl/ml), alone or in combination, for 24 hours. TNF-α (20ng/ml) and *Escherichia coli* derived LPS (Sigma: L2630) (1-10µg/ml) were used as positive controls. Media was harvested and stored at -80^{o}C for later analysis of cytokine release by ELISA or Meso Scale Discovery (MSD) assay. For selected experiments, cell counts were performed; cells were washed with 1xPBS and incubated with trypsin-EDTA to detach the cells. Once detached an equal volume of media (or trypsin neutralising solution) was added to neutralise the trypsin and the

cells pelleted at 300 g for 4 minutes. Cells were resuspended in 1ml 1xPBS and 10µl of the suspension counted on a Neubauer haemocytometer.

THP-1 cells were cultured in 48 well plates with the addition of PMA (5ng/ml) for 24 hours to induced cellular differentiation to an adherent cell population. Cells were treated with HI (0, 1.56, 3.125, 12.5, 25, 50µl/ml) or PA (0, 1.56, 3.125, 12.5, 25, 50µl/ml) for 24 hours. TNF-α (20ng/ml) and *Escherichia coli* derived LPS (Sigma: L2630) (1µg/ml) were used as positive controls. Media was harvested and stored at -80^{o}C for later analysis of cytokine release by ELISA.

3.8.4 Corticosteroid treatments

Dexamethasone (Sigma: D4902), Beclomethasone dipropionate (Sigma: B3022) and 17-Beclomethasone monopropionate (17-BMP, provided by Chiesi, Parma, Italy) were used for *in-vitro* steroid treatment experiments. The steroid preparations required solubilisation in DMSO; all 1mM stock dissolved in DMSO, to a maximum final DMSO concentration in cell culture media of 0.1%. Based on this, concentrations of 10^{-6} (1µM) to 10^{-15} M were used.

BEAS-2B and primary airway epithelial cells were seeded into 24 well plates for initial dose-response experiments. Cells were pre-treated for 4 or 24 hours prior to stimulation with TNF-α (20ng/ml) for a further 24 hours. 0.1% DMSO only, TNF-α only and steroid only controls were performed. Media was harvested and stored at -80^{o}C for later analysis of cytokine release by ELISA

Primary airway epithelial cells seeded in 6 well plates were pre-treated with Dexamethasone, or 17-BMP, both at 1nM final concentration in cell culture media, for 24 hours. Treatments including CSE (5%), HI (25µl/ml) and TNF-α (20ng/ml) were then added to the culture media for a further 24 hours. Media was harvested and stored at -80^{o}C for later analysis of cytokine release by ELISA.

3.8.5 Primary airway epithelial cell characterisation

For characterisation purposes cells were grown to 90% confluence and samples collected at each passage (without any treatment). Cells were cultured on coverslips in 24 well plates and fixed using 4% paraformaldehye (PFA) for later Haematoxylin and Eosin staining, and immunocytochemistry (see below). Cells cultured in T25cm^2 flasks were harvested by scraping into ice cold 1xPBS, centrifuged and resuspended in appropriate solutions for storage at -80^{o}C prior to further processing. For RNA extraction and RT-PCR cells were stored in Lysis Buffer with β-Mercaptoethanol (Absolutely RNA miniprep kit, Stratagene, Berkshire, UK: 400805). For protein assay and Western blotting cells were stored in Phosphosafe extraction buffer (Novagen: 712964).

3.9 FACS (fluorescence activated cell sorting) analysis of cell viability

Following treatment, cell media was removed and adherent cells detached by incubation with trypsin-EDTA (Sigma T3924). The cell suspension was added to the media to ensure collection of all cells present. Cells were pelleted at 300 g for 4 minutes and resuspended in 500µl of phenol red free media (DMEM 1x, Gibco: 11880) with 1µl propidium iodide (2.5mg/ml, Sigma: P4170). FACS analysis was performed on a BD FACS Canto (II), and data analysed using FACS DIVA software.

3.10 Assessment of cell proliferation by BrdU incorporation

The Cell Proliferation ELISA, BrdU (colorimetric) (Roche: 11 647 229 001) was used. This assay is based on the incorporation of BrdU (in place of thymidine) into the genomic DNA of proliferating cells.

Cells were seeded into 96 well plates (at densities described in section 3.8.2) and allowed to adhere. After 24 (HBE, BEAS-2B) or 72 (primary cells) hours, media was replaced with media containing CSE at concentrations of 0, 1, 2.5, 5, 7.5 and 10% for 24 hours. BrdU labelling reagent was added (10µl per well) and cells were incubated for a further 24 hours. Following removal of BrdU labelling medium, cellular DNA was denatured by addition of a FixDenat solution and the anti-BrdU-POD (peroxidase conjugated) antibody added. The amount of BrdU incorporated was then analysed by addition of a substrate reagent and quantification of immune complexes performed using the ELISA plate reader to measure optical density at 450 nm.

Control wells were plated with CSE media without cells, and cells with no BrdU added. The background absorbance was subtracted during data analysis.

BEAS-2B cells seeded in 12 well plates (as described above) were allowed to adhere for 24 hours and then media was replaced with the same CSE preparations used during one of the proliferation experiments as described above. The cells were cultured with CSE-containing media for 24 hours, followed by a further 24 hours (during the BrdU labelling step). The cells were then harvested for cell counts to be performed.

3.11 Haematoxylin and Eosin (H&E) staining

Primary cells were grown to confluence on collagen coated coverslips in 24 well plates. Cells were fixed using 4% PFA for at least 1 hour following which this was replaced by fresh 1xPBS. Plates were stored at 4^{o}C until staining.

For H&E staining cells were permeabilised with 0.1% Triton X-100 in 1xPBS for 4 minutes. This was removed and the cells were washed 3x with dH20 (1 minute washes). Filtered Haematoxylin (Thermo Scientific: 6765003) was added to each well for 4 minutes. Following removal of the Haematoxylin wells were washed with dH20 (2x1 minute washes, 1x5 minute wash). Alcoholic Eosin (Thermo Scientific: 6766007) was added to each well for 30 seconds and the same washing steps followed as previously. Coverslips were then left to dry and later mounted onto slides for imaging.

3.12 Immunocytochemistry (ICC)

Cells were prepared as for H&E staining. Glycine (100mM) was added to each well and left for 30 minutes to quench any remaining fixative. Cells were then permeabilised using 0.1% Triton X-100 in 1xPBS for 30 minutes*. This was discarded and wells were washed 2x with PBST (0.2% Tween 20 in 1xPBS) and 1xPBS for 5 minutes. Wells were blocked using 5% bovine serum albumin (BSA, Sigma A7906) in PBST for 60 minutes**. Primary antibodies were added at an appropriate dilution (see Table 5) in 5% BSA and the plates were incubated overnight at 4^{o}C.

**For CD31 antibody processing 0.2% Tween-20 was used to permeabilise the cells.*

***For CD31 and CD68 processing 1% BSA was used to block and for primary antibody preparation.*

Following overnight incubation at 4^{o}C, plates were placed at room temperature for 1 hour prior to further processing. The primary antibody was then removed and wells were washed 3x with PBST and 1x with 1xPBS (all 5 minute washes). Secondary antibodies were prepared at 1:100 dilution in 5% BSA and added for 90 minutes at room temperature in the dark. The antibody was removed and wells were washed 5x in PBST and 1x in 1xPBS. Slides were prepared with DAPI (Vecta Shield: H-1200) onto which the coverslips were mounted and incubated at 4^{o}C overnight. Images were taken using a Leica LSM 510 confocal microscope.

For each experiment controls were performed using appropriate isotype-matched antibodies in place of primary antibody; where concentrations of primary antibody were available, the same concentration of isotype control antibody was used. Controls with secondary antibodies alone were also performed. These controls were used to ensure that no non-specific binding had occurred, and allowed settings to be adjusted as required against background cellular autofluorescence.

3.13 RNA extraction

Total RNA was extracted using the Absolutely RNA miniprep kit (Stratagene: 400805). Cell lysates were stored in buffer containing guanine thiocyanate, a strong protein denaturant used to aid cell lysis and prevent ribonuclease degradation of RNA. This method then utilises a matrix which binds RNA and allows contaminants to be washed out using salt buffers. DNase treatment is used to prevent genomic DNA contamination. Highly purified RNA is eluted into a low ionic strength buffer and stored at -80^{o}C until further use.

RNA concentration was analysed using the NanoDrop to measure absorbance at 260nm. RNA purity was assessed by the A260/A280 ratio (absorbance at different wavelengths) to assess for contamination.

3.14 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RT-PCR for first strand cDNA preparation was performed using the AffinityScript Multiple Temperature cDNA synthesis kit (Stratagene: 200436). Between 1ng and 5µg of total RNA was used per reaction; this was standardised for each experiment for comparison between samples. Random primers were added and the reaction incubated at 65^{o}C for 5 minutes and then cooled to room temperature to allow the primers to anneal. Further components were added (including AffinityScript RT Buffer, dNTP mix, RNase Block Ribonuclease Inhibitor and AffinityScript Multiple Temperature RT) and the reaction was incubated at 25^{o}C for 10 minutes to extend the primers. The reaction temperature was increased for cDNA synthesis at 42-55^{o}C for 60 minutes. The reaction was stopped by incubation at 70^{o}C for 15 minutes. The prepared cDNA was then stored at -20^{o}C until further use.

3.15 Real time PCR

Real-time PCR was carried out using an Applied Biosciences (Step One Plus) machine. *Gapdh* was used as the endogenous housekeeping gene control in all experiments. SYBR Green JumpStart Taq ReadyMix (Sigma: S4438) was used with each reaction well containing 6.5µl SYBR Green Master Mix, 1µl forward and reverse primer mixture (10mM), 3.5µl H_2O and 2µl cDNA (20ng). ROX reference dye was added to each well. Each sample was processed in triplicate and a no template control for each primer set was used to check for primer dimer formation. Results were normalized to the expression of *Gapdh* and relative gene expression was calculated using the formula ΔΔCt = 2^(Ct of reference gene - Ct of gene of interest).

3.15.1 Primer assessment

Appropriate samples were selected for primer optimisation: housekeeping genes *Gapdh* and *18S*, and *CCSP*: BEAS-2B cDNA. TLR genes: PMA-differentiated THP-1 cDNA - this cell type known to express the relevant TLRs (Zarember and Godowski, 2002). An example of the results obtained is shown in Figure 4 for testing of GAPDH and 18S primers in BEAS-2B cell samples. To assess primer efficiency real-time PCR was performed using serial dilutions of the cDNA template and measured Ct values (threshold cycle) were plotted against a Log scale of the concentrations used. The equation of the straight line generated was calculated. Amplification efficiency was then calculated from the slope of the standard curve. Melting curves were reviewed for each reaction, to ensure an individual peak. In addition, PCR products were analysed by separation using a 2% agarose gel with 0.5µg/ml ethidium bromide and a DNA ladder (TrackIt 1Kb DNA ladder, Invitrogen: 10488072). Gels were run at 100V constant for approximately 30 minutes, until sufficient band separation was achieved, and imaging performed under UV light using the SynGene G:BOX (Figure 4C).

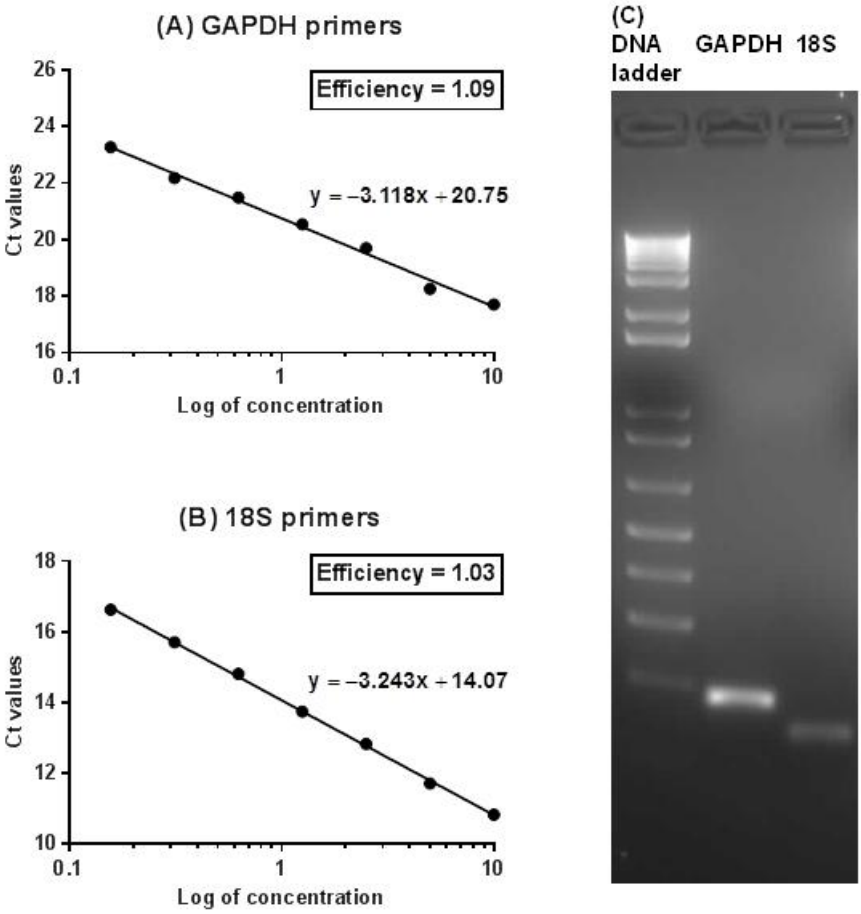


Figure 4 Primer optimisation
Real-time PCR was performed using serial dilutions of BEAS-2B cDNA template amplified with GAPDH and 18S primers in triplicate. Mean Ct values for (A) GAPDH and (B) 18S primer results were plotted against a log scale of the concentrations tested and a standard curve generated. Amplification efficiency was calculated based on the slope of the standard curve. (C) PCR products were separated on an agarose gel, to ensure presence only of one product.

3.15.2 Choice of housekeeping gene

Gapdh was the housekeeping gene routinely in use in our laboratory. However, this had not previously been studied in primary airway epithelial cells. To ensure that this was suitable for use these cells, in particular that expression was consistent across cell types (nasal, large and small airway), and that this was not affected by cell treatments (in particular CSE), a baseline analysis was undertaken. A second housekeeping gene, *18S*, was also analysed. No significant difference was detected in Ct values between cell types or treatments (2 way ANOVA) (Figure 5).

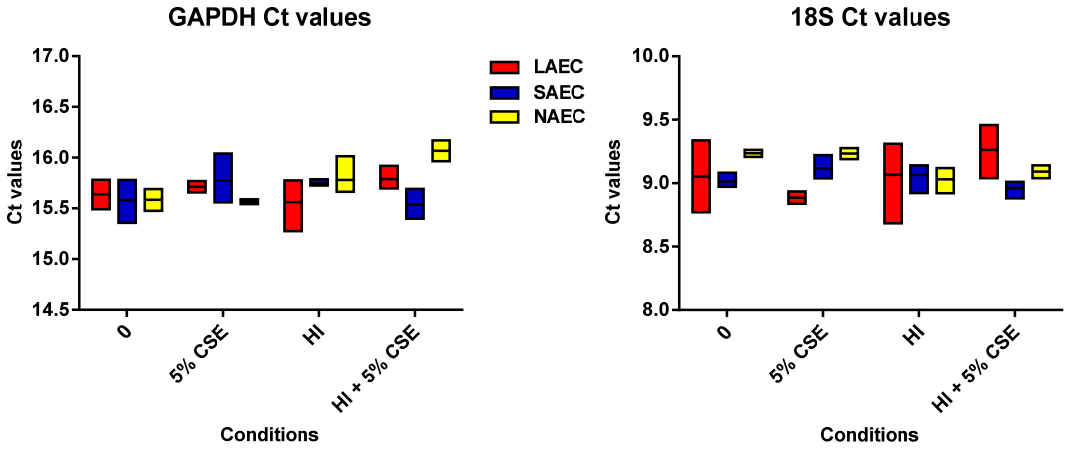


Figure 5 Choice of housekeeping gene for analysis of mRNA expression
Real-time PCR was performed using cDNA from NAEC, LAEC and SAEC from 1 COPD donor. Triplicate samples for each condition tested were amplified with GAPDH and 18S primers (with a mean of 3 Ct values generated for each sample *i.e.* in technical triplicates). Ct values for (A) GAPDH and (B) 18S were plotted (line at the mean).

3.16 Protein assay

Cells were harvested into phosphosafe extraction buffer (Novagen: 712964) and stored at -80^{o}C prior to analysis. For protein analysis cells were disrupted by sonication (x3) (Branson sonifier 150). Protein concentration was determined using a BCA protein assay kit (Thermo Fisher Scientific: 23225). Samples were prepared by adding 5µl of unknown sample to 80µl of sterile 1xPBS. Assay standards of BSA were prepared at concentrations of 0, 25, 125, 250, 500, 100, 1500 and 2000µg/ml in 1xPBS. Samples and standards were loaded in triplicate into a 96 well microplate (25µl/well) and 200µl of BCA working reagent added. The plate was incubated at 37^{o}C for 30 minutes prior to being read at 570nm. A standard curve was plotted. Protein concentrations of the unknown samples were calculated using the equation of the line generated by the standard curve.

3.17 Western blotting

Cell lysates at protein quantities of 10-60µg/well were used. Samples were prepared by adding appropriate volumes to NuPAGE sample loading buffer (Invitrogen, Paisley, UK: NP0007) with β-Mercaptoethanol (in a 2:1 ratio) and denatured at 100^{o}C for 5 minutes. Samples were loaded on a NuPAGE 4-12% Bis-Tris Gel (Invitrogen, NP0321). See-Blue Plus 2 Pre-Stained Standard (Invitrogen: LC5925) was used as a reference. Gels were run at 100V constant for 1.5-2 hours using 1x NuPAGE MES SDS running buffer (Invitrogen, NP0002).

For separation of lower molecular weight proteins a 16% gel was prepared with separating and stacking gel preparation:

Gel	Components	Volume
Separating (30ml)	30% acrylamide/0.8% bisacrylamide	16ml
	1M Tris-HCl, pH 8.9	7.5ml
	H_2O	6.2ml
	10% SDS	200µl
	10% ammonium persulfate	90µl
	TEMED	20µl
Stacking (15ml)	30% acrylamide/0.8% bisacrylamide	2ml
	1M Tris-HCl, pH 6.8	3.75ml
	H_2O	9.1ml
	10% SDS	37.5µl
	10% ammonium persulfate	67.5µl
	TEMED	22.5µl

Table 10 Acrylamide gel preparation (16%)

The separating gel was cast and saturated butanol used to cover the surface to ensure an even clean gel. Once set the butanol was washed off with water and the stacking gel added. Samples were prepared and gels run as outlined above. SDS-PAGE running buffer was used (0.25M Trizma HCl, 1.92M Glycine, 1% SDS, pH 8.3).

Gels were transferred for 2 hours onto Hybond-P PVDF membrane (GE Healthcare Life Sciences, Buckinghamshire, UK : RPN303F), in 1x transfer buffer (0.025M Tris, 0.192M Glycine) containing 20% methanol, at 200mA constant. Following transfer, membranes were blocked using 5% BSA in 1xTBST (Tris 20mM, NaCl 150mM, 0.05% Tween-20, pH 7.5) for 1 hour. Primary antibody was added in 5% BSA in 1xTBST overnight at 4^{o}C. Three 10 minute washes in 1xTBST were performed prior to addition of secondary antibody in 5% BSA in 1xTBST for 1.5 hours. Three further 10 minutes washes in 1xTBST were performed prior to placing membranes in ECL (SuperSignal West Pico Chemiluminescent Substance, Thermo Scientific, 34078) for 2 minutes. Images were acquired using a SynGene G:BOX with GeneSnap application.

3.18 Enzyme linked immunosorbent assay (ELISA)

Details of the antibodies and reagents used are shown in Table 11. 96 well microplates were prepared by coating the wells with capture antibody overnight. Wells were washed (x3) with wash buffer and blocking buffer added for 1 hour. Standards were prepared in reagent diluent at concentrations of 0, 31.25, 62.5, 125, 250, 500, 1000 and 2000pg/ml, to generate a standard curve. Samples were prepared by diluting collected media in reagent diluent. Wells were washed (x3) prior to loading of standards and samples (50µl/well). The plate was incubated for 2 hours at room temperature. Wells were washed (x3), detection antibody added, and the plate incubated for 2 hours at room temperature. Wells were washed (x3) and strepatividin-HRP added for 20 minutes in the dark at room temperature. Wells were washed (x3) and substrate solution was added for up to a further 20 minutes in the dark, before the reaction was stopped. Plates were read at 450 nm on the microplate reader to determine optical density. Sample cytokine concentrations were calculated using the equation of the straight line generated by the standard curve.

Antibody/Reagent/Cat. No.	Description/antibody plate coating
IL-8 Duoset (DY208)	Capture antibody: 4µg/ml in 1xPBS Detection antibody: 20ng/ml in reagent diluents
Wash buffer	1xPBS, 0.05% Tween-20
Blocking buffer	1xPBS, 1% BSA (Sigma: A7906)
Reagent diluents	1xTBST (Tris 20mM, NaCl 150mM, 0.05% Tween-20, pH 7.5), 0.01% BSA
Streptavidin-HRP (DY998)	1/200 dilution in reagent diluents
Substrate solution (DY999)	Hydrogen peroxide and Tetramethylbenzidine
Stop solution	2N H_2SO_4

Table 11 Antibodies and reagents used for ELISA
(From R&D systems, Abingdon, UK, unless otherwise stated)

3.19 Meso Scale Discovery assay cytokine analysis

Meso Scale Discovery (MSD®) assays were used to allow detection of multiple cytokines in BAL and serum samples, and in selected tissue culture media samples from specific cell treatments. These assays use a sandwich immunoassay format, with capture antibodies coated onto wells in a MULTI-SPOT® 96 well plate with a working electrode surface. Samples are added to the wells, followed by detection antibodies which are labelled with an electrochemiluminescent compound. A read buffer is added and the plates are read using an MSD SECTOR® instrument for analysis; voltage applied to the plate causes the labels bound to the electrode surface to emit light. The intensity of light emitted is used to quantify the concentration of cytokine(s) present in the sample. An 8-point standard curve is generated using known concentrations of cytokine calibrators (0, 0.61, 2.4, 9.8, 39, 156, 625, 2500 pg/ml).

The MSD assays and analysis were performed by myself, with support from Dr John Butler, MSD applications scientist. All of the necessary diluents and antibodies were provided with the MSD plates. 25µl of diluent 2 was added to each well and the plates were sealed and incubated for 30 minutes on a plate shaker at room temperature. 25µl of sample was added, the plates were resealed, and incubated for 2 hours on a plate shaker, at room temperature. The plates were washed x3 with PBS-T (1xPBS, 0.05% Tween-20), followed by addition of 25µl detection antibody solution. The plates were sealed and kept at 4^{o}C overnight. The following morning the plates were placed at room temperature on the plate shaker for 2 hours. The plates were washed x3 with PBS-T prior to the addition of 150µl of read buffer to each well. The plates were then read using the SECTOR® imager. The MSD DISCOVERY WORKBENCH® analysis software was used for generation of standard curves and calculation of cytokine concentrations. The lower limits of detection (calculated concentration which is 2.5 standard deviations above the zero calibrator) for each cytokine assay are detailed in the relevant results sections.

For the BAL and tissue culture samples, 1% BSA was added to the samples prior to analysis to minimise loss of analyte by adsorption to the labware being used. The

calibrators used for these samples were also were diluted in 0.9% Saline for the BAL samples, or appropriate media type for the tissue culture samples, with 1% BSA added. For BAL and serum analysis, MSD ultra-sensitive human cytokine assay 7-plex plates were used for detection of IL-8, IL-6, IL-1β, TNF-α, IL-10, IFN-γ, IL-12p70. For tissue culture media analysis, MSD tissue culture cytokine assay 4-plex plates were used for detection of IL-6, IL-1β, TNF-α and IFN-γ.

3.20 Statistical analysis of results

Clinical and biological data are described as median values with interquartile range; analysis was performed using non-parametric Mann-Whitney U tests. Correlation analysis of the bacterial microbiome data were performed using non-parametric Spearman tests. Data from cell culture experiments involving cell lines are all expressed as mean ± SEM, unless otherwise stated. Primary cell culture data is also expressed as mean ± SEM; results are expressed as relative to the "no treatment control" values for each sample/experiment to allow more accurate comparisons of data between individuals/groups. Changes in viability, proliferation and mediator release in response to treatments were compared to untreated controls. Paired T-tests were used to test for statistical differences between results unless otherwise indicated. A p value of <0.05 was considered significant. Bland and Altman plots were used as described to further illustrate the overall pattern in results observed (details of this are included in Chapter 6 in explanation of the relevant figures) (Bland and Altman, 1986). Statistical analysis was performed using GraphPad PRISM 6 software unless otherwise stated.

The pilot data generated from BAL parameters measured in this study was used for power calculations to inform future research planning. Following input from a statistician (Dr Kim Pearce, University of Newcastle), this was analysed for levels of IL-8, IL-6 and neutrophil %, for a 2 sample T test using the SAS 9.4 programme. The results from these calculations are shown and discussed in section 4.8.

Chapter 4. Assessment of airway and systemic inflammatory indices, and bacterial airway colonisation in samples from COPD and healthy control donors

4.1 Introduction

COPD is known to be associated with the presence of an increased inflammatory cell infiltrate and mediators, which have previously been investigated in sputum and BAL samples (Keatings et al., 1996, Riise et al., 1995). The presence of potentially pathogenic micro-organisms may contribute to the persistence of inflammation in the COPD airway despite smoking cessation (Soler et al., 1999). It has now been established that bacteria are present in the airway in health and disease (Zakharkina et al., 2013). New culture-independent microbiological techniques are allowing us to investigate the presence of bacteria in the airway, and to consider how this may impact on disease pathogenesis. The lung microbiome of never smokers, healthy smokers without evidence of airflow obstruction, and patients with COPD has been investigated (Erb-Downward et al., 2011). However, the relationship between bacterial presence as detected by culture-independent techniques in BAL samples, and airway and systemic inflammation has not been considered.

The aim of this work was to evaluate associations between airway bacterial colonisation and inflammation in a cohort of well characterised COPD patients compared to healthy controls. To gain further insight into this I have undertaken a detailed characterisation of a cohort of stable COPD patients and healthy controls. In particular, to investigate the association of bacterial presence with inflammatory indices in BAL and serum, and consider if additional information gained from culture-independent techniques is of importance to airway and systemic inflammation in the stable disease state. In order to investigate this, bronchoalveolar lavage samples (BAL) were analysed from patients with stable COPD and healthy control donors. Assessment included differential cell counts, cytokine profile, standard microbiology culture and culture-independent bacterial population analysis. Paired blood and serum samples were also analysed for differential white cell count, CRP and cytokine profile. Where possible, sputum samples were obtained for standard microbiology culture.

4.2 Study screening and recruitment outcomes

Patients were recruited through the Sir William Leech Research Centre at the Freeman hospital. The centre has an established database of COPD patients which was reviewed. Patient details would be added to this database with their consent having received information about the research centre from local outpatient clinics, through GP-led recruitment, and through response to poster advertisements in the local hospital trust. Prior to patient contact, pre-screening was performed of records where possible to check inclusion and exclusion criteria. Healthy controls were recruited through poster advertisements in the local hospital trust and at the university site.

The following flow chart (Figure 6) details the screening and recruitment process. Recruitment commenced on 7th February 2011 and the final participant was recruited on 3rd September 2013. Of 206 patients pre-screened for study participation, 76 were potentially eligible, and of this number 35 participants were enrolled in the study. In total 19 COPD patients and 11 healthy control participants completed the study. Table 12 also provides details of the reason why patients screened for the study were not eligible (based on the inclusion and exclusion criteria as detailed in the study protocol section).

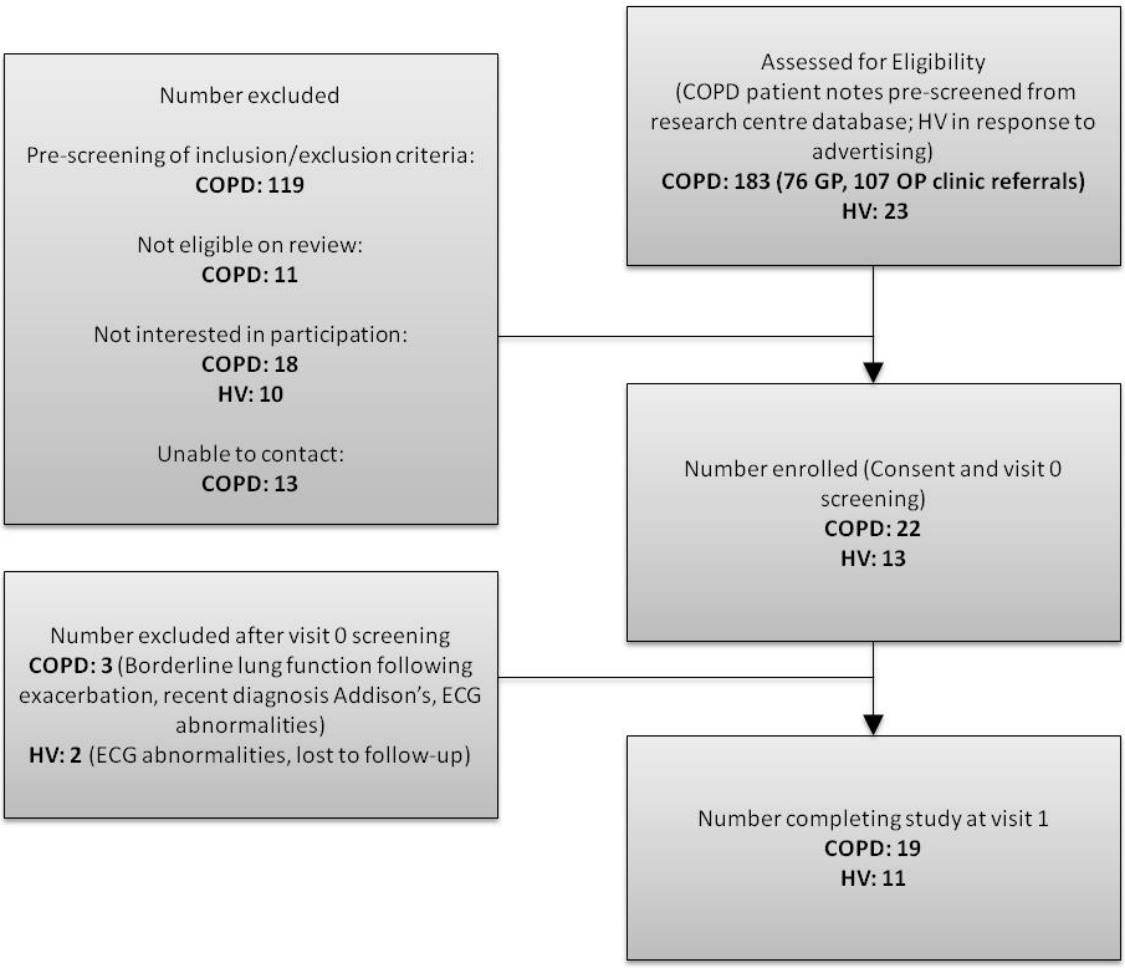


Figure 6 Study screening and recruitment summary flow chart
This flow chart outlines the number of COPD patients pre-screened from research database records and the number of healthy volunteers (HV) responding to advertisements. Details of numbers ineligible due to inclusion/exclusion criteria are shown. Following enrolment and completion of full study screening a further 3 COPD participants and 2 healthy controls were excluded as detailed, with GP/medical follow up planned as required.

Reason for ineligibility	Number of cases
FEV1<30% predicted	24
SpO2<92% or on LTOT	14
Other primary respiratory diagnosis	33
Co-morbidity - Cardiac disease	5
Co-morbidity - Malignancy	6
Co-morbidity - Other	12
In other research study	11
Age>80	25

Table 12 Reasons for potential participant ineligibility for study

Other primary respiratory diagnoses included: Asthma, Bronchiectasis, Fibrosis, lobar collapse, normal spirometry (4 GP patients), Asbestos-related pleural disease. Other co-morbidities included: active Crohn's disease on corticosteroids, active rheumatoid arthritis (RA)/psoriatic arthropathy on disease modifying anti-rheumatic drug treatment, RA with cervical instability/neck collar, diabetes on insulin, cerebrovascular disease on dual antiplatelet agents, recent cataract surgery, recent emergency aortic aneurysm repair, significant anxiety/depression.

4.3 Study participant demographics

Details of the participant demographic information are shown in Table 13 and Table 14. Both study populations were predominantly male (COPD 13M/6F; control 6M/5F), and well matched for age (COPD median age 63, range 47-75; control median age 55; range 42-72, $p>0.05$). Eight of the COPD population were current smokers (8 current, 11 ex), compared to only 1 of the control population (1 current, 5 ex, 5 never). Urine cotinine levels were checked to compare to reported smoking history (level >500µg/l consistent with current smoking); all correlated except for COPD subject 13 (reported ex-smoker, level 2809µg/l).

The COPD population had mostly moderate to severe airflow limitation based on GOLD criteria (2 mild, 10 moderate, 7 severe, see Table 15). Most also had evidence of hyperinflation with increased lung volumes due to airflow obstruction (16/19). Gas diffusion was reduced in 14/19 patients (TLCO) suggestive of the presence of underlying emphysema. Nearly all patients had exacerbated within the past 12 months (18/19), with 14 participants reporting 2 or more exacerbations. MRC dyspnoea scores ranged from between 1 and 5 (mode 2, see Table 16). Further quality of life assessment using the COPD assessment test (CAT) questionnaire revealed mainly medium and higher impact level of COPD-related symptoms (4 low, 10 medium, 1 high, 4 very high). Current COPD treatments included a short-acting β2 agonist (18/19), a long-acting muscarinic antagonist (15/19) and long-acting β2 agonist and corticosteroid combination inhaler (15/19).

COPD participant 16 was confirmed as having mild COPD as per the GOLD criteria with presence of symptoms and a reduced FEV1/VC ratio. Healthy volunteer participant 9 had the same pattern of spirometry but no symptoms and very minimal smoking history, and therefore did not meet criteria for a diagnosis of COPD.

Measures of small airway function reported include RV/TLC ratio and $FEF_{25-75\%}$. RV/TLC changes with age and gender and therefore the % predicted values are used to calculate this rather than the raw ratio, to give a more accurate result (Burgel, 2011). In assessment of lung volumes the “normal range” is often considered as between 80-

120%; however, consideration of upper 5th centile values shows that even healthy individuals can have predicted results which lie above this range based on reference equations (Ruppel, 2012). In the results shown, 3/18 COPD subjects had RV/TLC <120% (*i.e.* within the normal range), 5/18 had RV/TLC 120-140% (*i.e.* suggestion of air trapping), and 10/18 had values ≥140% (*i.e.* air trapping present). $FEF_{25-75\%}$ results show even greater variability with the lower limit of normal reported as <60% predicted at most ages (Burgel, 2011). In the COPD population studied 18/19 subjects had values of <40% predicted suggesting evidence of small airways disease.

Table 13 Study subject demographics and clinical characteristics - COPD patients

	Age	Gender	BMI	FEV1	FEV1 % pred	FVC	FVC % pred	FEV1 / FVC	GOLD	TLC	RV	RV /TLC	FEF 25-75%	TLCO	Smoking status	Pack years	Exacerbations in past year	MRC	CAT	LABA +ICS
1	63	M	29.5	1.14	37	3.09	79	37	Severe	109	175	161	10	65	Current	58	1	2	13	Y
2	65	M	29.2	1.91	52	3.77	80	51	Moderate	121	186	154	19	78	Current	48	2	2	14	N
3	69	M	24.5	1.2	47	2.43	74	49	Severe	101	121	120	18	71	Ex	40	3	2	17	Y
4	66	M	35.2	2.12	62	3.96	89	54	Moderate	134	182	136	29	74	Ex	42	2	3	19	Y
5	59	M	30.1	2.27	63	6.04	132	38	Moderate	125	143	114	21	77	Ex	20	3	3	18	Y
6	62	M	28.6	1.25	39	2.65	66	47	Severe	131	233	178	12	49	Ex	44	4	4	27	Y
7	70	F	23.5	1.14	57	2.59	107	44	Moderate	143	205	143	16	46	Ex	46	2	2	11	Y
8	62	F	38.5	1.34	60	2.35	88	57	Moderate	118	177	150	20	72	Ex	29	2	2	11	Y
9	52	M	31.9	1.25	37	3.03	73	41	Severe	129	252	195	14	49	Current	140	4	5	32	Y
10	57	M	29.7	1.32	46	3.3	92	40	Severe	121	182	150	15	81	Ex	40	5	2	34	Y
11	75	M	20.1	1.5	51	3.46	89	43	Moderate	102	138	135	15	82	Current	100	2	2	9	Y
12	50	M	18.8	2.41	77	4.53	115	53	Moderate	144	208	144	39	56	Current	76	4	4	40	Y
13	66	F	23.4	0.96	38	2.4	81	40	Severe	135	240	178	10	41	Ex	48	1	3	17	Y
14	48	M	37.8	2.85	72	4.2	86	68	Moderate	88	106	120	38	66	Current	40	3	3	31	N
15	62	M	31.6	2.63	81	4.16	100	63	Mild	98	113	115	33	119	Ex	34	1	1	6	Y
16	47	M	41.8	3.91	96	5.93	117	66	Mild	NA	NA	NA	62	80	Current	23	0	1	8	N
17	65	F	36	1.47	73	2.34	97	63	Moderate	111	145	131	20	49	Ex	54	8	4	19	Y
18	68	F	27.2	0.91	45	2.61	108	35	Severe	144	178	124	11	56	Ex	30	3	2	3	Y
19	65	F	27.8	1.25	65	2.16	94	58	Moderate	106	151	142	16	105	Current	33	1	2	14	N

Details: FEV1 (forced expiratory volume in 1 second), FVC (forced vital capacity), TLC (total lung capacity), TLCO (transfer factor for carbon monoxide - to assess diffusing capacity of the lungs for carbon monoxide), LABA (long-acting β2 agonist), ICS (inhaled corticosteroid). Note - both FEV1 and FVC measured post-bronchodilator (Salbutamol 200µg). Lung volumes NA for subject 16 as BMI to high for plethysmography body box used. TLC, RV, RV/TLC, $FEF_{25-75\%}$ and TLCO all reported as % predicted values.

Table 14 Study subject demographics and clinical characteristics - Healthy volunteers

	Age	Gender	BMI	FEV1	FEV1 % pred	FVC	FVC % pred	FEV1/FVC	Smoking status	Pack years	MRC
1	48	F	24.2	2.25	92	2.58	90	87	Ex	0.5	1
2	64	F	22.9	2.42	103	3.11	111	78	Never	0	1
3	55	M	28.8	3.55	106	4.25	102	84	Ex	4	1
4	62	F	38.1	2.13	96	2.65	101	80	Current	42.5	2
5	44	M	22.9	4.85	117	6.89	135	70	Never	0	1
6	57	M	32.2	3.72	126	5.15	140	72	Never	0	1
7	42	M	30.3	4.41	115	5.78	124	76	Never	0	1
8	55	F	37.1	2.02	86	2.28	82	88	Never	0	2
9	54	M	23.3	2.98	90	4.51	110	66	Ex	0	1
10	70	M	30.7	3.19	108	4.58	120	70	Ex	25	2
11	72	F	32.8	2.15	110	2.73	116	79	Ex	15	2

Details: FEV1 (forced expiratory volume in 1 second), FVC (forced vital capacity). Note - both FEV1 and FVC measured post-bronchodilator (Salbutamol 200µg)

Post-bronchodilator FEV1/FVC	FEV1 % Predicted	Severity of airflow obstruction
< 0.7	≥ 80%	Stage I - Mild*
< 0.7	50-79%	Stage II – Moderate
< 0.7	30-49%	Stage III – Severe
< 0.7	<30%	Stage IV - Very severe**

Table 15 GOLD criteria for assessment of severity of airflow obstruction
(*with symptoms, **or FEV1 < 50% with respiratory failure) (Global Initiative for Chronic Obstructive Lung Disease (GOLD), 2010)

Grade degree of breathlessness related to activities
1. Not troubled by breathlessness except on strenuous exercise
2. Short of breath when hurrying or walking up a slight hill
3. Walks slower than contemporaries on level ground because of breathlessness, or has to stop for breath when walking at own pace
4. Stops for breath after walking about 100 m or after a few minutes on level ground
5. Too breathless to leave the house, or breathless when dressing or undressing

Table 16 Medical Research Council (MRC) dyspnoea scale
Referenced in (National Institute for Health and Clinical Excellence (NICE), 2010)

4.4 Complications and adverse events

Of note 2 COPD patients had positive sputum samples at study screening but both were clinically stable with no signs or symptoms of acute infection; therefore it was still possible to proceed to bronchoscopy in these subjects (COPD donors 11 and 12, see Table 17). All bronchoscopy procedures were completed without any immediate complications. In COPD donor 3, an area of mucosal abnormality was observed (right main bronchus). This finding was referred back to the consultant responsible for this participant's medical care, to ensure the necessary further investigations and follow up could be arranged. On further investigation and review of this (including imaging and repeat bronchoscopy) there were no findings of concern which would influence use of this donor's samples in the study analysis. All participants were contacted on the day following the bronchoscopy procedure to ensure that no further complications had arisen. In addition, where microbiology samples were reported as positive participants were contacted on receipt of these results, to ensure that they had not developed any symptoms of possible exacerbation.

Two COPD patients attended the study centre for review following the bronchoscopy procedure:

- COPD donor 6 presented with increased cough and sputum production with some persisting streaks of haemoptysis 8 days post-bronchoscopy. The patient was clinically well with no new chest x-ray (CXR) changes on review. This participant was treated for a presumed infective exacerbation of COPD. This participant had later negative standard microbiology results. Clinic follow up was arranged with the consultant responsible for medical care.
- COPD donor 12 presented with fever, cough and pleuritic chest pain 1 day post-bronchoscopy. This participant had a positive sputum result (*S. pneumoniae*) at screening but had been clinically stable. Repeat sputum sampling on the day of bronchoscopy cultured *S. pneumoniae*, *H.influenzae* and *M.catarhallis*. BAL also cultured *S. pneumoniae*. CXR showed some new right sided infiltrates, thought most likely to represent post-BAL inflammatory change. This participant was managed with oral antibiotics and analgesia, and clinic follow up arranged with the consultant responsible for medical care, including repeat CXR.

4.5 Details of bronchoalveolar lavage processing

A standard BAL approach was taken instilling 3x60ml (total 180ml) 0.9% saline into the right middle/lower lobe and collecting the return on low suction. The only exceptions to this were left lower lobe samples from the following donors due to findings at bronchoscopy and also reports from previous CXRs: COPD donor 3 (right middle lobe possible endobronchial abnormality observed as detailed above); COPD donor 11 (history of previous tuberculosis with residual right sided pleural changes on previous CXR review); healthy control donor 1 (history of previous pneumonia with tethering of right hemidiaphragm noted on previous CXR report).

Results of BAL processing including total return volumes and differential cell counts are shown in Table 17 and Table 18. The BAL return in the COPD group was lower than in controls (COPD median 23ml (IQR 16.5-35); control median 50ml (IQR 40-61), $p<0.05$) though this may relate to severity of disease in the study cohort; BAL return may be correlated with the degree of emphysema as assessed by methods including TLCO (Lofdahl et al., 2005). In addition, epithelial cell counts were high in a number of the BAL samples processed. To assess BAL quality a count of epithelial cells (ciliated and squamous) is recommended; if this is greater than 5% of the total cell count, the sample is unlikely to be representative of alveolar contents (Haslam and Baughman, 1999). These factors need to be taken into consideration when interpreting this data.

Table 17 BAL (and sputum) sample results - COPD patients

Study No	BAL volume (total return ml)	Total cell count (x10^4 cells/ml)	Macrophages (%)	Neutrophils (%)	Lymphocytes (%)	Eosinophils (%)	% Epithelial cells	Sputum	Microbiology (standard C&S)
1	15	8.02	95.8	2.2	1.8	0.2	0.8	N	Negative
2	11	1.76	67.6	18.9	8.7	4.7	2.8	S	Negative
3	23	10.82	60.2	10.2	29.4	0.4	2.2	N	Negative
4	28	4.25	62.8	11.6	23.4	2.2	17.9	B	Sputum (B) *M.Catarhallis*
5*	13.5	0.9	61	22	11	7	49.2	S, B	BAL *M.Catarhallis*
6*	22	0.44	NS	NS	NS	NS	NS	S, B	Negative
7*	49	35.2	70.2	10.8	18.2	0.8	2.3	N	Negative
8	25	17.25	80.2	10.2	9.6	0	17.5	N	Negative
9	21.5	14.75	89	8.4	2.2	0.4	4.9	S, B	BAL *M.Catarhallis*
10	17	3.75	78.4	13	8.6	0	21.8	N	Negative
11	16.5	3.6	83.3	10.4	5.6	0.2	21.9	S, B	Sputum (S) *H.influenzae*
12*	10	2.29	38.4	50.9	8.8	1.9	85.8	S, B	Sputum (S) *S.pneumoniae*, Sputum (B) *S.pneumoniae*, *H.influenzae*, *M.catarhallis*; BAL *S.pneumoniae*
13*	52	2.1	58	35	7	0	21.5	N	BAL *H.influenzae* (scanty)
14	23	8.5	98.8	0.8	0.4	0	4.8	S, B	Negative
15	25	22.8	33.2	59.6	6.6	0.6	9.7	N	BAL *S.pneumoniae*, *H.influenzae*
16	60	21.75	99.2	0.6	0.2	0	3.8	N	Negative
17*	35	1.7	NS	NS	NS	NS	NS	B	BAL *E.coli* (scanty)
18	35	3.75	65	26	9	0	12.0	N	BAL *H.influenzae*
19	75	44.2	99.4	0.6	0	0	1.2	N	Negative

*Details: 5 - poor prep, few cells, haemorrhagic; 6 and 17 - too haemorrhagic to count, 7 and 13 - haemorrhagic, 12 poor prep, very difficult to count.
Sputum/microbiology results: N - no sample received, S - sample at screening visit, B - sample at bronchoscopy visit.

Table 18 BAL (and sputum) sample results - Healthy controls

Study No	BAL volume (total return ml)	Total cell count (x10^4 cells/ml)	Macrophages (%)	Neutrophils (%)	Lymphocytes (%)	Eosinophils (%)	% Epithelial cells	Microbiology (standard C&S)*
1	37	6.81	88.8	0	11	0.2	4.0	Negative
2	50	6.18	73.8	10.4	14.8	1	19.9	Negative
3	40	10.8	88	9.8	2	0.2	0.0	Negative
4	38	16.2	92	6.2	1.6	0.2	15.1	Negative
5	65	1.25	97	1.4	1.6	0	15.7	Negative
6	55	5.33	95.8	3.8	0.4	0	4.4	Negative
7	60	18.45	99.2	0	0.8	0	3.8	Negative
8	65	26.75	96	2	2	0	0.2	Negative
9	61	7.1	100	0	0	0	2.9	Negative
10	45	6.9	6.2	52.2	40.4	1.2	33.9	Negative
11	50	9.05	82	4.6	13.4	0	30.5	Negative

Details: Sputum sample received from donor 5 only (at screening visit). Remainder all BAL samples only.

4.6 Review of bronchoalveolar lavage, full blood count and microbiology results

Data is presented in the following tables as median values with interquartile range (IQR). Total cell counts ($x10^4$/ml) were not significantly different between the COPD and control groups (COPD median 4.25 (IQR 2.1-17.25), control median 7.1 (IQR 6.18-16.2), $p>0.05$) (Table 19). In the COPD samples, the BAL return volume correlated with the total cell count; greater return was associated with higher cell count (Spearman correlation, $r=0.493$, $p=0.032$). No correlations were found between either BAL return volume or total cell count and FEV1 or TLCO, suggesting that this observation was not related to these clinical measures of disease severity. In the control samples, there was no relationship between total BAL return and total cell count.

In terms of differential cell count there was no significant difference in macrophage, lymphocyte or eosinophil counts, but neutrophils were increased in the COPD samples compared to control samples (COPD median 10.8 (IQR 5.3-24), control median 3.8 (IQR 0-9.8), $p<0.05$). Analysis of data from ex-smokers only showed a lower macrophage % in the COPD group, with a higher neutrophil % compared to the control group (Table 20). Within the COPD group, current smokers had higher % macrophages with lower % neutrophils and lymphocytes compared to ex-smokers (Table 21). Positive microbiology was associated with higher neutrophil counts (Table 22). None of the control BAL samples were positive on standard microbiological culture.

In terms of blood white cell counts (WCC), total WCC was raised in COPD donor sample 14; total WCC $13.8x10^{-9}$/l, neutrophils 8.2, lymphocytes 3.9, monocytes 1.3, eosinophils 0.3, CRP 21mg/dl. Standard sputum and BAL cultures were negative from this donor. In the healthy control samples, two (donors 1 and 7) had low WCC with reduced neutrophil number (donor 1 total WCC $3.5x10^{-9}$/l, neutrophils 1.08; donor 7 WCC $3.1x10^{-9}$/l, neutrophils 1.32). No obvious cause was found for these results (both discussed with Haematology and referred back to the GP). The differential white cell counts for all other COPD and healthy control donors were in the normal ranges (data not shown).

Table 19 Details of BAL differential cell counts

	COPD (n=19)*	Control (n=11)	Significance
Total BAL return (ml)	23 (16.5-35)	50 (40-61)	**p=0.0006**
Total cell count (x10^4/ml)	4.25 (2.1-17.25)	7.1 (6.18-16.2)	p>0.05
Macrophage %	70.2 (60.6-92.4)	92 (82-97)	p=0.08
Neutrophil %	10.8 (5.3-24)	3.8 (0-9.8)	**p=0.03**
Lymphocyte %	8.6 (2-10.3)	2 (0.8-13.4)	p>0.05
Eosinophil %	0.2 (0-1.35)	0 (0-0.2)	p>0.05

*COPD group n=19 for total return and cell count, only n=17 for differential cell counts due to processing issues with haemorrhagic samples from COPD subjects 6 and 17. Data presented as median value (IQR).

Table 20 BAL Ex-smokers data only

	COPD (n=11)*	Control (n=10)	Significance
Total BAL return (ml)	25 (22-35)	52.5 (43.75-62)	**p=0.0003**
Total cell count (x10^4/ml)	3.75 (1.7-17.25)	7 (5.968-12.71)	p>0.05
Macrophage %	62.8 (59.1-74.3)	92.3 (79.95-97.55)	**p=0.0041**
Neutrophil %	13 (10.5-30.5)	2.9 (0-9.95)	**p=0.0027**
Lymphocyte %	9.6 (7.8-20.8)	2 (0.7-13.75)	p>0.05
Eosinophil %	0.4 (0-1.5)	0 (0-0.4)	p>0.05

*COPD group n=11 for total return and cell count, only n=9 for differential cell counts as above. Data presented as median value (IQR)

Table 21 BAL COPD current versus ex-smokers data

	Current smokers (n=8)*	Ex-smokers (n=11)*	Significance
Total BAL return (ml)	19 (12-50.75)	25 (22-35)	>0.05
Total cell count (x10^4/ml)	8.26 (2.68-20)	3.75 (1.7-17.25)	>0.05
Macrophage %	92.4 (71.53-99.1)	62.8 (59.1-74.3)	**p=0.0152**
Neutrophil %	5.3 (0.65-16.78)	13 (10.5-30.5)	**p=0.043**
Lymphocyte %	2 (0.25-7.925)	9.6 (7.8-20.8)	**p=0.0025**
Eosinophil %	0.2 (0-1.525)	0.4 (0-1.5)	p>0.05

*Numbers for total return and cell count, differential cell counts in n=8 current/n=9 ex-smokers. Data presented as median value (IQR).

Table 22 BAL COPD data for positive and negative microbiology samples

	Positive microbiology (n=9)*	Negative microbiology (n=10)*	Significance
Total BAL return (ml)	25 (15-35)	23 (16.5-48.75)	p>0.05
Total cell count (x10^4/ml)	3.6 (1.9-9.5)	9.660 (3.253-25.11)	p>0.05
Macrophage %	61.9 (43.3-78.73)	80.2 (68.9-99)	**p=0.0274**
Neutrophil %	24 (10.7-46.93)	10.2 (0.7-11.9)	**p=0.01**
Lymphocyte %	7.9 (5.85-10.5)	8.6 (0.3-13.9)	p>0.05
Eosinophil %	0.5 (0.05-2.125)	0 (0-0.6)	p>0.05
Current smokers	3	5	-

*Numbers for total return and cell count, differential cell counts in n=8 positive/n=9 negative. Data presented as median value (IQR).

4.7 Bronchoalveolar lavage and serum cytokine analysis

An MSD ultrasensitive 7-plex assay was used for analysis of cytokine profile in stored BAL supernatant and serum samples. Measurement of the described panel was chosen as this includes mediators which have been shown to be elevated in clinical samples from smokers and patients with COPD in previous research, and may therefore be important in disease pathogenesis (as discussed in Chapter 1, and reviewed in (Barnes, 2009) and (Thorley and Tetley, 2007). The assay used allowed detection of low levels of cytokines as detailed with the lower limit of detection (LLD) for each analysis parameter (based on 2.5 standard deviations above the zero calibrator generated for each experiment). Details of the results are shown in the tables and figures below; data is presented as median values (with interquartile range). Levels of IL-8, IL-6 and IL-1β were readily detectable in BAL. Data for TNF-α has also been included, though this was only within detection limits in 8 COPD BAL samples. In serum, IL-8, IL-6, TNF-α and IL-10 were detectable.

4.7.1 Detection of pro-inflammatory cytokines in bronchoalveolar lavage

In BAL, levels of IL-6 and IL-1β were significantly higher in samples from COPD patients compared to controls (see Table 23 and Figure 7). When analysing COPD ex-smoker and control (ex-smoker or never smoker) data only, levels of IL-8 and TNF-α were also significantly different (see Table 24 and Figure 8). For the COPD results only, taking into account smoking status, BAL TNF-α was increased in COPD ex-smokers compared to current smokers (Table 25). There was a trend for all BAL cytokine levels to be higher in ex-smokers compared to current smokers.

On review of positive versus negative standard microbiology results, no significant differences were observed in the COPD BAL samples mediator results (Table 26). The data displayed are for all positive samples including sputum and BAL cultures with only "scanty growth". Further analysis with the positive group as only those with positive BAL samples compared to negative BAL samples, or with the positive group including only BAL samples without scanty growth compared to all other samples, did not show any difference in findings compared to the results shown. On review of other disease features including FEV1, exacerbation frequency and CAT score, no relationships were found with BAL cytokine results.

No relationship was found between COPD BAL return or total cell count and either IL-8 or IL-6 levels. However, a positive correlation was found between COPD BAL return ($r=0.495$, $p=0.031$) or total cell count ($r=0.525$, $p=0.021$) and IL-1β levels, *i.e.* greater return/total cell count associated with greater IL-1β levels. The significance of these findings is unclear but it is possible that the increased levels of IL-1β observed were primarily due to the increased number of inflammatory cells collected in these samples, rather than the return volume.

A negative correlation was detected between control BAL return and IL-8 ($r=-0.731$, $p=0.013$) and IL-6 ($r=-0.699$, $p=0.02$) levels, *i.e.* greater BAL return associated with lower cytokine levels. This trend was also observed for IL-1β results but was not statistically significant. No correlations were found between cytokine levels and total cell count. These findings suggest a possible impact of dilution on the control sample results. However, this pattern was not observed in the COPD samples. There may be differences in both the epithelial lining fluid sampled by lavage and lung permeability in healthy compared to diseased lungs which may impact on the sampling results (Haslam and Baughman, 1999). Further consideration of methods of standardising BAL is discussed in section 4.12.5.

Of note there were a small number of COPD samples with particularly high cytokine levels as shown in Figure 7. On review of these data points the sample populations were similar for each of the cytokines tested; higher levels were detected in samples from a more selected population from the COPD group. On review of the BAL return and total cell counts from these samples, no relationship was found with the cytokine levels detected. However, a number of subjects in this group had haemorrhagic BAL noted on analysis; this may have influenced cytokine levels due to local airway epithelial injury on sampling (study numbers 6, 7, and 13). The subjects in the group had a range of disease severity with subjects in mild, moderate and severe GOLD categories. Other features included current smoking status (study numbers 11 and 19), and positive standard microbiology results (study numbers 11, 13 and 15). All except study subject 19 were on an inhaled corticosteroid.

BAL	LLD (pg/ml)	Samples below LLD	COPD (n=19)	Control (n=11)	Significance
IL-8	0.173	None	142.4 (45.7-280)	111.5 (24.1-154.7)	p>0.05
IL-6	0.375	None	5.3 (3.0-19.6)	3.0 (0.7-4.9)	**p=0.0293**
IL-1β	0.377	1 control	1.7 (0.9-6.6)	0.6 (0.4-2.1)	**p=0.014**
TNF-α	0.783	11 COPD, 8 control	0.7 (0.2-1.7)	0.3 (0.2-1.0)	p>0.05
IL-10	9.83	16 COPD, 11 control	3.8 (1.6-8.9)	3.0 (2.0-5.6)	NA
IFN-γ	2.17	15 COPD, 11 control	1.1 (0.2-1.7)	0.4 (0-0.9)	NA
IL12p70	4.58	14 COPD, 11 control	2.5 (0.7-5.1)	0.5 (0-1.6)	NA

Table 23 BAL cytokine data (MSD ultrasensitive 7-plex assay results)

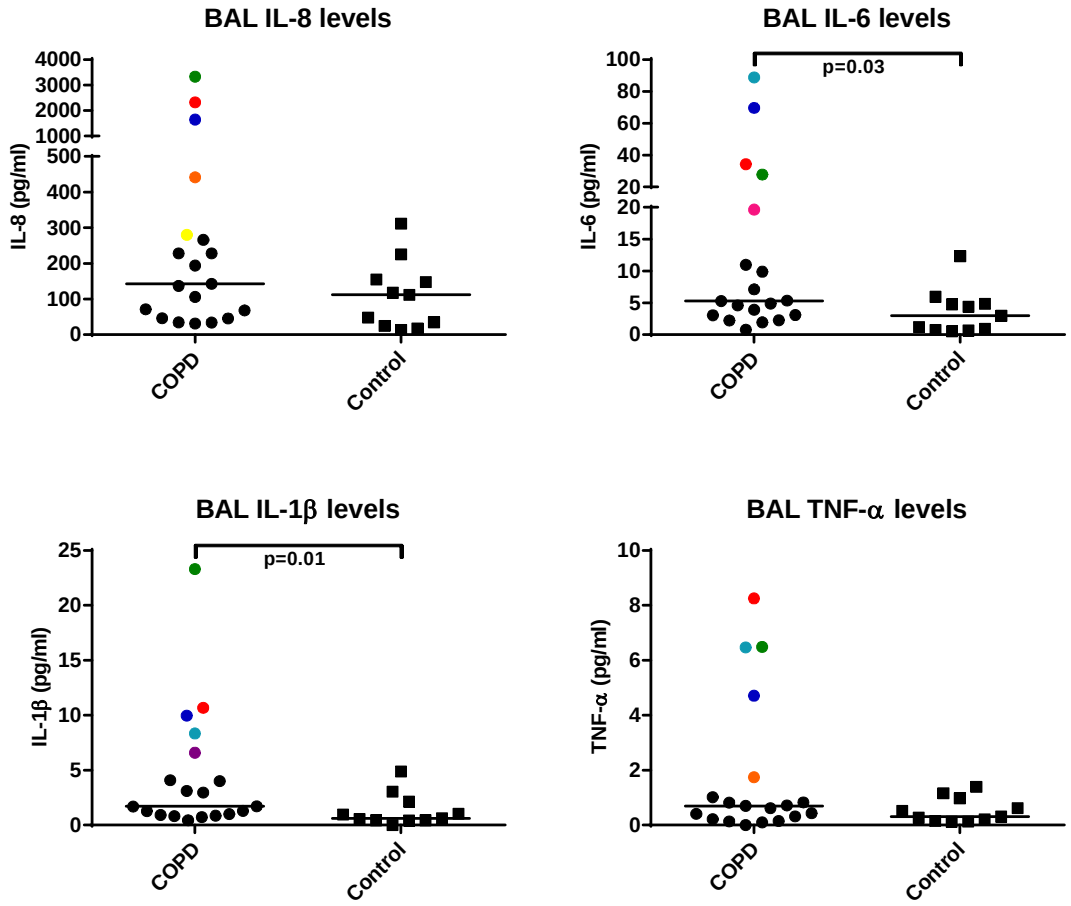


Figure 7 Cytokine levels detected in BAL samples from COPD and control donors

BAL supernatant samples were analysed using an MSD 7-plex ultrasensitive assay to detect levels of pro-inflammatory mediators present. These graphs show the results of this analysis from 19 COPD and 11 control samples; standards and samples were run in technical duplicates. Data are displayed as individual results with a line at the median value. The highest cytokine levels detected in COPD samples are labeled by study number to demonstrate that these are similar populations for each cytokine measured: study numbers 3 (red), 6 (royal blue), 7 (light blue), 8 (orange), 11 (pink), 13 (yellow), 15 (green), 19 (purple).

	COPD ex-smokers (n=11)	Control ex- or never smokers (n=10)	Significance
IL-8	266.2 (71.0-1646)	80.0 (22.3-149.3)	**p=0.02**
IL-6	5.3 (3.9-34.3)	2.1 (0.7-4.8)	**p=0.02**
IL-1β	3.0 (0.9-10.0)	0.6 (0.4-1.3)	**p=0.006**
TNF-α	0.8 (0.6-6.5)	0.3 (0.1-1.0)	**p=0.02**

Table 24 BAL cytokine data: Ex- or never-smokers only

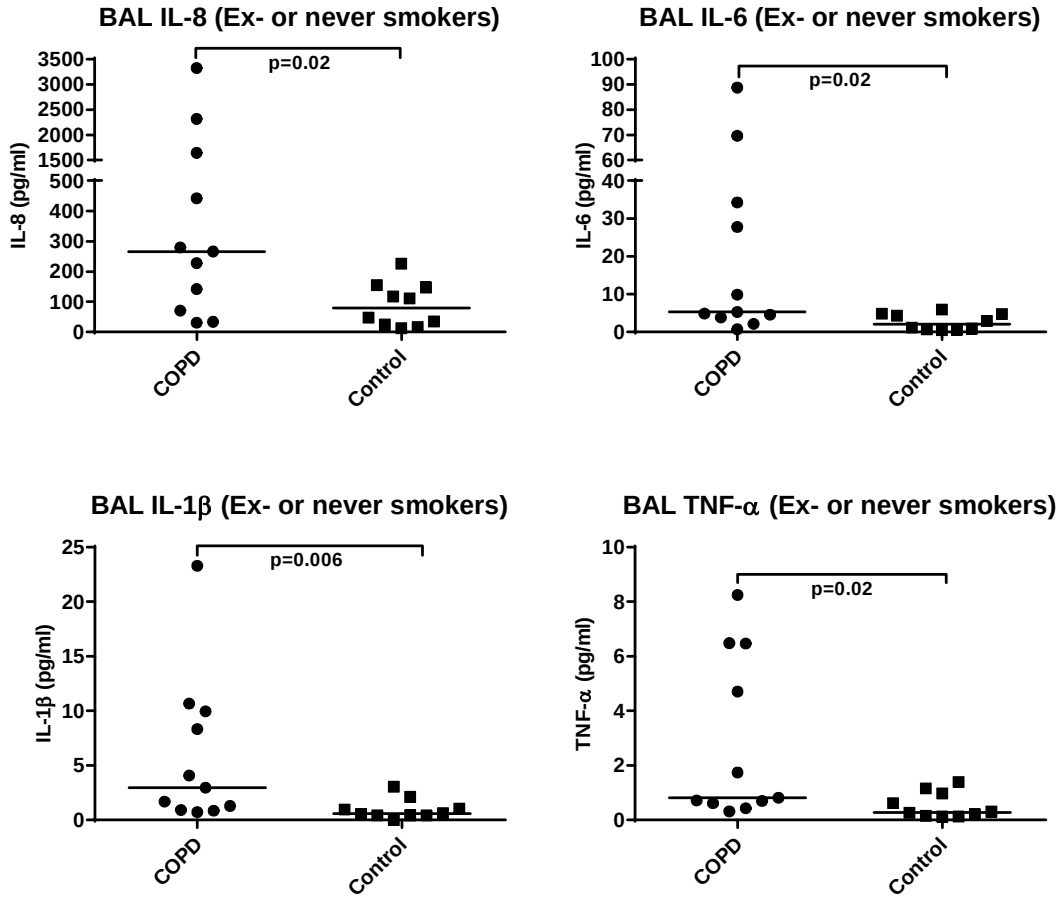


Figure 8 Levels of pro-inflammatory mediators in BAL samples from COPD and control donors using ex- or never-smokers data only

BAL supernatant samples were analysed using an MSD 7-plex ultrasensitive assay to detect levels of pro-inflammatory mediators present. This figure shows data from ex- or never (in the control group only) smokers only (to try to exclude any effect of current smoking on mediator levels); the data shown are from 11 COPD samples and 10 control samples. Standards and samples were run in technical duplicates. Data are displayed as individual results with a line at the median value.

Table 25 BAL cytokine data - COPD current and ex-smokers

	COPD current (n=8)	COPD ex (n=11)	Significance
IL-8	86.8 (45.6-180.0)	266.2 (71.0-1646)	p=0.09
IL-6	4.2 (2.4-10.0)	5.3 (3.9-34.3)	p>0.05
IL-1β	1.5 (0.9-3.8)	3.0 (0.9-10.0)	p>0.05
TNF-α	0.2 (0.1-0.7)	0.8 (0.6-6.5)	**p=0.009**

Table 26 BAL and serum cytokine data - COPD positive and negative microbiology

	COPD Positive micro (n=9)	COPD Negative micro (n=10)	Significance
BAL			
IL-8	194.2 (52.4-254.1)	139.7 (45.7-743.1)	p>0.05
IL-6	4.9 (3.1-15.3)	5.3 (2.8-43.1)	p>0.05
IL-1β	1.7 (0.8-3.5)	3.6 (0.9-8.7)	p>0.05
TNF-α	0.6 (0.4-0.8)	0.9 (0.1-5.1)	p>0.05
Serum			
IL-8	12.2 (10.7-13.5)	10.7 (8.1-13.6)	p>0.05
IL-6	1.9 (1.5-2.3)	2.3 (1.5-4.3)	p>0.05
TNF-α	4.3 (3.0-4.7)	3.4 (2.9-4.6)	p>0.05
IL-10	8.0 (6.3-12.6)	5.1 (3.7-9.3)	**p=0.04**

4.7.2 Serum results

In serum samples, IL-6 was significantly greater in COPD samples compared to controls (Table 27). In samples where serum CRP was raised (n=6 COPD samples, 3 current smokers, all moderate-severe COPD, one positive standard BAL culture for *Moraxella catarrhalis*), serum IL-6 was also elevated (raised serum CRP n=6 median IL-6 2.8 (IQR 2.1-8.8); normal/low serum CRP <5 n=12 median IL-6 1.8 (IQR 1.3-2.2); see Figure 10). Serum IL-6 levels correlated positively with CRP (Spearman correlation r=0.6, p=0.009) (Figure 10). No significant differences were observed in further analysis of COPD ex-smoker and control data only, or COPD current versus ex-smoker data only. Serum IL-10 was higher in samples from donors with positive standard microbiology cultures compared to the negative microbiology group (mean IL-10 8.0 (IQR 6.3-12.6) compared to mean IL-10 5.1(IQR 3.7-9.3), p=0.04) (Table 26). No other difference was observed in cytokine levels or CRP.

Serum	LLD (pg/ml)	Samples below LLD	COPD (n=19)	Control (n=11)	Significance
IL-8	0.108	None	11.7 (9.6-13.2)	9.2 (7.6-14.7)	p>0.05
IL-6	0.293	1 control	1.9 (1.5-2.8)	0.7 (0.4-1.1)	**p=0.0002**
IL-1β	0.709	All	0 (0-0.02)	0.02 (0-0.09)	NA
TNF-α	0.473	None	3.5 (2.9-4.5)	2.9 (2.6-4.1)	p>0.05
IL-10	2.68	1 COPD	6.7 (4.7-10.0)	5.5 (4.2-7.6)	p>0.05
IFN-γ	1.28	14 COPD, 9 control	0.5 (0.1-1.3)	0.5 (0.2-0.9)	NA
IL12p70	1.35	19 COPD, 9 control	0.6 (0-0.5)	0.3 (0-0.8)	NA

Table 27 Serum cytokine data (MSD ultrasensitive 7-plex assay results)

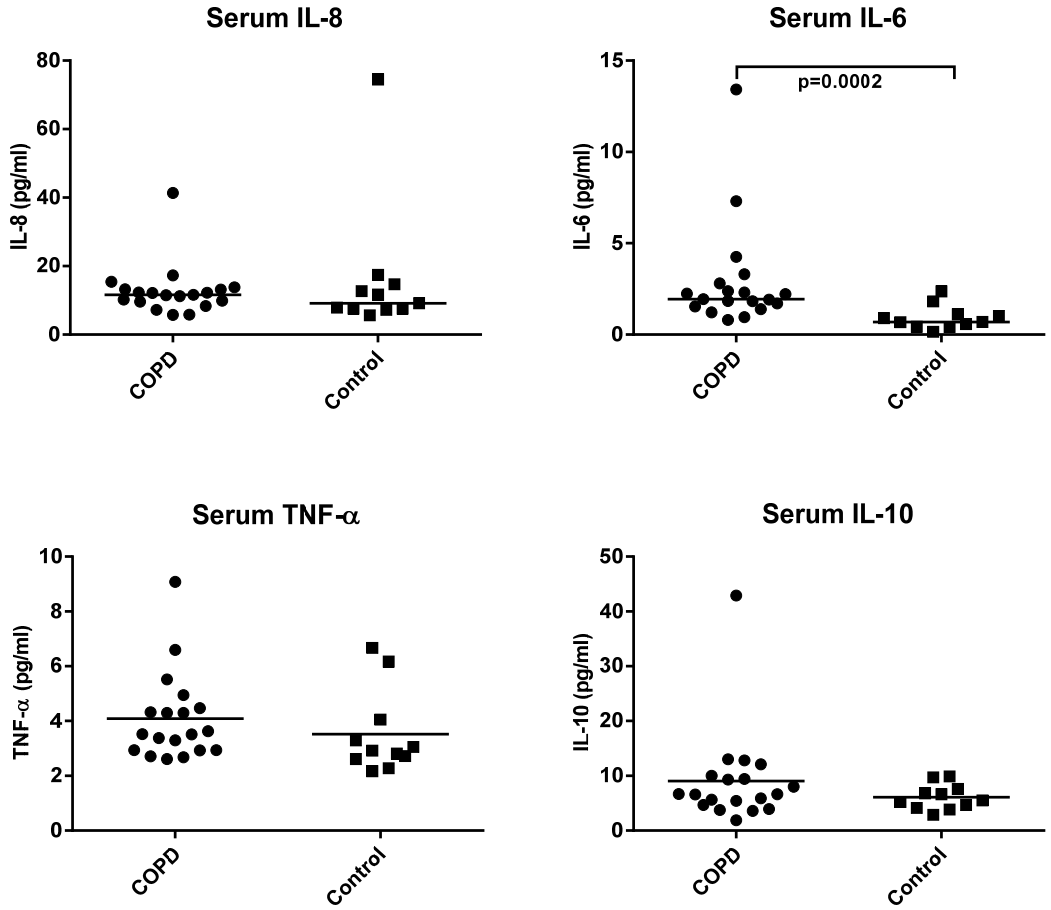


Figure 9 Cytokine levels detected in serum samples from COPD and control donors

Serum samples were analysed using an MSD 7-plex ultrasensitive assay to detect levels of pro-inflammatory mediators present. These graphs show the results of this analysis from 19 COPD and 11 control samples; standards and samples were run in technical duplicates. Data are shown as individual results with a line at the median value.

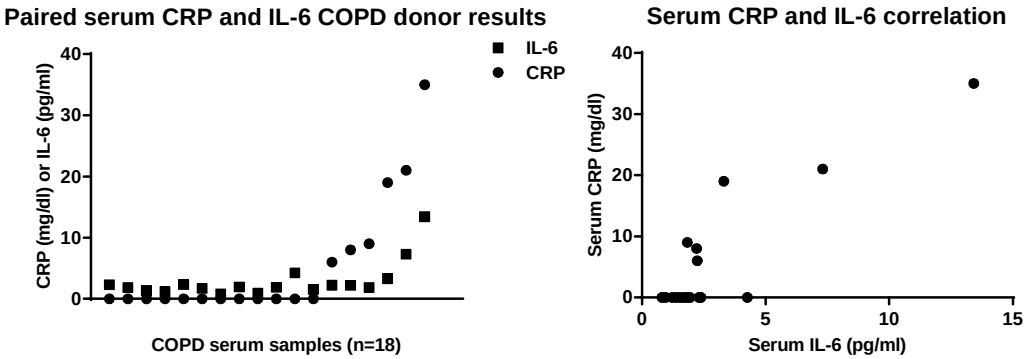


Figure 10 Serum IL-6 and CRP in samples from COPD donors
Serum samples were analysed for CRP levels (laboratory analysis) and IL-6 (MSD 7-plex assay). The data presented are results from n=18 COPD donors (1 sample not available due to haemolysis affecting CRP analysis). In donors with a raised CRP, serum IL-6 was also raised (left panel). A positive correlation was observed between CRP and IL-6 levels (Spearman correlation r=0.6, p=0.009) (right panel).

4.8 Using BAL pilot data to determine sample size required for future research

As a pilot study, the results from this work may be used to inform future studies in this area for calculation of the sample size required to demonstrate a significant difference between disease and control groups in the BAL parameters measured. Following input from a statistician (Dr Kim Pearce, University of Newcastle), this was analysed for the BAL data for levels of IL-8, IL-6 and neutrophil %, for a 2 sample T test. The SAS 9.4 programme was used determine possible sample sizes based on a range of potentially relevant detectable differences between disease and control results. The calculations were based on a power of 80% and p value of 0.05; these values were chosen as those commonly applied to power calculations in clinical research. As per the current study the input for the calculations included weighting of disease and control groups (approximately 2 COPD samples: 1 control sample). Standard deviations from results of the current study were applied individually for each group. This provided an output with N total (combined disease and control in a 2:1 ratio) and possible detectable difference.

The results are plotted as graphs in Figure 11. The output from the SAS 9.4 programme with exact p values and power are shown as tabulated data for IL-8 results in Table 28. The results generated demonstrate that there is a compromise between the size of the detectable difference and the sample number required to detect a small difference. For IL-8, a difference of 300pg/ml between groups requires a sample size which is more than 3 times greater than that required to detect a difference of 600pg/ml for example. Overall, the “power curves” generated all start to flatten out at around N=40, with limited increase in the detectable difference above this. This may be a reasonable compromise, and a feasible target for future study recruitment.

The impact of the sampling procedure used, and any variability in results over time or on repeated sampling within the individual need to be considered in planning further studies (Ward et al., 1995). Determining what is biologically and clinically relevant, in addition to results which are statistically significant, should also be highlighted.

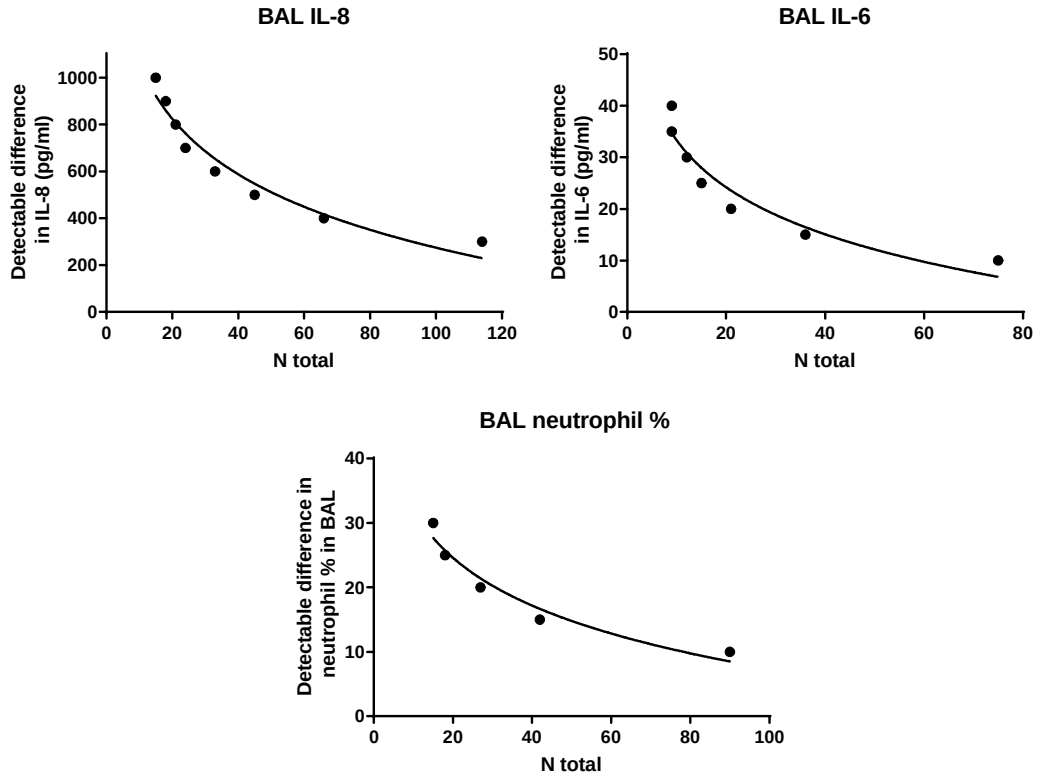


Figure 11 Detectable difference versus sample size for BAL indices
The graphs show results calculated for approximately 80% power and p=0.05 for BAL IL-8, IL-6 and % neutrophil count. N total is the total number of subjects required between disease and control groups in a 2:1 ratio (*i.e.* 2 COPD subjects to 1 control subject).

Mean difference (pg/ml BAL)	Actual alpha	Actual power	N total
300	0.05	0.804	114
400	0.05	0.808	66
500	0.05	0.824	45
600	0.0501	0.834	33
700	0.0501	0.816	24
800	0.0502	0.857	21
900	0.0503	0.874	18
1000	0.0504	0.868	15

Table 28 BAL IL-8 sample size calculation results
Using the standard deviations from the results generated by the current study, COPD (907) and control (96), the data displayed was generated for a 2 sample T test comparison using SAS 9.4 programme. The groups were weighted 2:1 as in the current study and N total reflect the total number of subjects required in this ratio *i.e.* N total 24, 16 COPD and 8 control subjects required.

4.9 Culture-independent microbiology analysis

In addition to the BAL analysis described above, a further unprocessed aliquot was stored at -80^{o}C for processing using a culture-independent method for detection of bacterial presence. This was performed for 14 COPD samples (donors 6-19), and 10 control samples (donors 1-3, 5-11). As described in the study protocol section, processing of BAL samples for DNA extraction, amplification of the 16S rRNA gene in the bacterial genome, and sequencing of products using the Illumina MiSeq platform (Kozich et al., 2013) was performed by Professor Stephen Cummings group at the University of Northumbria (with particular input from Hazel Ingram, Darren Smith and Andrew Nelson to be acknowledged).

The data generated were provided as details of operational taxonomic units (OTU) present in each sample processed, with normalised OTU results provided to take into account the number of reads (bacterial 16S rRNA sequences) for each bacterial taxa compared to the total number of reads in the sample (normalised value=sum of all reads for the sample/number of reads for each OTU in the sample). In total 221 OTUs were identified in the samples processed; those in the negative control were removed, and in addition any OTUs which had less than 100 reads across all samples; this left a total of 60 OTUs for further analysis.

4.9.1 Bacterial DNA identified in BAL samples from COPD and control donors

The results of this processing demonstrate the presence of numerous bacterial species in samples from both COPD and control donors, despite negative standard microbiology cultures. The figures below show the 20 most common taxa identified from COPD donors and from controls. In COPD samples the most common bacteria identified were Proteobacteria (including Enterobacteriaceae, *Haemophilus*, *Pseudomonas* and *Moraxella*), Bacteroidetes (including *Prevotella*), and Firmicutes/Bacilli (including *Streptococcus* and *Staphylococcus*) (Figure 12). In control samples the most common bacteria identified were *Prevotella*, Firmicutes/Bacilli (including *Streptococcus* and *Veillonella*), Proteobacteria (including Enterobacteriaceae, Pasteurellaceae, *Pseudomonas* and *Neisseria*) (Figure 13). The proportions of the most common bacteria isolated (all those genera with >1% of all bacterial DNA isolated) are also shown (this accounts for 94.3% of bacterial DNA

isolated from COPD samples, and 96.2% of bacterial DNA isolated from control samples) (Figure 14). This highlights the predominance of certain species, particularly in the COPD samples with evidence of a particular bacteria making up more than 50% of OTUs present in the samples; 3 samples dominated by Enterobacteriaceae (57%, 95%, 73%), 2 samples dominated by *Haemophilus* (95%, 80%), 1 sample dominated by *Moraxella* (62%), 1 sample dominated by *Staphylococcus* (88%). This is compared to only one control samples with *Streptococcus* making up 51% of OTUs (Control donor 1).

Principle co-ordinates analysis was performed to assess the similarity of bacterial communities present between healthy and COPD groups using data about all the bacterial taxa present in each sample; a distance/matrix score is generated for each sample and plotted (Figure 15). If groups are dominated by particular bacteria then this should separate them. As can be seen from the graph generated, although some COPD patient scores lie separately, there is significant overlap between healthy control and COPD samples, therefore demonstrating that the disease and control groups could not be separated on the basis of the bacterial species present.

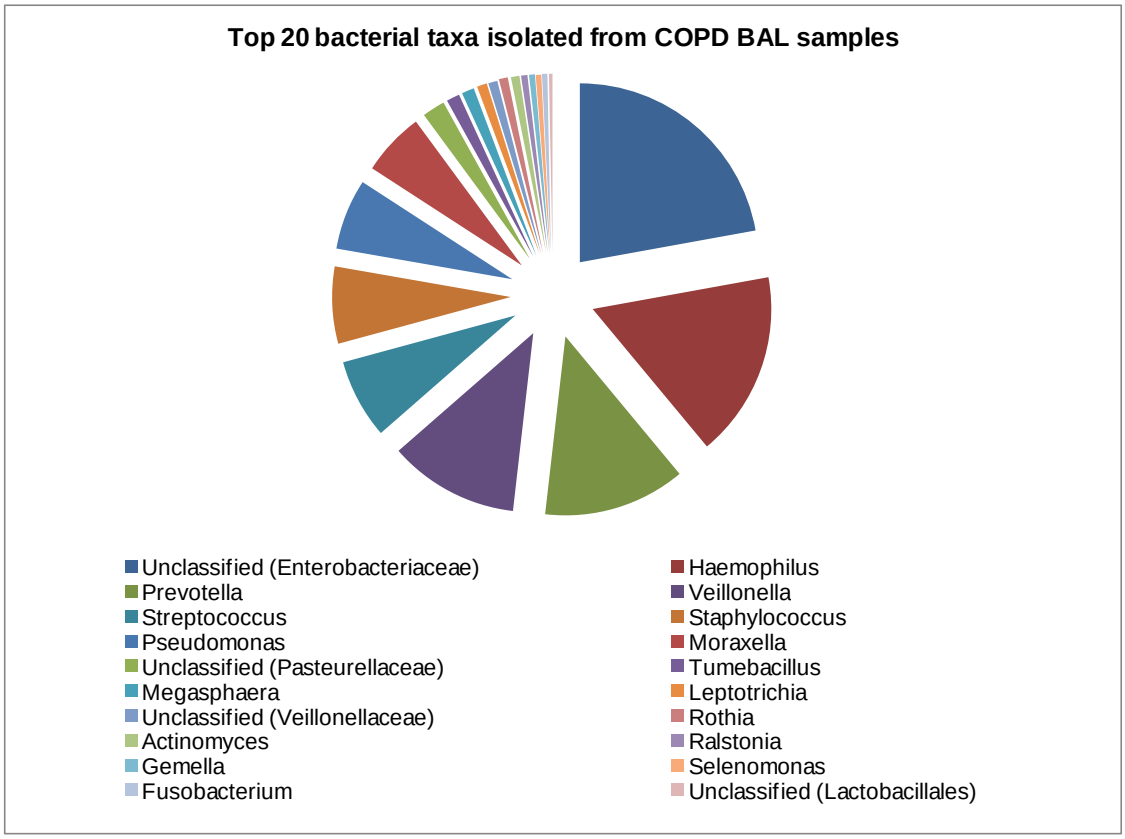


Figure 12 Details of bacterial DNA isolated from BAL samples from COPD donors
BAL samples were processed for bacterial 16S rRNA gene sequencing as described and the results above show the most common taxa identified in samples from COPD donors (n=14). This is based on normalised OTU results which takes into account the number of reads (bacterial 16S rRNA sequences) for each bacterial taxa compared to the total number of reads in the sample.

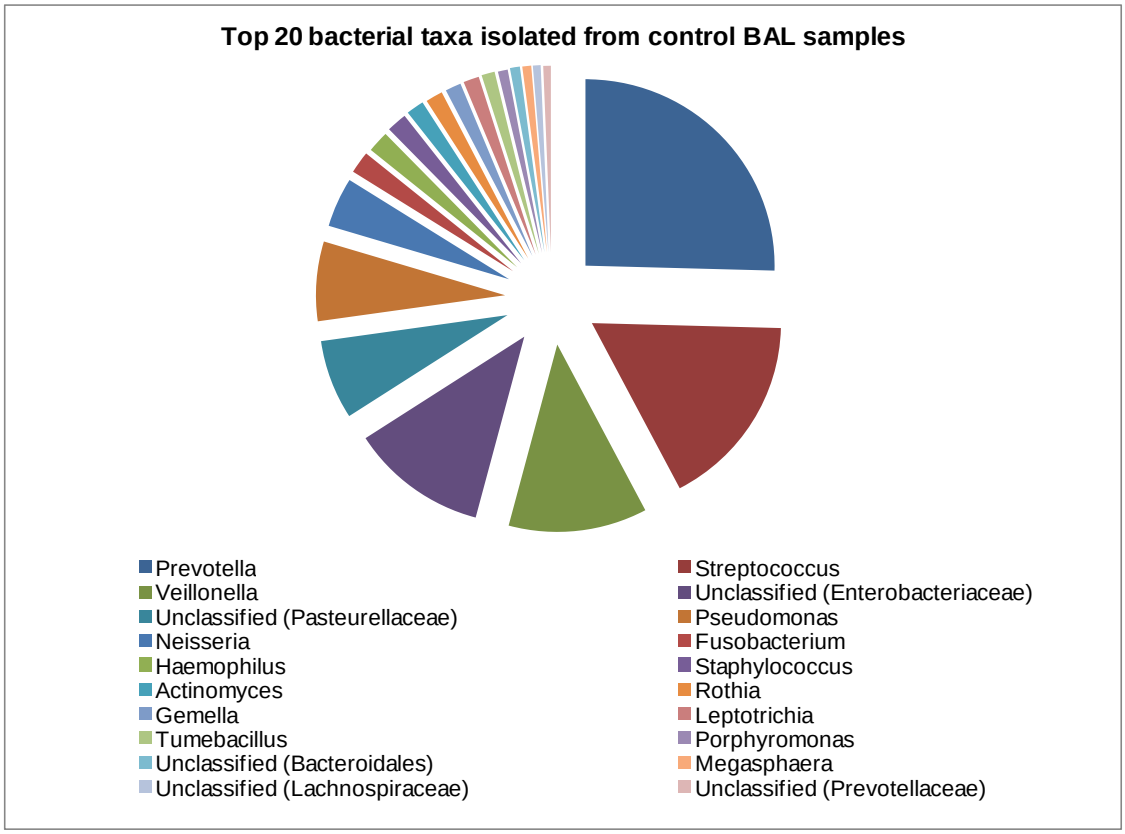
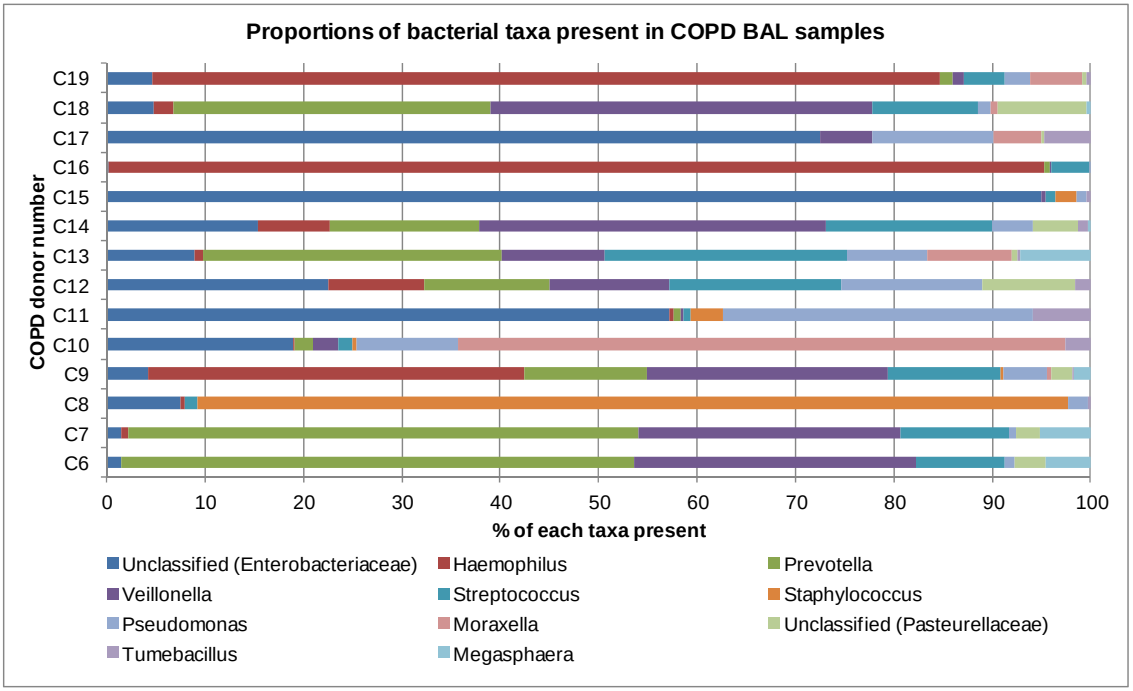


Figure 13 Details of bacterial DNA isolated from BAL samples from control donors
BAL samples were processed for bacterial 16S rRNA gene sequencing as described and the results above show the most common taxa identified in samples from healthy control donors (n=10). This is based on normalised OTU results which takes into account the number of reads (bacterial 16S rRNA sequences) for each bacterial taxa compared to the total number of reads in the sample.



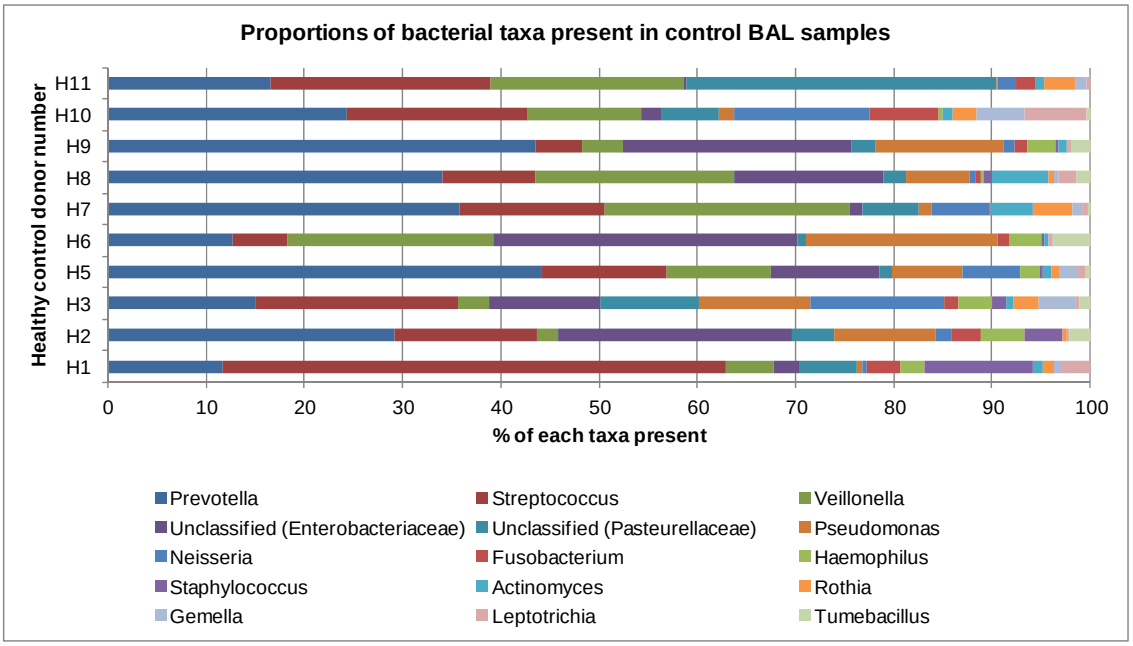


Figure 14 Details of proportions of most common bacterial DNA isolated from BAL samples from COPD and control donors

These panels show the proportions of the most common bacterial DNA isolated from COPD (top panel) and control (lower panel) BAL samples (note different legends for each panel). Low diversity is noted is some COPD samples *e.g.* C8, C16.

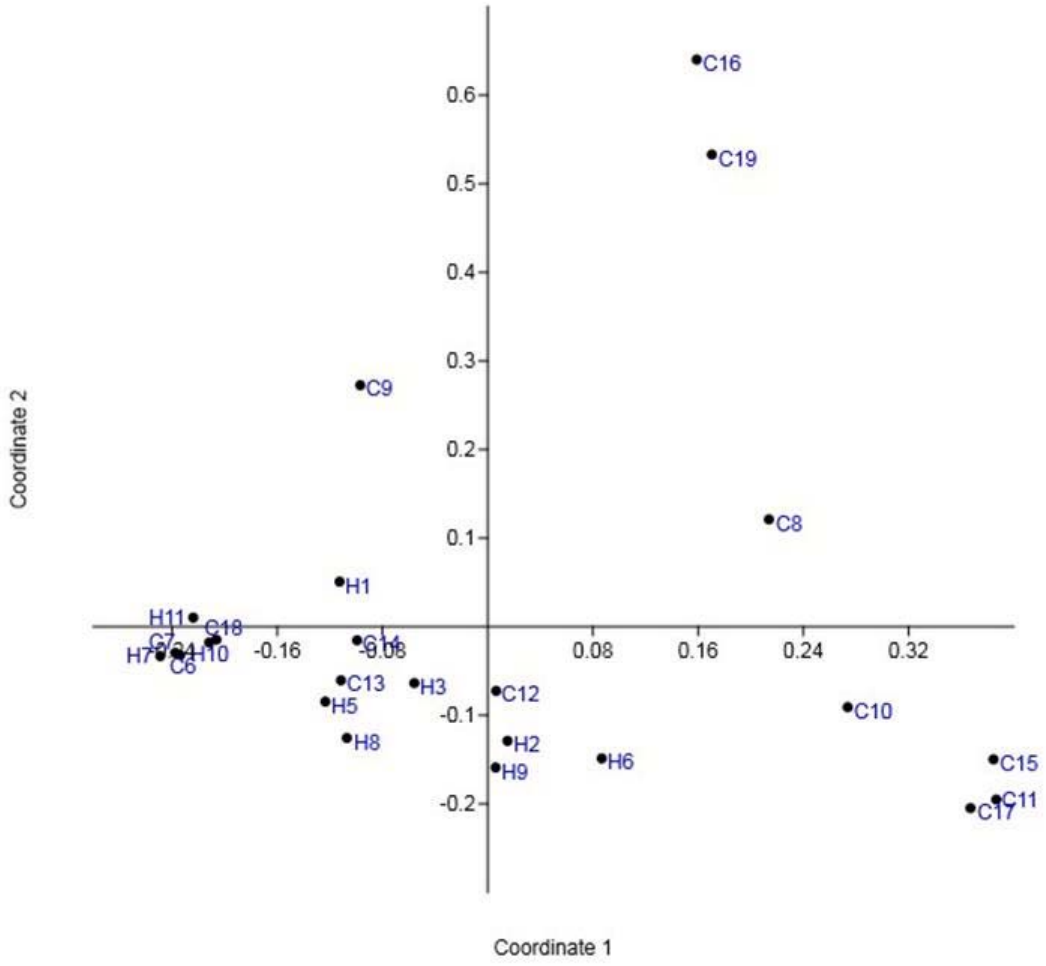


Figure 15 Principle co-ordinates analysis of bacterial OTU data
The similarity of the bacterial communities present in COPD (n=14) and healthy control (n=10) samples was analysed using principle co-ordinates analysis. This graph shows the results of the distance/matrix scores is generated for each sample and shows that although some COPD patients lie out with the central group, it is not possible to separate healthy and COPD donors on the basis of the bacterial taxa present.

4.10 Comparison of results from standard culture and culture-independent microbiology analysis

Of note, on review of the positive standard microbiology results, and comparison with the bacterial DNA isolated from the COPD BAL samples, there was only a predominance of Enterobacteriaceae DNA in one sample which grew *Escherichia coli* (COPD donor 17) (see Table 29). Otherwise, positive standard culture results were not reflected in a high percentage of the cultured bacterial DNA being detected in the samples processed. Some of the bacteria isolated using the culture-independent approach may have been considered as normal "mouth flora", *Prevotella* and *Veillonella* for example, and therefore would not necessarily be reported in culture results. In addition, the results from the culture-independent approach may only be considered as semi-quantitative, and cannot be substituted as a measure of bacterial load which may be relevant in terms of the positive standard culture results observed.

COPD donor number	Standard BAL microbiology culture result	Features of bacterial DNA isolated from BAL
C6	Negative	52% *Prevotella*, 29% *Veillonella*
C7	Negative	52% *Prevotella*, 27% *Veillonella*, 11% *Streptococcus*
C8	Negative	88% *Staphylococcus*
C9	*Moraxella catarrhalis*	38% *Haemophilus*, 24% *Veillonella*, 13% *Prevotella*, 12% *Streptococcus*, **0.5% *Moraxella***
C10	Negative	62% *Moraxella*, 19% Enterobacteriaceae
C11	Negative	57% Enterobacteriaceae, 31% *Pseudomonas*
C12	*Streptococcus pneumoniae*	23% Enterobacteriaceae, 13% *Prevotella*, 12% *Veillonella*, **17% *Streptococcus***, 14% *Pseudomonas*
C13	*Haemophilus influenzae*	30% *Prevotella*, 25% *Streptococcus*, **0.8% *Haemophilus***
C14	Negative	15% Enterobacteriaceae, 15% *Prevotella*, 35% *Veillonella*, 17% *Streptococcus*
C15	*Streptococcus pneumoniae*, *Haemophilus influenzae*	95% Enterobacteriaceae, **1% *Streptococcus*, 0% *Haemophilus***
C16	Negative	95% *Haemophilus*
C17	*Escherichia coli*	**73% Enterobacteriaceae**, 12% *Pseudomonas*, 0% *Haemophilus*
C18	*Haemophilus influenzae*	38% *Veillonella*, 32% *Prevotella*, 11% *Streptococcus*, **2% *Haemophilus***
C19	Negative	80% *Haemophilus*

Table 29 Comparison of standard and culture-independent microbiology results

4.11 The diversity of bacterial taxa identified and COPD and control BAL samples

The bacterial microbiome may be described in the context of *richness* (the number of different OTUs present), and *evenness* (the degree to which the OTUs present are of equal abundance). A combined assessment of the richness and evenness of the species present is used to measure *diversity*; the Shannon diversity index ranges from 0 if only one species is present, to higher values if multiple species of lower abundance are present. Further analysis shows that the richness and evenness of the microbiota detected is greater in control samples (see Figure 16). As a combination of these indices, the diversity is also greater in the control samples (Shannon diversity index). However, there is a wider spread of the number and relative abundance of bacterial taxa within the COPD samples analysed. In analysis of current compared to ex-smokers in the COPD group there was no significant difference in bacterial diversity demonstrated (current smokers n=6, ex-smokers n=8, $p>0.05$, data not shown).

Taking the COPD samples alone, the number of taxa correlated negatively with FEV1 *i.e.* higher FEV1/milder disease correlated with fewer taxa present (Spearman correlation, $r=-0.57$, $p=0.03$) (see Figure 17). The same trend was seen for the diversity index and FEV1 (Spearman correlation, $r=-0.49$, $p=0.08$), but not evenness and FEV1 (Spearman correlation, $r=-0.23$, $p=0.44$). The effect was also lost if COPD and control samples were combined. This however suggests greater richness and diversity of samples in more severe COPD in the study sample population; this is displayed based on GOLD stage (see Figure 18). No correlation between age and either richness or diversity was observed in the COPD population, or both COPD and control samples combined (data not shown). It was not possible to accurately assess for an effect of inhaled corticosteroid treatment due to the limited number of subjects on no ICS treatment (3) compared to those on this therapy (11), but no obvious pattern in results was observed.

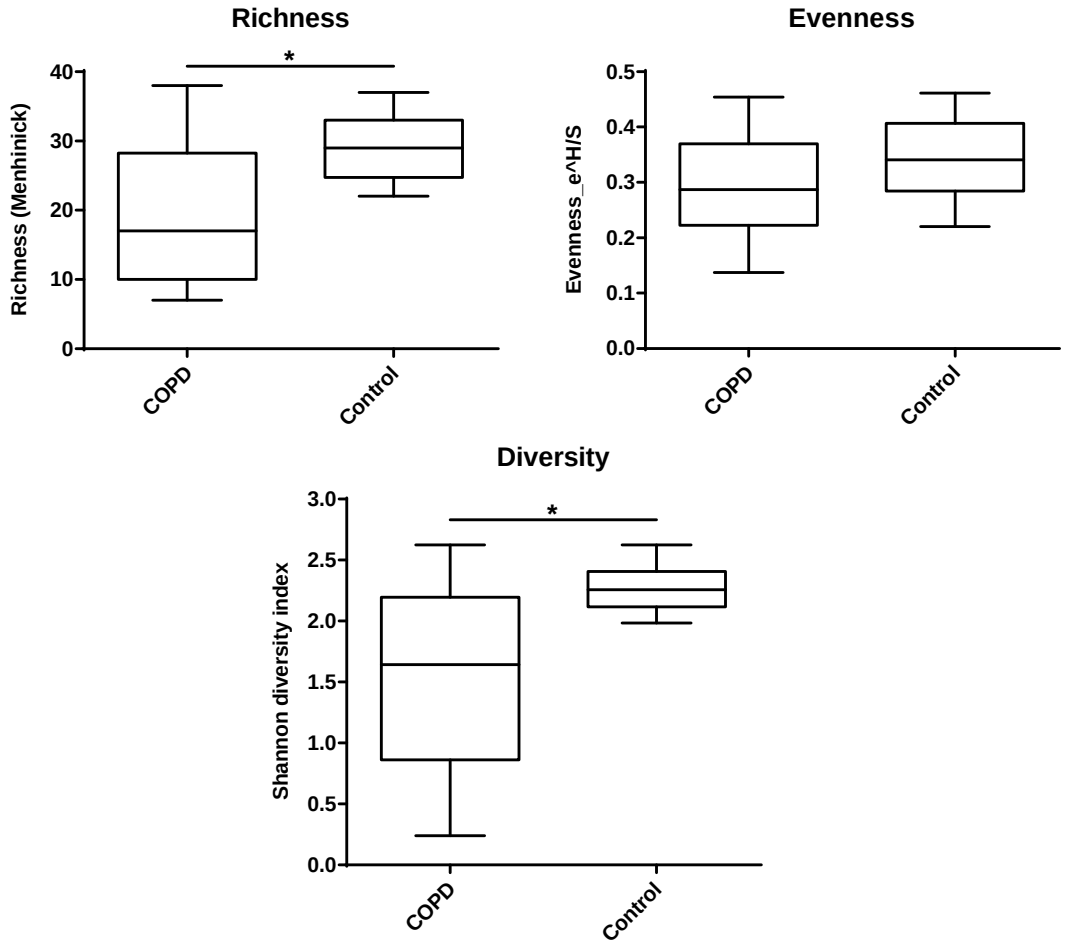


Figure 16 Analysis of bacterial DNA isolated from COPD and control BAL samples
Displayed data show the richness, evenness and diversity of bacterial DNA identified in BAL samples from COPD (n=14) and control (n=10) donors (line at the median with minimum to maximum range plotted). All parameters were greater in control compared to COPD samples (*$p<0.05$).

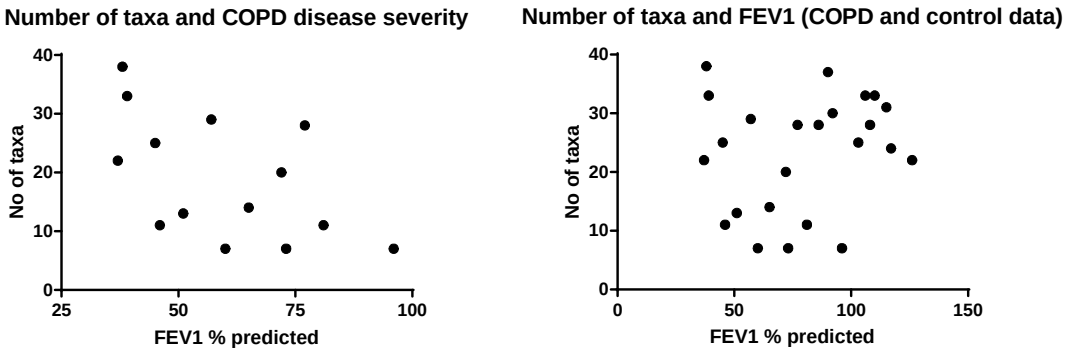


Figure 17 Correlations between number of bacterial taxa present and disease severity/FEV1

The richness of the bacterial taxa (*i.e.* number of different species present) was analysed for COPD (n=14) and control (n=10) BAL samples. For COPD samples the number of taxa present correlated negatively with FEV1 (Spearman correlation, r=-0.57, p=0.03) (left panel). However, on analysis of COPD and control data combined this effect was no longer observed (Spearman correlation, r=0.14, p>0.05) (right panel).

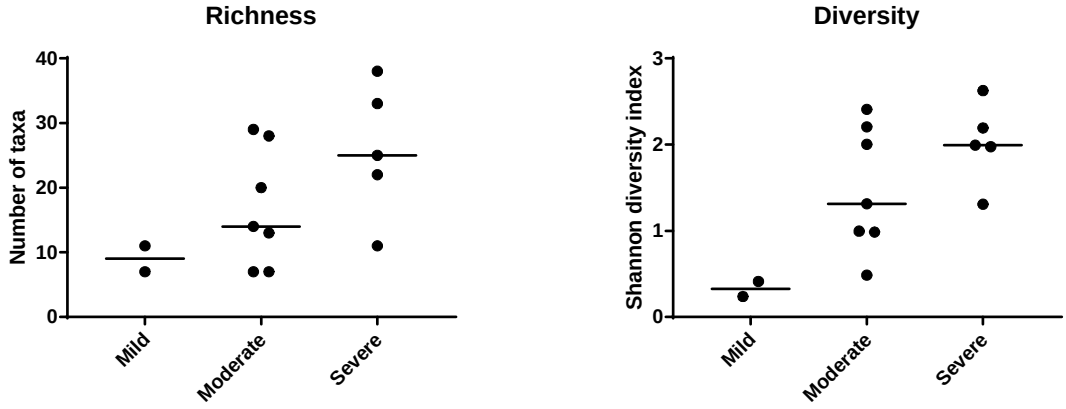


Figure 18 Richness and Diversity by GOLD stage for COPD samples

The richness and diversity of the bacterial taxa isolated from COPD samples (n=14) is displayed by GOLD stage (stage I mild, stage II moderate, stage III severe).

4.12 Discussion

4.12.1 Summary of the data presented

Of the 35 patients recruited, 19/22 COPD patients and 11/13 healthy controls completed the study. The groups were well matched for age but only a small number of current smokers were recruited (8 COPD, 1 control). The COPD group had predominantly GOLD stage II-III disease (moderate-severe), and most patients were on inhaled therapy.

COPD donors had higher BAL neutrophil % compared to controls, but current smoking in the COPD group was associated with a higher macrophage %. BAL IL-6 and IL-1β were higher in COPD samples compared to controls. Analysis of ex-smoker data only in the COPD group also showed higher BAL IL-8 and TNF-α compared to controls. Overall, there was a trend towards higher mediator levels in COPD ex-smokers compared to current smokers. Positive standard microbiology cultures from COPD donors were associated with higher neutrophil % but no difference in the inflammatory mediators measured. Serum IL-6 was increased in COPD samples compared to controls, and correlated positively with CRP levels in the COPD group. Serum IL-10 was higher in BAL with positive standard microbiology results.

Culture independent analysis of bacterial DNA in BAL showed a wealth of species present in both COPD and control samples. There was significantly greater diversity of bacteria in control compared to COPD samples, with some COPD samples showing dominance from a particular bacterial species. However, healthy control and COPD groups could not be separated on the analysis of bacterial taxa identified. The number of taxa present correlated with FEV1 in the COPD group, with greater richness and diversity found to be related to more severe disease. Standard and culture-independent microbiology results did not identify the same bacteria on comparison of culture results to the bacterial DNA present. Using either technique, no associations were observed between positive cultures, or bacterial DNA present, and inflammatory indices in BAL.

4.12.2 Characteristics of COPD BAL and serum compared to control samples

The differential BAL cells counts shown here are similar to data reported previously in the literature; COPD has been associated with a higher BAL neutrophil count, and current smoking with a higher macrophage count (Rutgers et al., 2000, Wen et al., 2010). Increased levels of pro-inflammatory mediators have been described in BAL from COPD patients compared to samples from smokers with normal lung function and non-smokers (Riise et al., 1995). A trend towards higher BAL cytokine levels was observed in ex-smokers with COPD; this is in contrast to previous reports of increased mediator levels in current healthy smokers compared to non-smokers (Kuschner et al., 1996). However, this finding has also been observed by others in COPD patients; Wen *et al* reported increased IL-8 and associated increased neutrophil % in BAL in ex- compared to current smokers with COPD (Wen et al., 2010). The nature of inflammation in ex-smokers with COPD may differ from that induced by current smoking.

The increased serum IL-6 and CRP observed, with evidence of positive correlation reflects previous findings in COPD patients (Ferrari et al., 2013). These indices have been described as markers of systemic inflammation, and have been correlated with disease severity and functional impairment in COPD (Gan et al., 2004, Broekhuizen et al., 2006). Serum IL-10 was higher in the COPD group with positive standard microbiology cultures, but the significance of this finding is unclear, particularly at the low levels detected. This anti-inflammatory cytokine has been shown to be lower in sputum in COPD (Takanashi et al., 1999), and reduced in serum and sputum samples from healthy smokers and COPD patients compared to non-smokers (Zhang et al., 2013), with these findings suggested to be related to the increased inflammation observed. It is possible that IL-10 could be increased in the context of chronic bacterial colonisation but this has not previously been investigated.

4.12.3 Standard versus culture-independent microbiology analysis

Despite only 9/19 BAL samples with positive standard microbiology results, bacterial DNA was isolated from all COPD and all control BAL. This is most likely to be related to the sensitivity of culture techniques for different types of bacteria. In addition,

standard culture techniques are more geared towards isolation of a restricted number of pathogens, and many bacteria are difficult to culture due to specific growth conditions (Hilty et al., 2010). Analysis of the same sputum samples using culture, and non-culture techniques including bacterial DNA and RNA analysis (Rogers et al., 2009), and quantitative PCR (qPCR) (Bafadhel et al., 2011), has previously highlighted the limitations of standard culture approaches, with significantly fewer bacterial species identified using culture only (86% identified on qPCR compared to 28% on standard culture in the Bafadhel study).

The species identified in the present study reflect patterns described in the literature. Hilty *et al* reported on BAL samples from 5 COPD, 11 asthma and 8 healthy control patients (Hilty et al., 2010). The Bacteroidetes, *Prevotella* and *Veillonella*, were most common in control subjects. These are recognised as common Gram-negative anaerobic flora of the oropharynx, but may not be recognised in culture due to their requirement for anaerobic conditions. Proteobacteria (including *Haemophilus* and *Moraxella*) strongly associated with airways disease in both asthma and COPD. Similar patterns of the most common bacteria identified have been reported in other studies of COPD patients and healthy controls (Erb-Downward et al., 2011, Zakharkina et al., 2013), with the largest study to date by Pragman *et al* (22 COPD patients and 10 controls) reporting the main phyla in all samples analysed to include Actinobacteria, Firmicutes and Proteobacteria (Pragman et al., 2012).

Pragman *et al* suggest an increase in diversity with the development of COPD, though their results were also thought to be influenced by subject age (Pragman et al., 2012). In the current study, the overall COPD group had lower bacterial diversity than the control group, but richness and diversity correlated with FEV1 suggesting an increase in diversity with increasing disease severity, which has some similarities to the Pragman group findings. There was no association between age and bacterial diversity in the present study. An increase in bacterial diversity in more severe disease could relate to a change in the lung microenvironment with increasing lung damage. It could also be affected by an increase in frequency of disease exacerbations, and treatment for these including systemic steroids.

In contrast to these findings previous studies have also reported reduced bacterial diversity in selected COPD patients (Erb-Downward et al., 2011, Zakharkina et al., 2013). Zakharkina *et al* reported that 2/9 patients that had low diversity were shown to have a predominance of *Pseudomonas* present (Zakharkina et al., 2013). It may be that smaller sample sizes are responsible for the differences observed between different studies, or issues including geographic location and differences in patient management. It is possible that a minority of COPD patients have lower diversity and this may represent a specific disease phenotype which could be important to further investigate.

In assessing a possible relationship between changes in the lung microbiome and disease progression in COPD, determining cause and effect is difficult. Compared to a healthy lung microbiome, an initial reduction in diversity could be associated with the development of COPD, which then increases in later stage disease. Homeostasis in the lung microenvironment and the balance between "good" versus "bad" bacteria may also be important, *i.e.* the pattern of bacteria detected in terms of mouth flora as possible "bystanders", compared to potentially pathogenic bacteria. DNA from potentially pathogenic bacteria such as Proteobacteria was more commonly isolated from COPD patients than controls *e.g. Haemophilus* 16% COPD compared to 1.8% control in the current study, which has also been found previously (Hilty et al., 2010).

Interestingly, standard and culture-independent microbiology results did not necessarily identify the same bacteria; it may have been expected to see a higher proportion of the bacterial DNA present in a sample with positive standard culture to be the same as the bacterial species cultured in the lab. This was only observed in one sample (COPD donor 17) as described above. This highlights that the culture-independent technique used is at best semi-quantitative. Low numbers of actively replicating bacteria in the airways could still propagate further in the appropriate culture conditions.

The association of higher neutrophil count with positive standard microbiology cultures provides evidence for inflammation in the context of bacterial presence

despite clinical stability in the COPD patient group investigated here. Association of airway inflammatory indices with bacterial colonisation have been reported previously. Soler *et al* studied 52 COPD patients, 8 non-smoking and 18 smoking controls, and shown increased neutrophil counts and higher TNF-α BAL levels in COPD patients and smokers colonised with potentially pathogenic micro-organisms (PPM) (Soler et al., 1999). To avoid issues with contamination a cut off value of ≥100 colony forming units (cfu) per ml was chosen to indicate positive BAL cultures. The presence of PPM in "significant amounts" (≥100 cfu) was associated with these findings, but not the presence of PPM in insignificant amounts (<100 cfu), or "non-PPM" (such as *Streptococcus viridians*). Sethi *et al* studied 26 ex-smokers with COPD, 20 ex-smokers without COPD, and 15 healthy non-smokers (Sethi et al., 2006). PPMs at levels of ≥100 cfu/ml were associated with increased neutrophil counts, IL-8, IL-6 and MMP-9 in BAL. These observations may not have been reported in the current study due to the smaller sample size studied, heterogeneity of the COPD population studied including current and ex-smokers, and lack of quantitative culture results. Some of the BAL culture results were reported as showing "scanty" growth. However, even on comparison of positive BAL culture, with those with no or scanty growth in a "negative culture group", no difference was observed in inflammatory mediator levels, though the difference in neutrophil count was still present. In addition to these issues, our clinical laboratory would also not routinely report non-PPMs, and this data is therefore also lacking.

The above observations may also be relevant to the negative findings reported here regarding the characterisation of bacterial DNA present with inflammatory indices in BAL; no associations were found with the sample richness or diversity, or with the percentage of bacterial DNA identified from specific PPMs. The bacterial DNA analysis technique used looks at microbial diversity but is not quantitative, and bacterial load may be important to consider *in-vivo* as shown by the earlier BAL studies described above (Soler et al., 1999, Sethi et al., 2006). Furthermore, a recent study of qPCR of sputum samples showed that higher PPM bacterial load was associated with increased levels of inflammatory mediators; a "threshold effect" of PPM load to cause

inflammation was proposed (Singh et al., 2014). *Haemophilus influenzae* was associated with increased airway inflammation at all bacterial loads.

In the healthy lung, which has now been shown to have a microbial population, to maintain homeostasis immune responses to pathogens must be controlled, as in the healthy gut environment. Increase in cytokine levels implying an active immune response would be potentially detrimental and if persistent could result in epithelial injury. Adaptation to microbial stimuli, with tolerance for a certain level of bacterial load or composition may be the norm; on smoking and in COPD loss of this balance could influence this but possibly not during "steady state" conditions. However, if significant overrepresentation of a potentially pathogenic organism is detected, this could induce inflammation, as suggested by the research described above regarding levels of *Haemophilus influenzae* (Singh et al., 2014).

4.12.4 Review of study recruitment and participant demographics

Recruitment to the study was successful and use of the research centre database, including patients from both primary and secondary care, allowed inclusion of a more general COPD population, with a range of disease severity, rather than a specifically selected secondary care group. Overall the procedures involved, including bronchoscopy, were acceptable to the study participants, and no serious adverse events occurred. Bronchoscopy and associated sampling is generally a safe and well tolerated procedure and was felt to be appropriate in this research setting, with full ethics committee approval and written informed consent of all study participants. The COPD and control groups were well matched for age, which is particularly important in allowing comparisons between health and disease, especially in light of the previous bacterial microbiome research discussed above with the possibility of skewed data due to age differences (Pragman et al., 2012). Lack of specific healthy smoking controls is a weakness of the study which must be taken into consideration in data analysis; determination of effects due to disease or related to smoking is therefore more difficult. However, comparisons can be drawn between current smokers with COPD, ex-smokers with COPD and controls. Additionally, most of the COPD group were on maximal inhaled therapy including long acting bronchodilator and corticosteroid treatment; further analysis of the effect of inhaled therapy on the results observed

was therefore not performed. It is possible that current medication could have impacted on inflammatory indices measured in the COPD group.

In considering the importance of small airways disease, results of RV/TLC and $FEF_{25-75\%}$ have been reported in the COPD study population. These may be used as estimates of small airways function but are affected by significant variability and resultant effects on the accuracy of such recordings (Burgel, 2011). The results recorded suggest that small airways disease was present throughout the COPD group. Other methods which could have been implemented to further assess this and more clearly "phenotype" participants include review of CT imaging; however, requirement for recent CT would have significantly limited participant recruitment. In addition, there is also currently no standardised method for analysis of small airways disease on CT (Stewart and Criner, 2013).

As described, a broad range of COPD patients was chosen to aid recruitment and to make the study results generalisable and more applicable to a real world population. In the setting of a small pilot study, the selection criteria used were felt to offer the best compromise, to ensure adequate recruitment and to direct future research in this area. However, clearly the limited number of patients in different disease severity groups and smoking status does impact on the conclusions which can be drawn from this study. Future considerations of research in this area may therefore either require an increase in numbers in all groups or focus on a particular disease phenotype. A number of different phenotypes are described, which can be based on features including physiological and radiological findings, frequency of exacerbations or evidence of systemic inflammation (Han et al., 2010). It has been proposed that we should try to identify and focus on phenotypic features with relevance to disease progression and longer term outcomes; this may allow the greatest impact from future research (Han et al., 2010). In the context of future research planning, the sample size calculations generated from the results of the current study in section 4.8 may provide some basis for numbers required to detect differences in BAL sample results. However, as these results are based on a small heterogeneous COPD sample, if greater

numbers of subjects can be recruited from different disease categories then these numbers may not be applicable.

4.12.5 Further consideration of the research approach used

Issues with BAL sampling, sample storage and processing could have affected the current study results. This is discussed in the following sections, alongside consideration of ways to improve the method used, or alternative approaches to addressing the research question posed.

Standardisation of BAL procedure

The BAL return reported showed significant variability. This may in part relate to the degree of underlying emphysema as discussed above (Lofdahl et al., 2005). The BAL results have been expressed as differential cell counts and mediator levels as concentration per ml of BAL. It is possible that the variability in BAL return and effect on dilution of BAL components affected the observed results. Differential cell counts are routinely reported as these proportions are unaffected by any dilution factor (Haslam and Baughman, 1999). There is currently no recommended standard available for adjusting BAL return for comparison of acellular components (Haslam and Baughman, 1999). Use of an internal standard such as urea or albumin is often reported. However, these proteins may not be a constant level in body fluids, and the amount detected in BAL is affected by lung permeability and diffusion into the lavage fluid. Lung permeability in turn may be affected by underlying lung disease. Instead, a standardised procedure for collection of BAL is recommended to include use of a standard volume, number of aliquots and collection site (Haslam and Baughman, 1999), as described in the current study.

Some of the cytokines tested were below the detection limits of the assay used including TNF-α, IL-10, IFN-γ and IL-12p70. As described in the methods section, 1% BSA was added to the BAL samples prior to analysis to minimise loss of analyte by adsorption to the labware used as recommenced in the assay protocol. Mediator levels may have been affected by sample storage; defrosting/refreezing and longer term storage have been shown to impact on this (Strieter et al., 1999). BAL samples in the current study were stored in aliquots at -80^{o}C for individual analysis. In addition

the dilutional effect of BAL sampling will impact on the results observed. Repeat testing using individual ELISAs with potentially greater sensitivity than a multiplex platform could be considered. Methods of "concentrating" the protein present in the BAL samples could also be used *e.g.* freeze-drying, precipitation methods or membrane filtration using centrifugation (Plymoth et al., 2003, Heng et al., 2013). However, consideration of further loss of analyte during processing, and possible interference with the assay used for measurement is important. Use of control samples with known protein/cytokine concentration for comparison would also be required.

Issues with using BAL sampling to assess the microbiome

A wide range of bacteria detected using 16S rRNA amplification and sequencing was observed. The use of BAL samples here is of relevance as this raises the possibility of oropharyngeal contamination. BAL was routinely collected as the first sample during the procedure. The possibility of carry-over of oropharyngeal bacterial DNA into the BAL samples could have been assessed by concurrent analysis of a sample from this area with the BAL samples (Charlson et al., 2011). Further methods could have been employed to minimise the risk of contamination during the BAL procedure; a previous study has described "scope lavage" prior to collection of BAL, where once the bronchoscope is wedged in position in the airway, saline is first instilled, aspirated and discarded, prior to formal BAL sampling (Cabrera-Rubio et al., 2012).

Use of sampling using a protected specimen brush (PSB) in more proximal airways could also have been considered. Comparison of samples from the level of the glottis using one bronchoscope (with tip and scope lavage), followed by serial BAL and PSB using a second bronchoscope has been described in healthy volunteers (Charlson et al., 2011). Bacterial DNA was isolated from all samples, and this group suggested carryover of upper respiratory tract DNA into BAL samples on the basis of a reduction in biomass detected on serial BAL sampling compared to oropharyngeal samples. However, the methods describe an increasing volume of saline instilled over serial BAL samples which clearly may have affected these results. In addition the bacterial communities detected were comparable in all samples, including those of BAL and PSB;

the healthy lung microbiome may reflect microaspiration of oral contents rather than this being an issue with sampling methodology.

A further group has also reported similar results from PSB and BAL sampling with comparison of data from different sites within the lung in healthy volunteers (Dickson et al., 2015). This group reported differences between the lung microbiota and that of the upper airway, but did observe that the richness of the bacterial community identified decreased with distance of sampling from the upper respiratory tract in comparing supraglottic to lower airway samples, again suggesting that the healthy lung microbiota may be affected by microbial immigration from the upper airway.

The above studies do suggest that sampling of the lower airway using either BAL or PSB can give comparable results in healthy lungs. These results are however in contrast to the variability reported in the microbiome of COPD patients, between the upper and lower airway (Cabrera-Rubio et al., 2012), and explant lung biopsy sites in severe COPD (Erb-Downward et al., 2011).

The relevance of bacterial DNA analysis and inflammation in different lung compartments

With the heterogeneity of the disease process and microbiome in COPD, and sampling from one area of the lung only in the current study, this may also have affected the results reported. Analysis of BAL from more than one area of the lung would be interesting to investigate, and also the repeatability of measurements from the same place in the same individual over time. In the current study BAL was used as a method of sampling the local microbiome which may potentially influence the small airways. However, this sampling method is aimed towards collection of epithelial lining fluid from the alveolar compartment (Walters and Gardiner, 1991). Consideration of sampling actually within the small airways using for example a PSB may provide results which are more relevant to the small airways. This could be analysed alongside airway epithelial cells collected by brushing from the same level, to investigate associated immune/inflammatory gene expression; this may be particularly relevant as not all mediators are necessarily released into the epithelial lining fluid but may be secreted

basally into the local cellular microenvironment. Collection of epithelial lining fluid for analysis of locally produced mediators in the airways themselves is not widely performed, though may be possible using an absorption probe (Ishizaka et al., 2001). Comparison of results between airway and alveolar samples in the same part of the lung would give clearer direction to future research in this area, to allow planning of sampling methods for investigation depending on the area of interest.

Another potential pitfall of the approach used with DNA as opposed to RNA extraction from samples is the detection of non-viable organisms; however, the presence of even dead bacteria is still relevant as a potential trigger for the immune response (Martinez et al., 2013). It is difficult to know how significant an impact the findings of bacterial DNA in BAL samples may have at the airway level. Research using this non-culture technique in addition to a quantitative culture method, with concurrent measurement of inflammatory indices could help to elucidate this further. Further review of the effect of low bacterial diversity, and patterns of dominance by PPMs in the bacterial DNA isolated, on airway inflammation may also be important to consider. Overall, investigation using larger numbers, including healthy smoking and COPD smoking controls is required. Analysis of patients at each different stage of disease could also help to elucidate associations between changes in the lung microbiome and the disease process.

Chapter 5. Primary airway epithelial cell culture and characterisation

5.1 Introduction

There is wealth of research using primary airway epithelial cells isolated from bronchial brushings or from explant lung tissue, as a source from which to develop *in-vitro* culture models to investigate chronic respiratory disease. The premise of their use, as opposed to cell lines, is to investigate cellular responses using those cells which may be directly involved in the disease process *in-vivo*. Previous models developed in COPD have primarily utilised cells from the larger airways (trachea and main bronchi). However, many of the changes observed in COPD arise in the small airways and this is an area which has been much less extensively studied. This is in part due to issues with sampling the more distant portion of the airway. Large airway and even nasal airway epithelial cells have been used as surrogates to investigate epithelial cell responses from a "united airway". However, given changes seen specifically in the larger (bronchitis, mucus gland hyperplasia) and the smaller airways (peribronchial fibrosis), this suggests that the disease process may be different depending on location within the airway.

Airway epithelial cells have been shown to express TLRs which are important in the initiation of the immune response to respiratory pathogens (Parker and Prince, 2011). TLR expression at different airway levels is also therefore important to consider prior to assessing the response of these cells to respiratory pathogens in an *in-vitro* cell culture model. Nasal TLR4 expression has been shown to be reduced in healthy smokers and patients with severe COPD; correlation of TLR4 mRNA expression between nasal and large airway epithelial cells was demonstrated (MacRedmond et al., 2007). Previous research has however demonstrated differences between nasal and large airway epithelial cell responses to stimuli including LPS following CSE treatment (Comer et al., 2012). Whether the same is true for large versus small airway epithelial cell responses, and the potential impact of this on the disease process at different airway levels, and effect of anti-inflammatory treatments, has not been investigated.

To investigate differences in cellular responses in cells from different airway levels which may be important in the disease process in COPD, primary cell cultures were established from nasal, large and small airway brushings. These cells were characterised to check appropriate cell morphology and expression of epithelial cell markers. Baseline TLR expression was analysed in all cell types cultured.

5.2 Establishment of cell cultures

Cultures were successfully established from brushings collected from all airway levels. Details of sampling, success of cultures in terms of passage number and issues with infection are shown in Table 30 and Table 31. Early infection occurred in only 3 out of 30 sets of brushings obtained (10%): 2 sets of COPD samples (SAEC and LAEC) and 1 set of control samples (SAEC and NAEC). Based on the timing of infection within days of collection these instances were thought to be related to contamination during the sampling and initial cell seeding process; appearances were in keeping with superimposed fungal infection. All of the associated BAL cultures were negative; only sputum from COPD donor 11 cultured *H.influenzae*. During later passages of cells in culture, 4 sets of samples were affected by infection as indicated. As soon as any infection was detected all affected cells were discarded and appropriate incubator and cell culture hood cleaning and hygiene measures addressed.

Table 30 Details of primary airway epithelial cell cultures established from brushings from COPD subjects

		SAEC		LAEC		NAEC		
No	**Date**	**Sample**	**Culture**	**Sample**	**Culture**	**Sample**	**Culture**	**Details**
1	15/02/2011	Y	P4	Y	P4	Y	Limited growth, discarded at P1	-
2	01/03/2011	Y	Infection P0	Y	Infection P0	Y	Limited growth, discarded at P1	Early fungal infection
3*	22/02/2011	Y	P5	Y	P5	Y	Limited growth, discarded at P1	-
4	08/03/2011	Y	P4	Y	P3 (Morphological change from P2)	Y	P2 then limited growth	-
5	31/05/2011	Y	P4†	Y	P4†	Y	P3 then limited growth†	†Late fungal infection
6	20/04/2011	Y	P3†	Y	P3 (Morphological change from P2)†	Y	P3 then limited growth†	†Late fungal infection
7	16/08/2011	Y	P4	Y	P4	Y	P2	-
8	01/11/2011	Y	P5	Y	P5	Y	P2	-
9	29/11/2011	Y	P4	Y	P4	Y	P2	-
10	31/01/2012	Y	P5	Y	P4	Y	P2	-
11*	27/11/2012	Y	Infection P0	Y	Infection P0	Y	P2	Early fungal infection
12	22/05/2012	Y	P3†	Y	P3†	Y	P2	†Late fungal infection
13	23/10/2012	Y	P2	Y	P2	Y	P2	-
14	23/11/2012	Y	P2	Y	P2	Y	P2	-
15	12/02/2013	Y	P2	Y	P2	Y	P2	-
16	08/02/2013	Y	P2	Y	P2	Y	P2	-
17	18/06/2013	Y	P2	Y	P2	Y	P2	-
18	09/07/2013	N	No sample	Y	P2	N	No sample	Bronchoscopy not well tolerated, limited sampling
19	01/10/2013	Y	P2	Y	P2	Y	P2	-

Details: All SAEC/LAEC samples from RLL, except *from LLL. †Indicates late fungal infection. Highlighted cells show those used in the cell culture experiments detailed in Chapter 6: Pale grey n=3 HI dose response work; darker grey n=6 CSE and HI treatment work.

Table 31 Details of primary airway epithelial cell cultures established from brushings from healthy control subjects

		SAEC		LAEC		NAEC		
No	**Date**	**Sample**	**Culture**	**Sample**	**Culture**	**Sample**	**Culture**	**Details**
1*	15/11/2011	Y	P4	Y	P4	Y	P2	-
2	22/11/2011	Y	P5	Y	P5	Y	P3	-
3	13/12/2011	Y	P5	Y	P5	Y	P3	-
4	03/07/2012	Y	Infection P0	N	No sample	Y	Infection P0	Bronchoscopy not well tolerated, limited sampling, early fungal infection
5	16/10/2012	Y	P2	Y	P2	Y	P2	-
6	06/11/2012	Y	P2	Y	P2	Y	Limited growth, discarded at P2†	†Late yeast infection
7	12/03/2013	Y	P2	Y	P2	Y	P2	-
8	01/02/2013	Y	P2	Y	P2	Y	Limited growth, discarded at P0	-
9	22/03/2013	Y	P2	Y	P2	Y	P2	-
10	09/04/2013	Y	P2	Y	P2	Y	P2	-
11	13/09/2013	Y	P2	Y	P2	Y	P2	-

Details: All SAEC/LAEC samples from RLL, except *from LLL. †Indicates late fungal infection. Highlighted cells show those used in the cell culture experiments detailed in Chapter 6: Pale grey n=3 HI dose response work; darker grey n=6 CSE and HI treatment work.

5.3 Primary airway epithelial cell culture and passage

After seeding all cell types took between 10-14 days to reach confluence in a T25cm^2 flask with similar growth rates overall. LAEC and SAEC continued to grow well after passage but initial NAEC cultures were more difficult to sustain; following passage cell growth rates were poor with morphological changes including cell enlargement and more elongated and spindle shaped cells with apparent extracellular matrix deposition (see Figure 19). Initially all cell types were cultured in SAGM (*i.e.* media developed for small airway epithelial cell culture), as this was already in use for primary bronchial epithelial cell cultures in our laboratory. Following initial problems with nasal cell culture, media was changed to BEGM media from COPD subject 6 onwards and in all control NAEC cultures. Subsequent cultures were maintained at least to P2, though later passage remained limited compared to LAEC and SAEC cultures.

This issue led to consideration and review of the appropriate media for LAEC culture. Of note in two of the early LAEC cultures morphological changes were noted (see Figure 20) suggestive of epithelial to mesenchymal transition *in-vitro*. This has been shown to occur during *in-vitro* cell culture in response to TGF-β (Borthwick et al., 2009). Cell stress in *in-vitro* culture may have contributed to changes observed; phenotypic changes in cells *in-vitro* is a recognised issue following prolonged culture (Ward et al., 2005). To ensure optimal culture conditions for LAEC, these cells were also cultured in BEGM media. Cell characterisation was performed for LAEC cultured in BEGM (LAEC BEGM) and LAEC cultured in SAGM (LAEC SAGM) to ensure no effect of the media on marker expression. Early cell stimulation experiments were also performed using both culture media; this was undertaken to try to ensure that any differences observed between LAEC and SAEC could be related to cell type rather than culture conditions, as described in Chapter 6.

At passage 0 cells were imaged by light microscopy to demonstrate the typical cobblestone appearance of an epithelial cell monolayer in culture (see Figure 21 and Figure 22). At passage 2 cells were further imaged by light microscopy as shown, and fixed for H&E staining (see Figure 23).

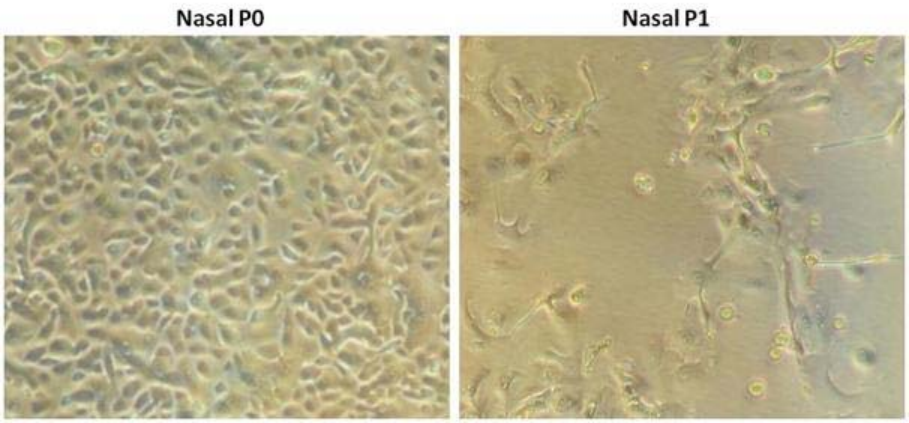


Figure 19 Light microscopy of NAEC at P0 and P1
The images shown are from cell cultures established from brushings taken from COPD donor 1 in the study, cultured in SAGM media (x4 magnification). At P0 confluent epithelial cell monolayers were observed (left panel). However at P1 growth rates slowed and changes in cell morphology with apparent extra-cellular matrix deposition were seen (right panel).

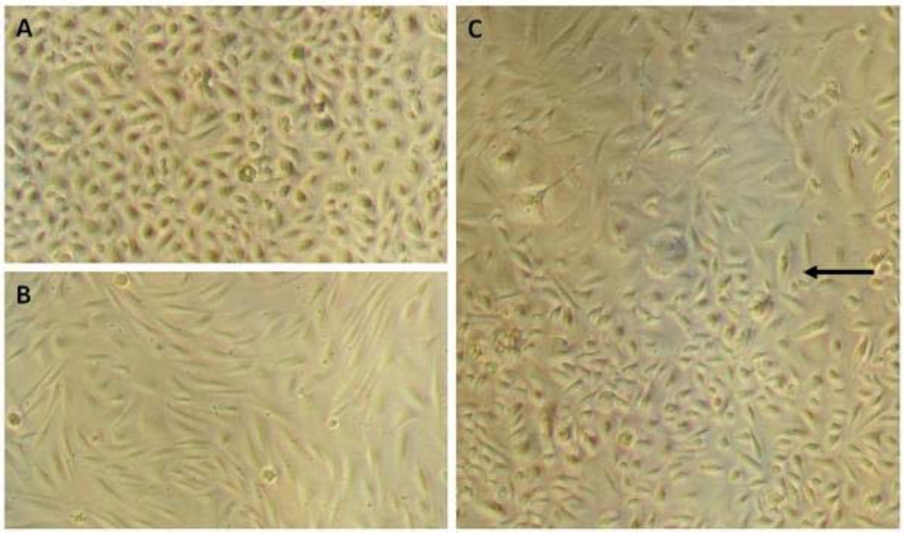


Figure 20 Light microscopy of LAEC at P3, with possible EMT changes
The images shown are from cell culture established from brushings taken from COPD donor 4 in the study (x4 magnification). Areas of cells with epithelial morphology (A), changing into fibroblast morphology (B), are visible (C, see arrow).

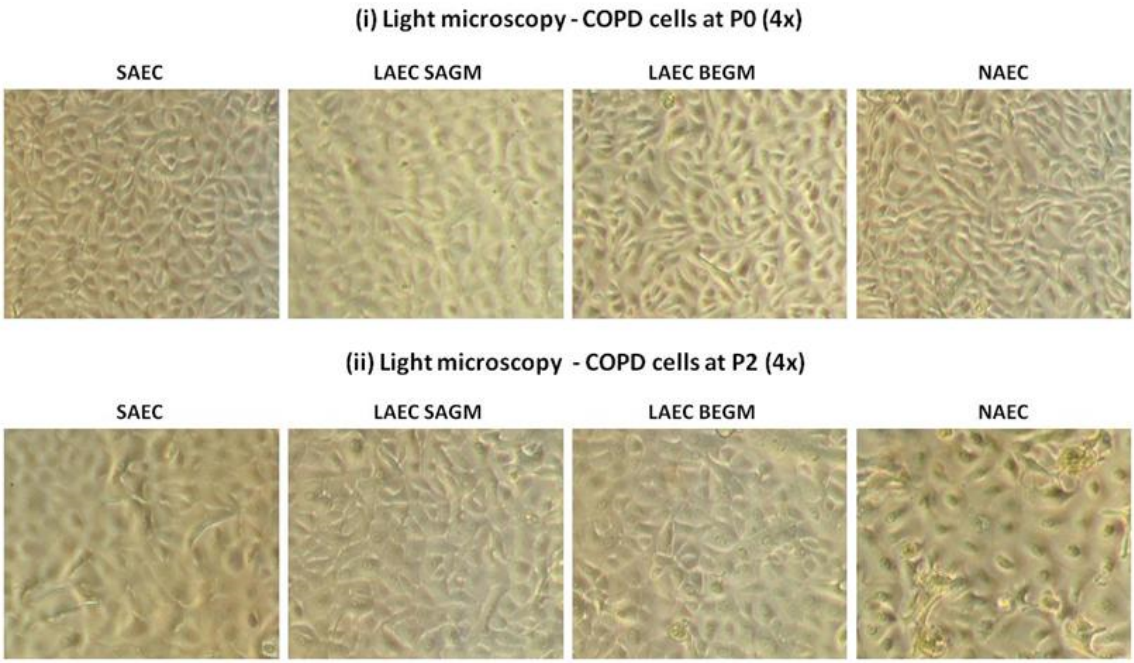


Figure 21 Light microscopy images of COPD cells in culture
Cells were imaged by light microscopy after reaching confluence at P0 and then later at P2. Images are x4 magnification. The images shown are a representative example; all images are from cultures established from one COPD donor. BEGM and SAGM - media types as described in main text.

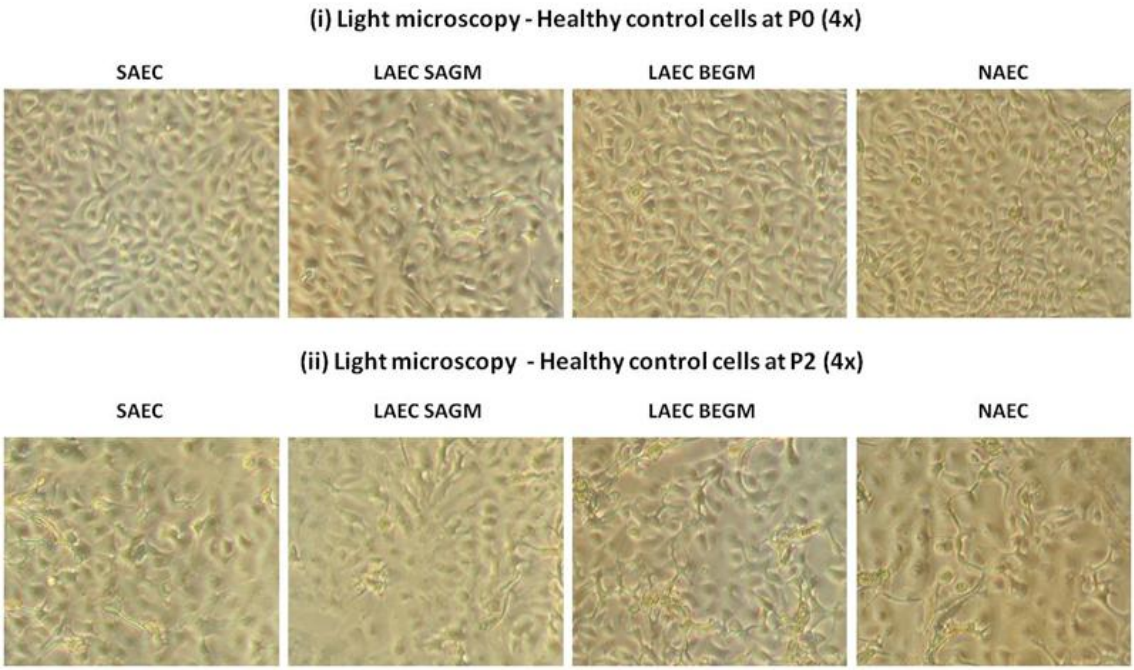


Figure 22 Light microscopy images of healthy control cells in culture
Cells were imaged by light microscopy after reaching confluence at P0 and then later at P2. Images are x4 magnification. The images shown are a representative example; all images are from cultures established from one healthy control donor. BEGM and SAGM - media types as described in main text.

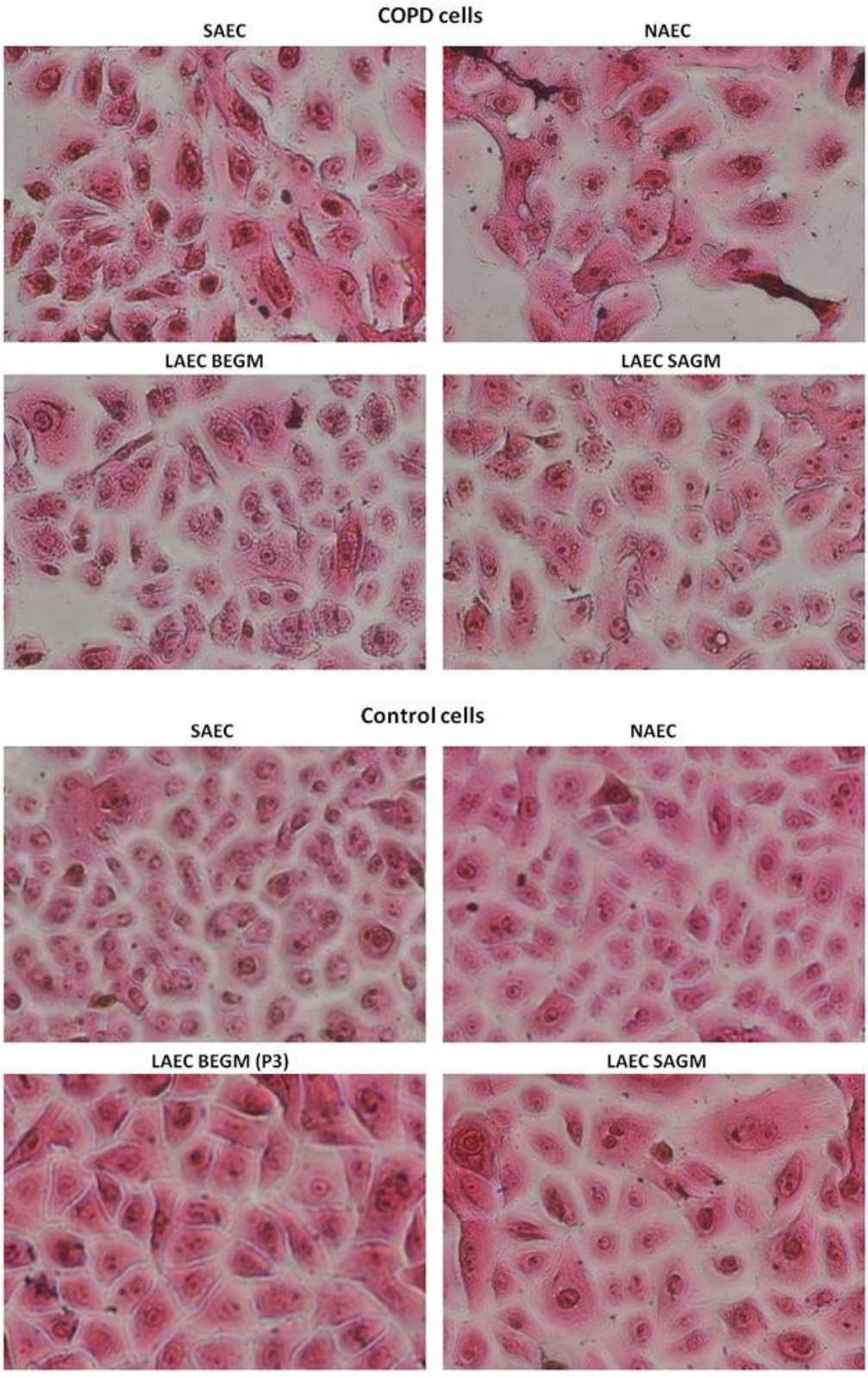


Figure 23 H&E staining of COPD and healthy control cells fixed at P2
Cells were cultured on collagen coated coverslips and fixed in 4% PFA when confluent for H&E staining. The images shown are a representative example taken at x10 magnification; the top panel shows images from cultures established from one COPD donor, the lower panel shows images from cultures established from one control donor. BEGM and SAGM - media types as described in main text.

5.4 Airway epithelial cell marker expression

In order to further characterise the cells cultured from brushings and ensure that these were the relevant cell types for study, immunocytochemistry (ICC) was performed to qualitatively assess epithelial cell marker expression (see Figure 24). Cytokeratins 17 (CK17) and 19 (CK19), and E-cadherin (Ecad) were used as epithelial cell markers; CK19 can be detected in all airway epithelial cells but CK17 only in basal cells (Nakajima et al., 1998). Antibodies to markers characteristic of other cell types which could have contaminated the cultures were also assessed as negative controls (see Figure 25). These were tested in the appropriate cells to ensure that the antibodies were able to bind to the markers of interest. Negative controls included: Fibronectin (Mesenchymal), CD45 (Leukocyte), CD68 (Alveolar macrophage) and CD31/PECAM (Endothelial). Images from cells at P1 from one COPD donor are shown; these are representative of those obtained from both COPD and control cell cultures.

The pattern of Cytokeratin expression detected was as expected with cytoplasmic staining of these intermediate filament proteins. However, the E-cadherin distribution was unexpected with cytoplasmic staining, and particularly a granular perinuclear appearance; this glycoprotein is localised in the adherens junctions of epithelial cells. This finding could be related altered cell growth and adhesion patterns in the culture conditions used, with coverslips for ICC sample preparation. In addition, disruption of cellular attachments and cell wall breakdown through processing of the samples for ICC, including the permeabilisation step used with Triton-X, may have impacted on this protein. For CD31, a transmembrane protein, cells were only permeabilised with Tween to ensure detection of this marker; however, this was not performed for Ecad.

At P2 only Cytokeratin 17 and Fibronectin were assessed (see Figure 26). Minimal positive staining for Fibronectin was observed in cells at this passage; this may relate to cellular stress in culture, and was observed in both COPD and control cells as shown.

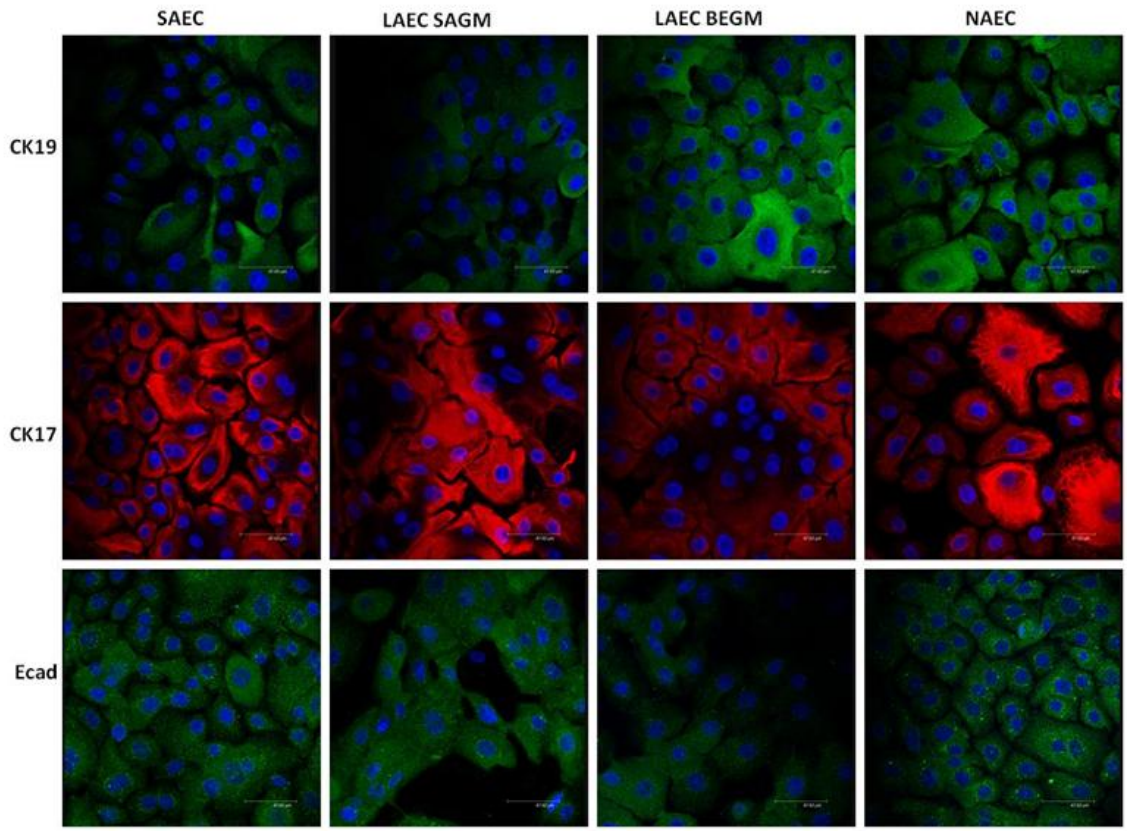


Figure 24 ICC analysis of epithelial cell marker expression in cells at P1
Cells were cultured on collagen coated coverslips and fixed in 4% PFA when confluent to allow processing for immunocytochemistry. Primary antibodies to CK19, CK17 and Ecad were used. Conjugated secondary antibodies allowed detection of CK19 and Ecad positive (green fluorescence - FITC) and CK17 positive cells (red fluorescence - TRITC). DAPI mounting media was used for nuclear staining (blue). Negative controls were performed using only secondary antibodies to ensure no non-specific binding and to set background cellular autofluorescence (data not shown). Details of all antibodies used are provided in the materials section. The cells shown were isolated from one COPD donor.

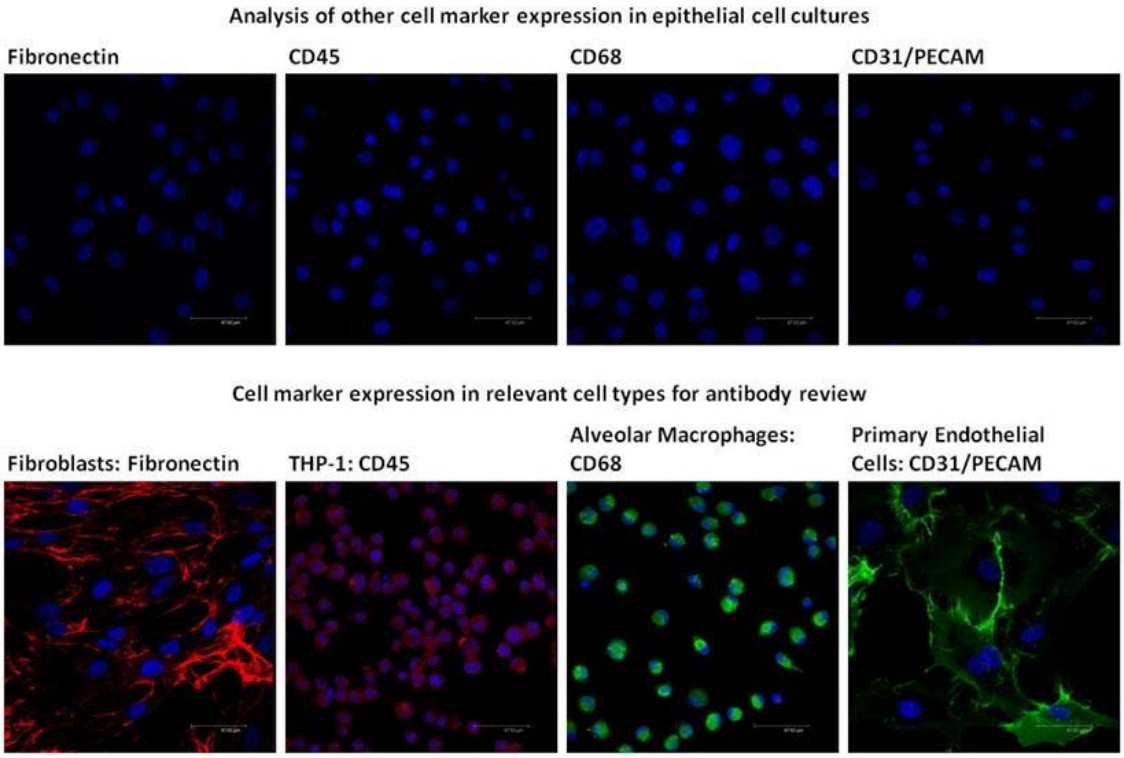


Figure 25 ICC analysis of non-epithelial cell marker expression
Cells were cultured on collagen coated coverslips and fixed in 4% PFA when confluent to allow processing for immunocytochemistry. Primary antibodies to Fibronectin, CD45, CD68 and CD31 were used. Conjugated secondary antibodies allowed detection of positive cells: Fibronectin and CD45 (red fluorescence - TRITC), CD68 and CD31 (green fluorescence - FITC). DAPI mounting media was used for nuclear staining (blue). Negative controls were performed using only secondary antibodies to ensure no non-specific binding and to set background cellular autofluorescence (data not shown). Details of all antibodies used are provided in the materials section.

The top panel shows the results obtained from the above analysis in LAEC at P1 from one COPD donor - this is a representative example of the findings in all cell types from both COPD and control donors. This demonstrates that the cells cultured do not express non-epithelial cell markers and that there was no contamination from other cell types.

The lower panel demonstrates each of the cell markers analysed in an appropriate positive control cell type to show that the antibodies were able to bind to the target proteins being analysed.

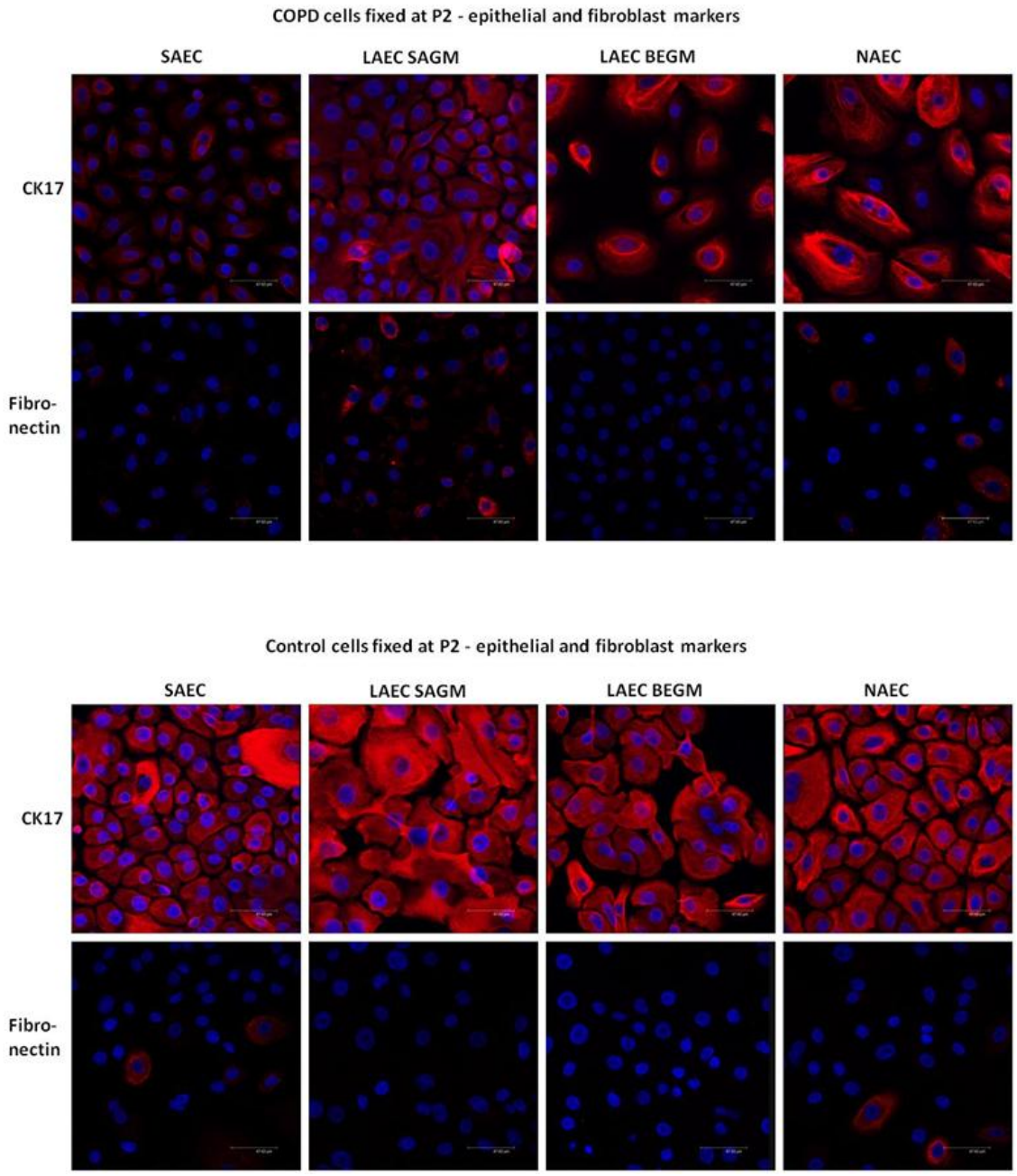


Figure 26 ICC analysis of cell marker expression in cells at P2
Cells were cultured on collagen coated coverslips and fixed in 4% PFA when confluent to allow processing for immunocytochemistry. Primary antibodies to CK17 and Fibronectin were used (see materials). Conjugated secondary antibodies allowed detection of CK17 and Fibronectin positive cells (red fluorescence - TRITC). DAPI mounting media was used for nuclear staining (blue). Negative controls were performed using only secondary antibodies to ensure no non-specific binding and to set background cellular autofluorescence (data not shown).

Top panel: Cells from one COPD donor at P2. Lower panel: Cells from one control donor at P2.

5.5 Clara cell secretory protein (CCSP) expression

In an attempt to differentiate between large and small airway epithelial cells, Clara cell secretory protein (CCSP) expression was investigated. Initially this was assessed using ICC (see Figure 27). However, no positive staining was detected. Similar results were obtained from cell lines tested (HBE and BEAS-2B) (data not shown).

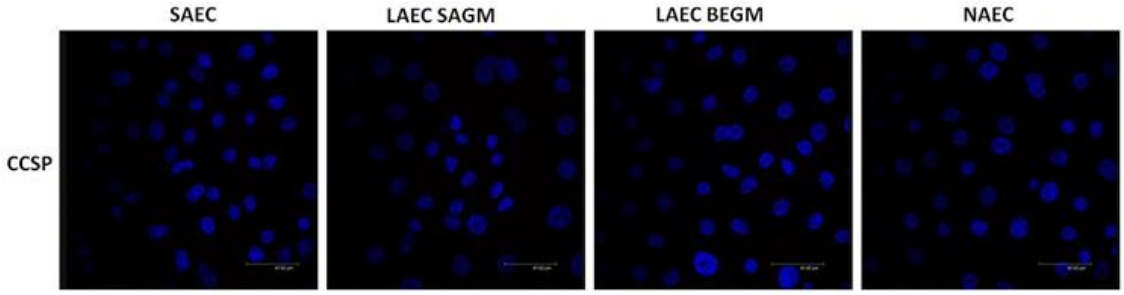


Figure 27 ICC analysis of CCSP expression in cells at P1
Cells were cultured on collagen coated coverslips and fixed in 4% PFA when confluent to allow processing for ICC. Primary antibody to CCSP was used (see materials). Conjugated secondary antibody allowed detection of CCSP-positive cells (red fluorescence - TRITC). DAPI mounting media was used for nuclear staining (blue). Negative controls were performed using only secondary antibodies to ensure no non-specific binding and to set background cellular autofluorescence (data not shown). The images displayed were obtained from cells of one COPD donor; this is representative of results from both COPD and control donor cells with no positive staining identified.

The CCSP antibody was therefore tested on Western blot and this showed positive bands at the expected molecular weight in samples from HBE and BEAS-2B cells; the protein is an 8Da monomer which forms 16 kDa homodimers (see Figure 28). The antibody also reacted positively with recombinant human CCSP on Western blot (R&D: 4218-UT) but this required a protein concentration of 0.2µg. Detection of CCSP protein expression on Western blot in primary cell samples was not observed; this may relate to lower protein levels in these samples and issues with antibody detection of this.

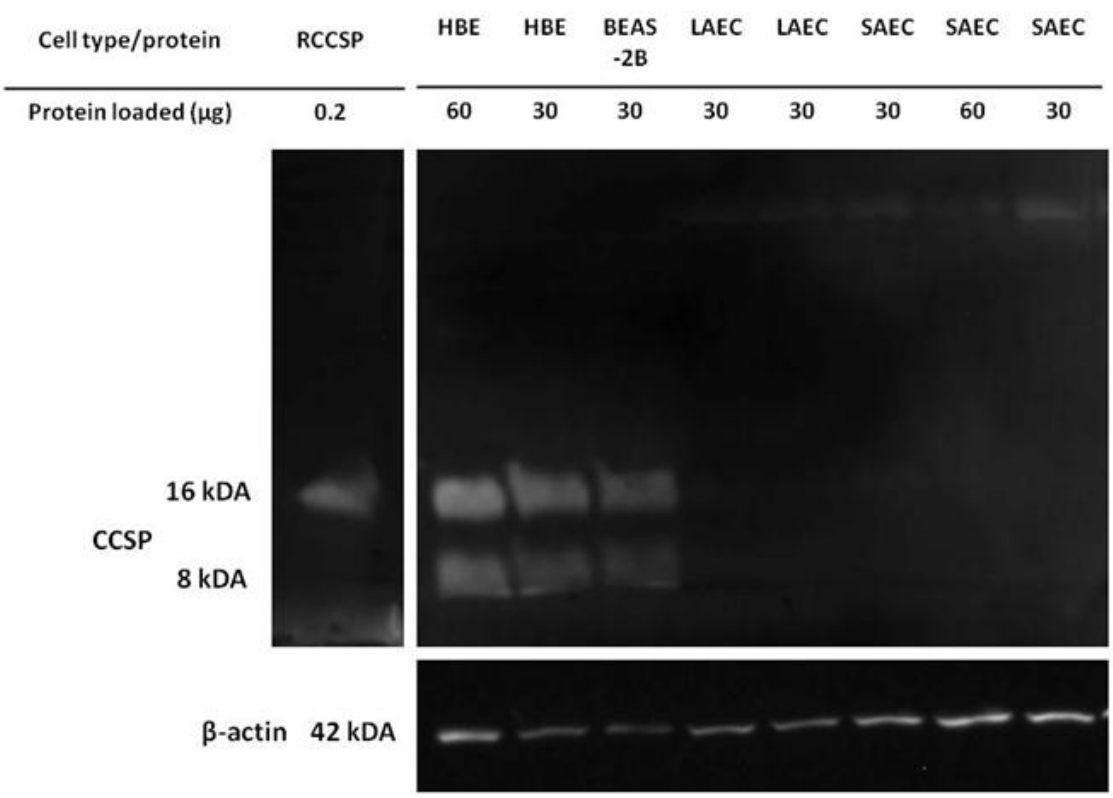


Figure 28 Western blot analysis of CCSP expression
HBE, BEAS-2B and primary airway epithelial cells were cultured to confluence in T25cm2 flask and harvested for epithelial cell marker expression by Western blot. β-actin was using as a loading control. Recombinant CCSP (RCCSP) was used as a positive control to test the antibody used. This figure shows an example of the Western blot results obtained. CCSP protein expression in HBE and BEAS-2B cells is confirmed by positive bands at the appropriate molecular weight. This was not observed in primary cell samples, though some non-specific higher molecular weight bands are noted, of unclear significance.

To further investigate CCSP expression between cells cultured from different airway levels, mRNA expression was assessed from cell samples harvested at P2 (see Figure 29). The graphs show all raw data results. Analysis of available paired sample results (4 sets for each group) using paired T tests showed a significant difference between control SAEC and LAEC cultured in SAGM only.

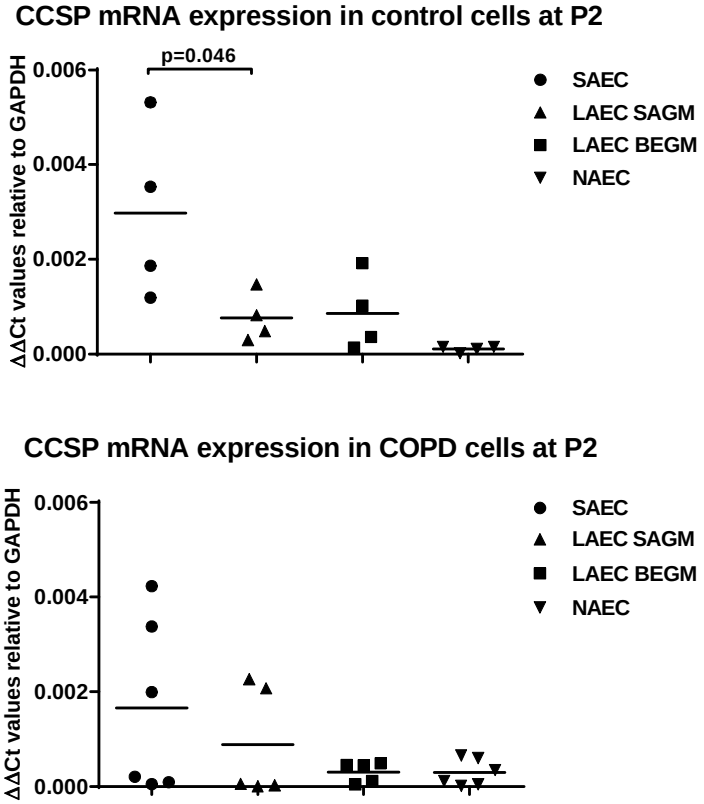


Figure 29 CCSP mRNA expression in primary airway epithelial cells at P2
CCSP mRNA expression was analysed by real time PCR using 20ng cDNA per sample in technical triplicates (note control samples all n=4; COPD samples SAEC and NAEC n=6, LAEC n=5). Expression levels were all low (average threshold cycle 29.4 compared to 19.7 for GAPDH). However, the pattern of results shows higher expression levels in SAEC compared to LAEC or NAEC. Paired data was analysed using a paired T test; a significant difference was found between control SAEC and LAEC SAGM only ($p<0.05$).

5.6 Primary airway epithelial cell Toll-like receptor expression

In addition to characterisation of relevant epithelial cell markers in the primary cultures established, baseline TLR expression was also investigated. The purpose of this was to assess for any significant differences at baseline which may have informed further *in-vitro* cell culture work.

The data are shown in Figure 30, with results from n=4 control paired sets of samples, and n=6 COPD paired sets of samples. This analysis was only performed in LAEC cultured in BEGM and this is the data shown labelled as LAEC. Overall expression levels of all TLRs were low compared to the housekeeping gene *Gapdh* (*e.g.* GAPDH threshold cycle 19, TLR2 25 cycles, TLR4 29 cycles). There was a trend towards higher expression levels in SAEC compared to other cell types, but variability between individual sample results overall. No significant difference was observed between control and COPD cells. TLR5 expression was higher in control SAEC compared to LAEC as shown in Figure 30G (n=4, $p<0.05$).

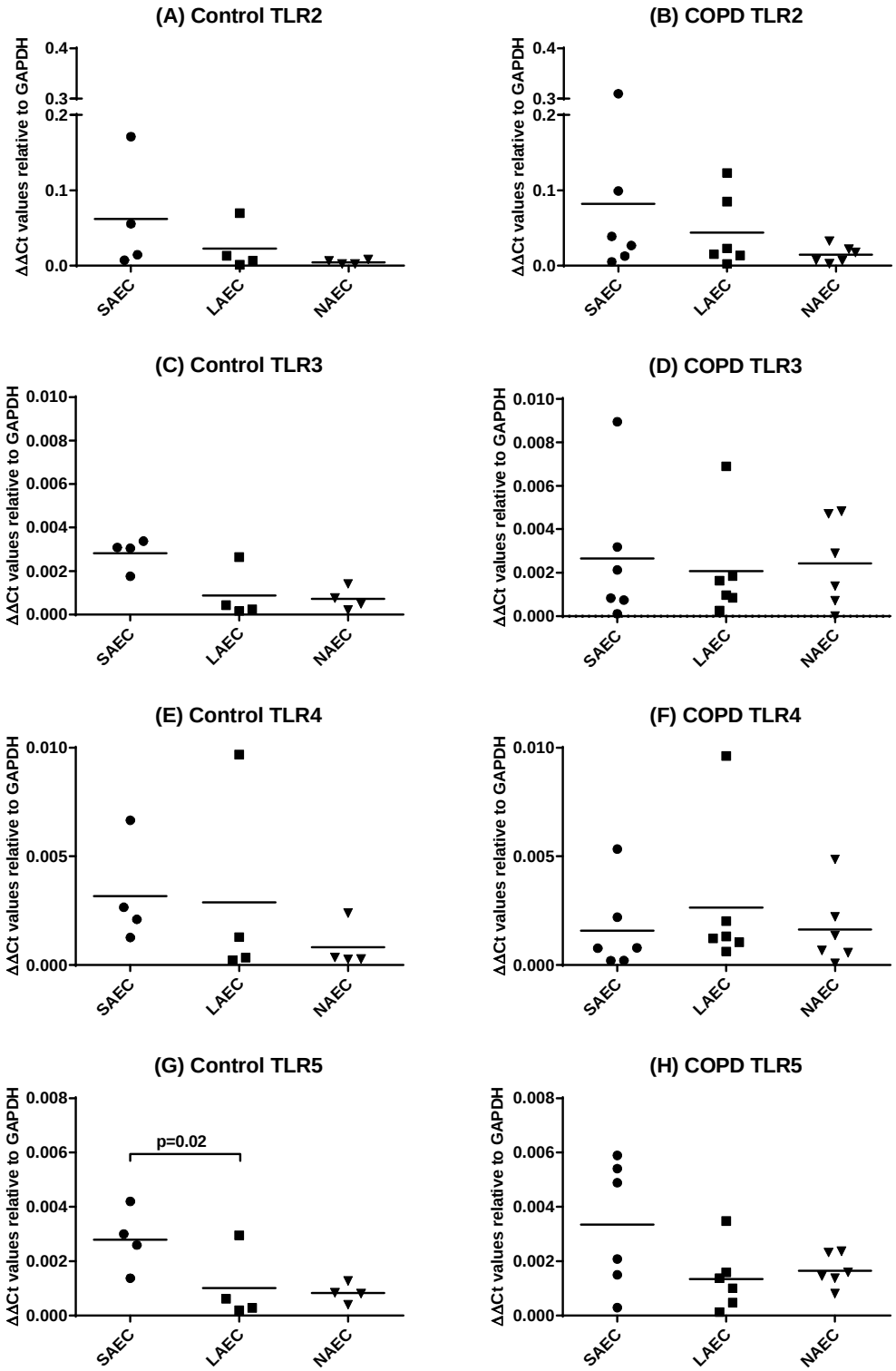


Figure 30 Baseline TLR expression in primary airway epithelial cells in culture
TLR mRNA expression was analysed by real time PCR using 20ng cDNA per sample in technical triplicates (control samples all n=4; COPD samples n=6). Data are expressed as ΔΔ Ct values relative to the house keeping gene GAPDH. Overall expression levels were low with the highest values observed for TLR2 and lowest values for TLR5. No significant differences were noted in expression between the different cell types, except for control cell TLR5 expression (data analysed using paired T tests).

5.7 Discussion

5.7.1 Summary of data presented

The results presented here demonstrate successful establishment of primary airway epithelial cell cultures from the nasal, large and small airways. Overall 90% of initial cultures reached confluence and could be passaged for ongoing investigation. These cells were maintained in submerged culture with characterisation showing the expected cobblestone appearance of an epithelial cell monolayer, and expression of appropriate epithelial cell markers. CCSP expression was difficult to detect at the protein level, but mRNA expression of this potential small airway epithelial marker was greatest in this cell type. Expression of TLRs 2-5 was demonstrated in cells from all airway levels, but with generally very low levels of expression noted in the resting state. TLR5 expression was greater in SAEC compared to LAEC, but this was significant in the control cells analysed only.

5.7.2 Success of primary airway epithelial cell culture

Methods for establishment of cultures of large airway epithelial cells from bronchial brushings have been described previously (Forrest et al., 2005); cells are collected from the large airways, at the 2nd-3rd airway generation. This has also been extended to collection of small airway epithelial cells using a smaller diameter brush (≤2mm) and collecting samples under fluoroscopic screening from more peripheral, smaller generation airways (Harvey et al., 2007, Banerjee et al., 2009). The success of primary airway epithelial cell culture has improved over time. In 1994, Tanaka *et al* sampled cells from the small airway using an ultrathin bronchoscope; 28% of cells collected from small airways and 27.2% of cells collected from central airways reached confluence in 24-48 well plates, with culture rates limited by infection (Tanaka et al., 1994). In 2005, Forrest *et al* reported only 39% success rate for primary large airway epithelial cell culture from post-lung transplant patients; this was mainly due early patient-derived infection in this group (Forrest et al., 2005). Banerjee *et al* later described rates of 84% and 79% of cultures established from large and small airway brushings in transplant patients (Banerjee et al., 2009).

The culture success rates in the current study are similar to those published by Banerjee *et al*. The improvements observed may be related to less patient derived infection and developments in culture media, including antibiotic supplementation. Other groups have also successfully cultured SAEC. Takizawa *et al* cultured cells from the small airways of 12 smokers and 7 non-smokers to analyse spontaneously released IL-8 and soluble ICAM-1 levels; culture rates were not specifically reported but the authors comment that cells could be passaged from 3-5 times, and also stored in liquid nitrogen for later use (Takizawa et al., 2000). However, the culture of cells from three different airway levels in controls and COPD patients has not previously been described. This provides a unique opportunity to investigate differences between airway levels in *in-vitro* cell work, to further understand the pathogenesis of COPD.

5.7.3 Airway epithelial cell characterisation

In the study by Harvey *et al* detailed characterisation of cell types isolated from large and small airway brushings were described (Harvey et al., 2007). A higher proportion of ciliated cells, but fewer basal, undifferentiated, and secretory cells were demonstrated in samples from small airways. The small airway cell samples also contained Clara cells; this cell type predominates in smaller airways in place of goblet cells seen in large airways (Jeffrey and Li, 1997). Non-ciliated Clara cells can be distinguished through their expression of Clara cell secretory protein (CCSP) which is located within cytoplasmic granules. The exact function of CCSP is not clear but it may have an anti-inflammatory role in regulating inflammation (Singh and Katyal, 1997). Previous studies have demonstrated CCSP expression almost exclusively in small airways based on immunohistochemistry of airway tissue samples (Boers et al., 1999). Harvey *et al* used fresh brushings cell samples to detect Clara cells from small airways by transmission electron microscopy (Harvey et al., 2007). Additionally surfactant protein A (SPA) was used as a marker for detection of Clara cells. Cytospins of epithelial cells obtained from small airway brushings have shown CCSP expression at the mRNA level at P0 and at P2, which was not observed in large airway epithelial cells or the 16HBE14o$^-$ cell line (Banerjee et al., 2009).

Characterisation of SAEC by use of CCSP in the current study proved challenging. This issue highlights some of the limitations of *in-vitro* cell culture work, and the use of

submerged cultures. The low expression levels of CCSP detected may be due to the cell culture conditions; when placed in submerged culture there is loss of cell differentiation and absence of ciliated cells (de Jong et al., 1994). Detection of even a small difference in CCSP expression between the different cell types tested suggests that the phenotype of cells is not completely lost in culture. SPA has also previously been used as a marker for Clara cells (Harvey et al., 2007), but other work suggests that this is not detected in this cell type (Nakajima et al., 1998). In the complex continuous airway epithelium, with gradual transition between cell types, there is unlikely to be any single marker which can define small versus large airway epithelial cells; Clara cells may be recognised even in larger airways (Knight and Holgate, 2003). Most importantly for the work presented here, sampling of cells was undertaken from the distal smaller airways as accurately as possible, with successful culture of cells from three airway levels for further investigation.

On commencing work on the current study, our laboratory had experience of PBEC culture from brushings from lung transplant recipients but not COPD patients and healthy volunteers. NAEC and SAEC culture had also not been attempted previously. To ensure that seeding density was sufficient for successful culture, the established local protocol was to seed cells into T25cm^2 flasks (section 3.5.3). Ethical approval was obtained for the study protocol which included samples of 2 brushings at each airway level (section 2.3.3). Therefore 2 T25cm^2 flasks were seeded at P0. This limited the number of cells available for characterisation work at this early stage. In order to ensure adequate cell number for subsequent experiments cells were cultured to P2 (see Chapter 6). This was felt to be the best compromise between adequate cell volume for meaningful experimental work and to minimise loss of the *in-vivo* cell phenotype.

In terms of cell characterisation, the assessments reported may have been affected by cell culture and passage. To clarify this both freshly brushed cells and cells at earlier passage could have been assessed. This was not possible within the current study due to the above limitations. Harvesting of a small aliquot of freshly brushed cells was attempted from a sample of brushings, for subsequent RNA isolation and analysis of

mRNA expression; however insufficient RNA was isolated for this. Previous research has reported comparison of cell marker expression at P0 and P2, using immunocytochemistry of cytospins, and RNA extraction for analysis of mRNA expression; epithelial cell marker expression was preserved between P0 and P2 (Banerjee et al., 2009). In planning future work in this area, additional brushing sampling to allow for characterisation of cells before culture, alongside cells at different passage numbers is important to consider. On assessing a freshly brushed cell sample it would also be essential to review the cell population harvested to assess for possible contamination with other cell types *e.g.* AM, fibroblasts, as described in the characterisation work of cultured cells in the current study. The culture success rates reported are better than those which had previously been observed in our laboratory (overall annual rates of 50-60%). In addition to the above, review of required seeding density for culture propagation could be further assessed. This may allow a greater volume of cells to be available for experimentation at an earlier passage *e.g.* P1.

5.7.4 Toll-like receptor expression in cultured airway epithelial cells

TLR mRNA expression has been widely reported in epithelial cells isolated from the nasal (Lin et al., 2007), large (Mayer et al., 2007), and small airway (Ritter et al., 2005). The relationship of TLR mRNA expression to protein expression is important to consider; previous research in this area has commented on discrepancies between levels of mRNA compared to protein expression detected in both airway and intestinal epithelial cells (Guillot et al., 2004, Cario et al., 2002). TLR expression and localisation at the cell surface or at intracellular sites has been shown to be dependent on cellular differentiation in intestinal epithelial cells (Cario et al., 2002). Guillot *et al* showed the absence of cell surface expression of TLR4 in BEAS-2B cells but demonstrated the presence of this TLR4 in an intracellular subapical cytosolic compartment in this cell line and in primary bronchial epithelial cells (Guillot et al., 2004). The mRNA expression level and intracellular location of TLR4 was not affected by stimulation with the TLR4 ligand LPS. It has been suggested that intracellular location of TLRs may be a mechanism by which the airway epithelium controls its response to airborne stimuli to which it is continuously exposed (Guillot et al., 2004); this could differ depending on airway level.

Differences in expression patterns observed may be related to the cell type studied and whether the cells are resting or stimulated. Mayer *et al* report that limited response of BEAS-2B cells and primary LAEC to lipoteichoic acid (a bacterial cell wall component and known TLR2 ligand) was related to low TLR2 expression, and that of the associated CD36 co-receptor (Mayer et al., 2007). Ritter *et al* demonstrated effects of Poly I:C , a TLR3 ligand, in primary SAEC; increased TLR3 mRNA and protein expression, as well as increased expression of TLR1, TLR2 and associated signalling proteins was reported (Ritter et al., 2005). Wang *et al* showed apical expression of TLR5 in differentiated healthy control LAEC (Wang et al., 2012). The mRNA expression of this TLR was increased by flagellin stimulation, and this affect was reduced by blocking antibody or use of siRNA in BEAS-2B cells. TLR expression was also detectable in submerged basal epithelial cell cultures in this study; however the level of expression was much lower in these undifferentiated cells, compared to those at air-liquid interface, and the response to flagellin was also less in terms of effect on TLR5, IL-6 and IL-8 mRNA expression. In the current study, TLR5 was noted to be increased in SAEC compared to LAEC or NAEC. In addition to the work described above, Wang *et al* reported that SAEC TLR5 was downregulated in smokers both with and without COPD compared to healthy controls in a microarray analysis of this cell type (Wang et al., 2012); this raises the possibility of a smoking-related effect which could be relevant in the response to bacteria which express flagellin.

This previous work shows the importance of considering the baseline TLR expression results in context. It is perhaps not surprising that in an unstimulated state, TLR expression levels are low in undifferentiated airway epithelial cells in submerged culture. In addition, mRNA expression does not account for TLR functionality. The importance of the effect of cell stimulation on TLR expression, at both the mRNA and protein levels, location at the cell surface or within the cell, and downstream signalling are all essential to consider.

As discussed above for cell characterisation and epithelial cell marker expression, TLR expression may also be affected by culture and passage. Investigation of expression in freshly brushed cells and cells at different passage numbers could help to evaluate this.

Given the low expression levels reported, to ensure an adequate concentration of good quality RNA can be analysed, extraction from a larger cell volume may also be beneficial. This should be performed alongside review of sample processing, storage and the extraction method used. Use of a gene array technique, to analyse the whole panel of TLR genes, could provide an initial overview of expression to direct further work, for example where differences are observed between cell types. Further discussion of methods for investigation of TLR expression and function are reviewed in Chapter 6.

Chapter 6. Development of an *in-vitro* cell culture model to assess the effects of cigarette smoke and bacterial pathogens on innate immune responses in airway epithelial cells

6.1 Introduction

The COPD disease process is primarily thought to be triggered by smoking, but in addition, the presence of other factors which can stimulate the immune system including bacterial presence is also now implicated in this. *In-vitro* cell culture work has been used to demonstrate the effects of cigarette smoke at the cellular level, using different preparations including cigarette smoke extract or condensate. Bacterial components, such as lipopolysaccharide, have been used to model the effect of pathogens, as PAMPs which can be recognised by TLRs to initiate an immune response. However, depending on which cell type is used (*i.e.* cell line or primary cells) a number of different responses have been reported. To date, the principal cell types used have been tracheal or bronchial epithelial cells. Nasal epithelial cells have also been used in comparison to bronchial epithelial cells to see if the disease process can be modelled in cells from this site. The responses of the small airway epithelium, which is a key site of the disease process in COPD, and comparison with large or nasal epithelium, have been much less well investigated.

In order to investigate the response of the airway epithelium from different levels, and the effect of cigarette smoke and bacterial pathogens, culture of primary nasal, large and small airway epithelial cells were established from recruited COPD and healthy control donors for investigation in an *in-vitro* cell culture model. Alongside this, cellular responses in airway epithelial cell lines were investigated, to delineate appropriate treatment conditions as a baseline, and to determine if primary cell responses could be modelled in cell lines. Cellular responses to clinically relevant stimuli, non-typeable *Haemophilus influenzae*, a common respiratory pathogen in COPD, and cigarette smoke extract were investigated. To consider the effect of corticosteroid treatment on epithelial cell responses corticosteroid pre-treatments were also performed.

6.2 Approach to *in-vitro* cell culture work

Initial experiments focussed on finding appropriate culture conditions and stimulation parameters, taking into account cell viability and proliferation results, to assess cellular responses in a cell line model. This was then used to guide primary cell culture work.

Cigarette smoke extract (CSE) was chosen to model cigarette smoke exposure, as a technique which was already established in our laboratory, and to allow comparison with previous research using this method. Concentrations of CSE of up to 10% were used, on the basis of previous work using cell lines in our laboratory, including assessment of the effect on cell viability as discussed below. The aim of treatment was to induce a cellular response without significant cell toxicity/death, at the lowest level possible, to try to model the potentially low levels of cigarette smoke components which reach the airway epithelium through the layer of epithelial lining fluid and mucus in the airways.

Bacterial whole cell lysates were chosen to model bacterial infection, as being potentially more reflective of bacterial presence *in-vivo* with multiple PAMPs, rather than focussing on individual TLR ligands in the initial investigation of cellular responses. As for CSE, the doses of bacterial whole cell lysate used were also based on concentrations which could be shown to induce a cell response without significant cell death. The lysates used were tested in both airway epithelial cell lines and in a monocytic cell line as detailed below.

Lysates of non-typeable *Haemophilus influenzae* (HI) and *Pseudomonas aeruginosa* (PA) were used in the cell line work described. *Haemophilus influenzae* is a Gram-negative bacteria which is differentiated into typeable strains which possess a polysaccharide capsule, and non-typeable strains (NTHi) which do not (King, 2012). NTHi are a common cause of respiratory tract infections and are implicated in COPD exacerbations. NTHi express a lipo-oligosaccharide (LOS) on the cell wall (a lipopolysaccharide which lacks the O side chains); this can activate TLR4 (King, 2012). NTHi lipoproteins, including P6 an outer membrane protein, can activate intra-cellular signalling pathways via TLR2 (Shuto et al., 2001). In addition NTHi small cytoplasmic

molecules, rather than LOS or envelope proteins, have been shown to induce IL-8 release from A549 cells (Wang et al., 2003). *Pseudomonas aeruginosa* is a Gram-negative rod which expresses PAMPs including LPS, flagellin (a TLR5 agonist) and peptidoglycan (a TLR2 agonist) (Zhang et al., 2005).

Cells were treated for a 24 hour time period prior to harvesting of samples for analysis. This duration was chosen based on feasibility of treating and harvesting a large number of cells within this time period, and also to expose cells to stimuli over a longer period in keeping with chronic exposure *in-vivo*. Cell responses were first assessed by measuring IL-8 release by ELISA. IL-8 was chosen as the initial mediator to study, as a potent neutrophil chemoattractant known to be elevated in clinical samples from COPD patients, and on the basis that research has shown changes in IL-8 following CSE exposure in *in-vitro* airway epithelial cell culture work as described in Chapter 1. TNF-α was used as a positive control as this is known to stimulate IL-8 release from airway epithelial cells in culture (Cromwell *et al.*, 1992). LPS was used as a further positive control as a known TLR4 ligand. LPS doses of 1 and 10µg/ml were chosen as it is know that higher doses of this TLR ligand may be required to stimulate epithelial cells compared to other innate immune cells, possibly related to low expression of TLR4 and the co-receptor MD2 (Becker *et al.*, 2000). 16HBE14o$^-$ (HBE) cells were used as an established cell line in use in our laboratory. Following review of initial results, BEAS-2B cells were also used, as a comparison to assess if the same responses were observed in an alternative airway epithelial cell line.

6.3 Airway epithelial cell line culture model results

6.3.1 Effect of cigarette smoke extract and bacterial whole cell lysate on cell line viability and proliferation

Experiments to investigate the effect of increasing concentrations of CSE on cell viability in airway epithelial cell lines were previously completed during my MRes project (in HBE and A549 cells), and therefore this work was not repeated as part of the current study. In this previous work, cells were cultured with concentrations of CSE from 0-10% for 24 hours, and viability assessed by propidium iodide uptake on flow cytometry; no effect of CSE up to 10% concentration was observed (data not shown).

To further assess potential toxic effects of CSE on cellular function, the effect on proliferation was investigated. CSE concentrations of ≥1% significantly reduced proliferation of both HBE and BEAS-2B cells (each n=3, $p<0.05$, paired T tests) (see Figure 31A and B). This effect was confirmed by concurrent cell counts performed for one of the BEAS-2B experiments (Figure 31C and D).

Experiments to assess the effect of non-typeable *Haemophilus influenzae* whole cell lysate (HI) preparations on cell viability were performed. The effect of increasing concentrations of HI was assessed; doses up to 25µl/ml did not have any significant effect on cell viability (n=2, $p>0.05$, unpaired T tests) (see Figure 32).

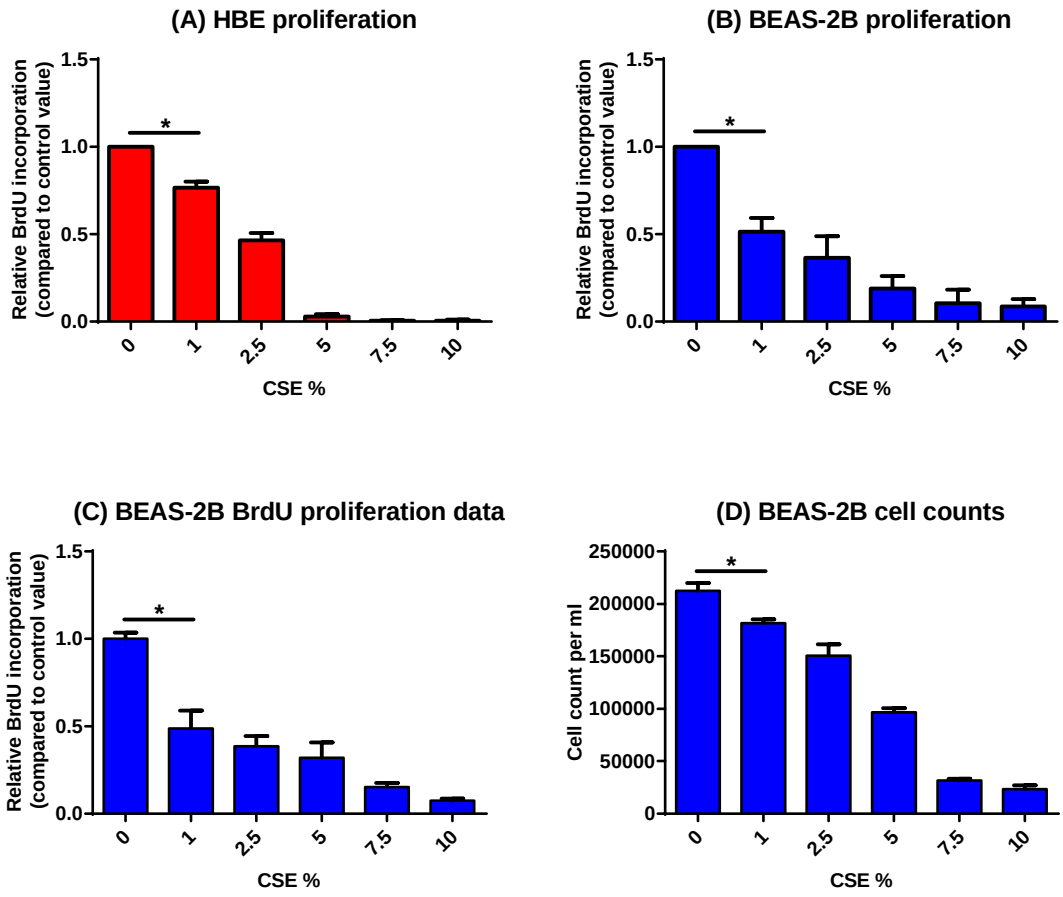


Figure 31 Cell proliferation following CSE exposure in airway epithelial cell lines
Cells were treated in triplicate with increasing concentrations of CSE (0-10%) and the effect on proliferation assessed by BrdU incorporation (cells cultured for 24 hours in media containing CSE, following by 24 hours of BrdU labelling time).

Displayed data show the mean results (± SEM) from 3 separate experiments in (A) HBE, and (B) BEAS-2B cell lines, expressed as values relative to the control sample mean BrdU incorporation. In both cell lines proliferation decreased with increasing CSE concentration (*$p<0.05$, paired T tests).

For one of the BEAS-2B experiments (C) cells were seeded separately in 12 well plates (at 200,000 cells per well), treated in triplicate under the same conditions, and then harvested at the same time point as BrdU incorporation to perform concurrent cell count analysis (D); data displayed as mean cell count per well (± SEM) (*$p<0.05$, unpaired T test). This confirmed a reduction in cell number in line with the reduced proliferation observed.

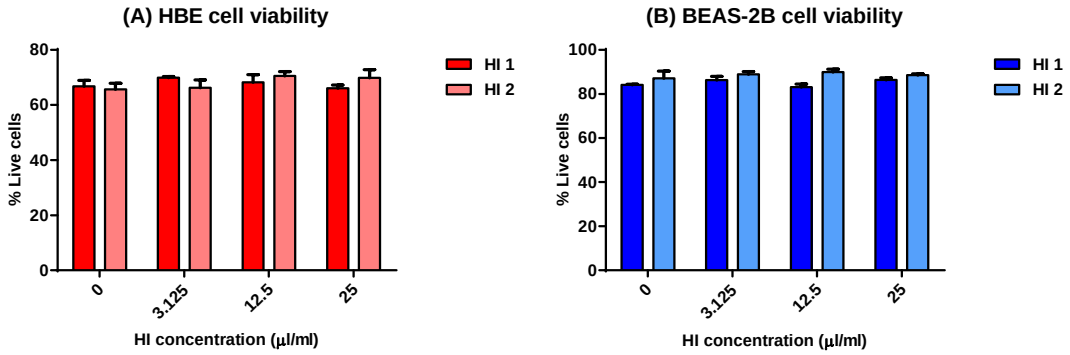


Figure 32 Effect of HI whole cell lysate preparations on airway epithelial cell line viability

Cells were treated in triplicate with media containing increasing concentrations of HI (0-25µl/ml) for 24 hours, and cell viability assessed by uptake of propidium iodide on flow cytometry. Data shown are the results from 2 experiments with different HI preparations (from the same strain), displayed as mean values (± SEM). No significant effect on viability was observed at the concentrations tested.

6.3.2 Effect of cigarette smoke extract and bacterial whole cell lysate on pro-inflammatory cytokine release from airway epithelial cell lines

Having assessed cell viability and proliferation as above, doses of up to 5% CSE and up to 25µl/ml HI, alone and in combination, were used for subsequent cell stimulation experiments.

Cigarette smoke extract, but not bacterial whole cell lysate, increased IL-8 release from HBE cells

A significant increase in IL-8 release was observed in HBE cells treated with increasing concentrations of CSE (baseline IL-8 440±7 pg/ml; 1% CSE IL-8 686±13 pg/ml; n=3, $p<0.05$) (Figure 33A and B). However, no change in IL-8 levels was seen in response to HI, despite use of preparations from 3 different clinical isolates (overall mean values from 3 experiments: baseline IL-8 1171±45 pg/ml; 25µl/ml HI IL-8 1099±118 pg/ml; n=3, $p>0.05$) (Figure 33C). To ensure that this was not an issue with lysate preparation, or specific response to HI, lysate preparations from 3 clinical isolates of *Pseudomonas aeruginosa* (PA) were also tested (Figure 33D); no change in IL-8 levels was observed following PA treatment (overall mean values from 3 experiments: baseline IL-8 1358±10 pg/ml; 25µl/ml PA 1212±100 pg/ml; n=3, $p>0.05$). It should be noted that the baseline IL-8 release in these experiments was noted to be higher than that observed in the initial CSE exposure experiments. However, a positive response to TNF-α as a control was still observed above a higher baseline level (baseline IL-8 823±58 pg/ml; TNF-α IL-8 1560±69 pg/ml; n=3, $p<0.05$) (Figure 33F). The cells also responded to LPS at a dose of 10µg/ml though this was less marked than the TNF-α induced IL-8 release observed (baseline iL-8 823±58 pg/ml; LSP10 1276±52 pg/ml; n=3, $p=0.05$) (Figure 33F).

The lysate preparations were all tested in THP-1 cell line experiments (see Figure 34), to ensure that an appropriate response was observed in this monocytic cell line as has been observed previously (Paul-Clark et al., 2008), and to ensure that the lysate preparation had not inactivated the bacterial PAMPs present. A significant increase in IL-8 levels was noted with all doses tested from 1.56µl/ml HI (overall mean values from 3 experiments: baseline IL-8 151±8 ng/ml; 1.56 HI IL-8 325±10 ng/ml; n=3, $p<0.05$).

The same pattern of results was observed for PA (baseline IL-8 164±8 ng/ml; 1.56 PA IL-8 448±28 ng/ml; n=3, $p<0.05$).

To assess for a possible combined effect of CSE and HI treatment in HBE cells, a further experiment was performed with both treatments (Figure 33E). However, no additional effect compared to that of CSE alone was observed.

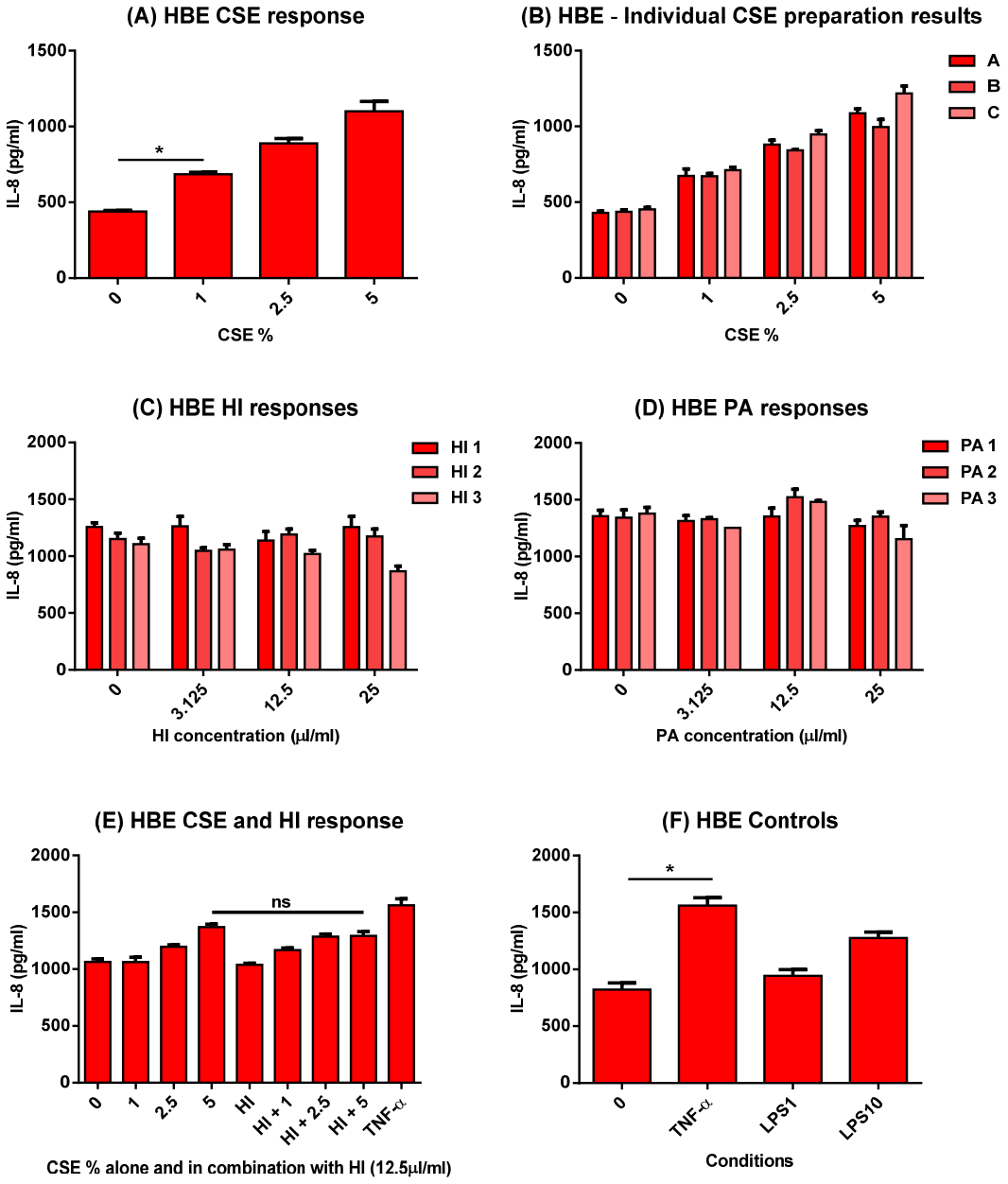


Figure 33 HBE responses to CSE and bacterial whole cell lysate preparations
Cells were treated in triplicate with CSE (0-5%), HI or PA (0-25ul/ml) alone, or combined CSE/HI treatment. TNF-α (20ng/ml) and LPS (1µg/ml and 10µg/ml) were used as positive controls. After 24 hours media was harvested for analysis of IL-8 levels by ELISA. (A), (B) An increase in IL-8 release was observed with increasing CSE concentration (n=3, $p<0.05$, paired T test). (C), (D) HBE cells did not release IL-8 in response to HI or PA (n=3, $p>0.05$, paired T test of mean values). (E) Combined CSE/HI treatments had no further effect on IL-8 levels compared to CSE treatment alone (n=1, $p>0.05$, unpaired T test). (F) A positive response to TNF-α was observed (n=3, $p<0.05$, paired T test), but the LPS response was less marked. Data displayed as mean (± SEM) (*$p<0.05$).

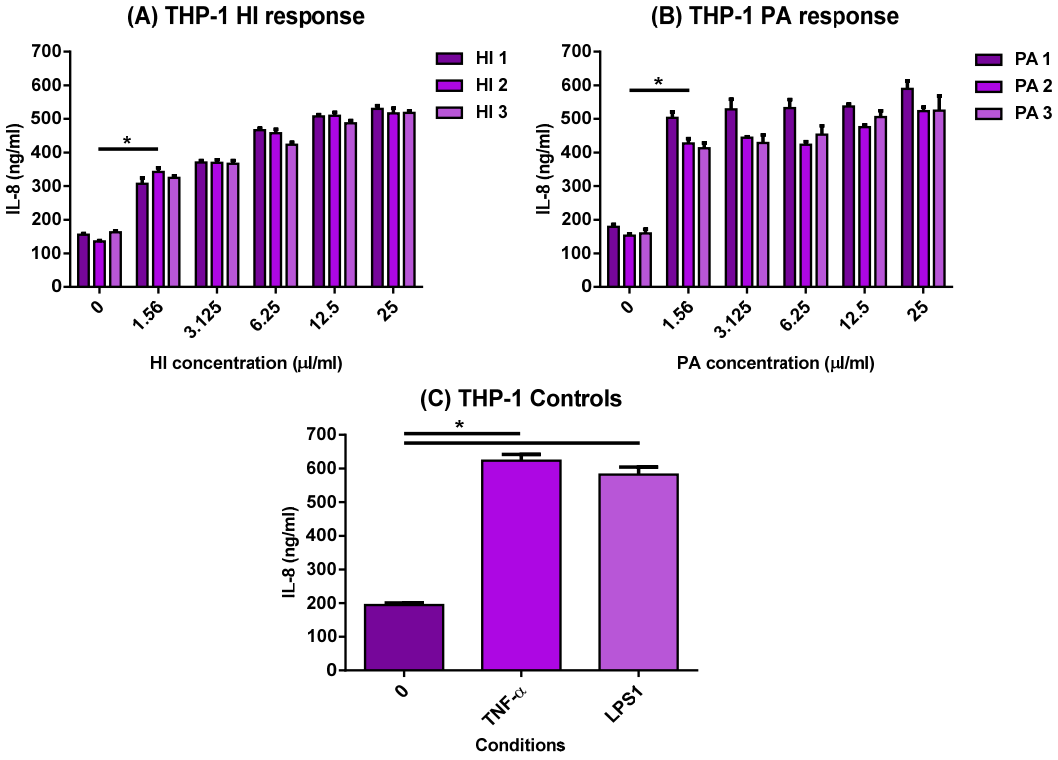


Figure 34 THP-1 responses to bacterial whole cell lysate preparations
Cells were treated in triplicate with HI or PA (0-25ul/ml). TNF-α (20ng/ml) and LPS (1μg/ml) were used as positive controls. After 24 hours media was harvested for analysis of IL-8 levels by ELISA. (A), (B) Levels of IL-8 increased with increasing HI or PA concentration (n=3, $p<0.05$, paired T tests of mean values). (C) A positive response to TNF-α and LPS was also observed (n=3, $p<0.05$). Data displayed as mean (± SEM) (*$p<0.05$). Note units in ng compared to pg for airway epithelial cell lines.

Cigarette smoke extract had no effect on IL-8 release from BEAS-2B cells, but an increase in IL-8 was observed after bacterial whole cell lysate treatment

In contrast to the HBE cell line, CSE had no significant effect on IL-8 release from BEAS-2B cells (baseline IL-8 121±17 pg/ml; 5% CSE IL-8 168±44 pg/ml; n=3, $p>0.05$) (Figure 35A). In case of any effect of CSE on cell number (based on proliferation results) and possible associated reduced IL-8 levels due to this a further experiment was performed with cell counts to adjust IL-8 levels based on cell number present (Figure 35B-D). Taking cell counts into consideration there was no still impact observed of CSE concentrations up to 5% on IL-8 levels (baseline IL-8 276±31 pg/ml; 5% CSE IL-8 325±28 pg/ml; n=1, $p>0.05$ unpaired T test). However, unlike the HBE cell responses observed, BEAS-2B cells responded with significant increase in IL-8 levels to all doses of HI tested (overall means from 3 experiments: baseline IL-8 414±143 pg/ml; 3.125 HI IL-8 3268±201 pg/ml; n=3, $p<0.05$) (Figure 36A). Positive responses were also seen to control treatments including TNF-α and LPS (baseline IL-8 230±65 pg/ml; TNF-α IL-8 3755±418 pg/ml; LPS1 IL-8 3189±459 pg/ml; LPS10 IL-8 5279±188 pg/ml; n=3, $p<0.05$) (Figure 36C). Combined treatment with HI and CSE showed no difference compared to either treatment alone (n=3, $p>0.05$) (Figure 36B).

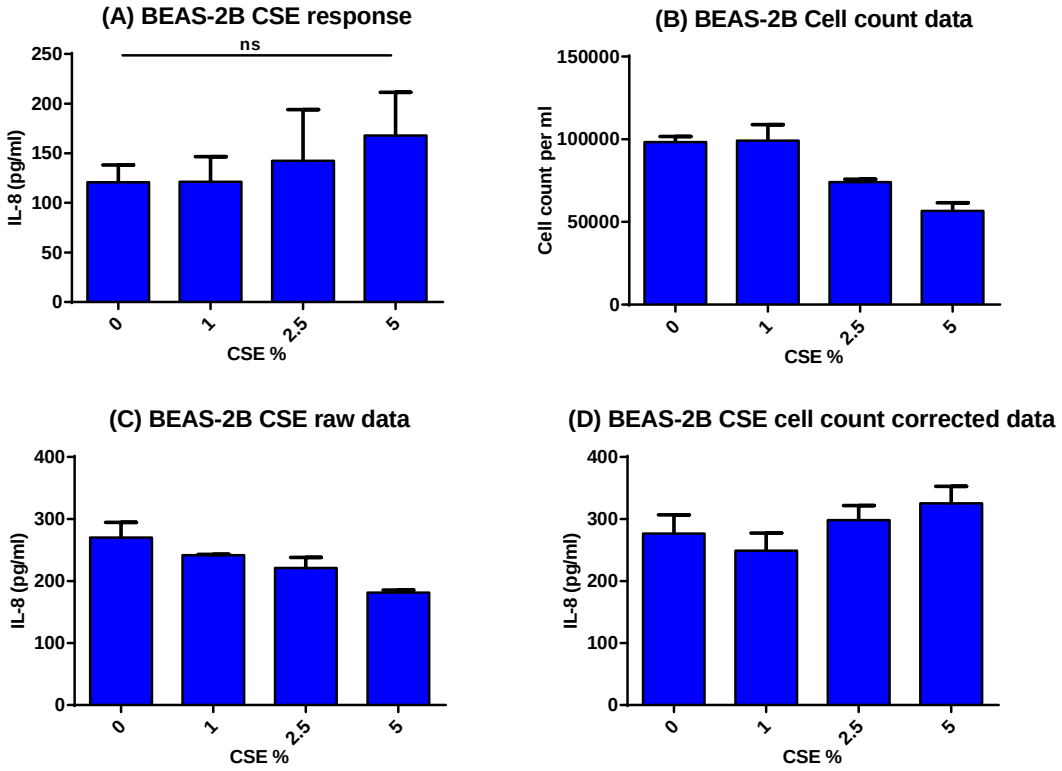


Figure 35 BEAS-2B responses to CSE

Cells were treated in triplicate with CSE (0-5%). After 24 hours media was harvested for analysis of IL-8 levels by ELISA. (A) No significant change in IL-8 release was observed with treatment up to 5% CSE (n=3, $p>0.05$, paired T test). To ensure that the results observed were not affected by a potential effect of CSE on cellular proliferation, with possible reduced cell number impacting on levels of IL-8 release, a further experiment was performed with cell counts to correct the IL-8 levels (C-D): these results show that both cell number and IL-8 levels decreased with increasing CSE% and so when correcting IL-8 levels for cell number no significant increase in release was observed (n=1, $p>0.05$, unpaired T test). Data are displayed as mean (± SEM).

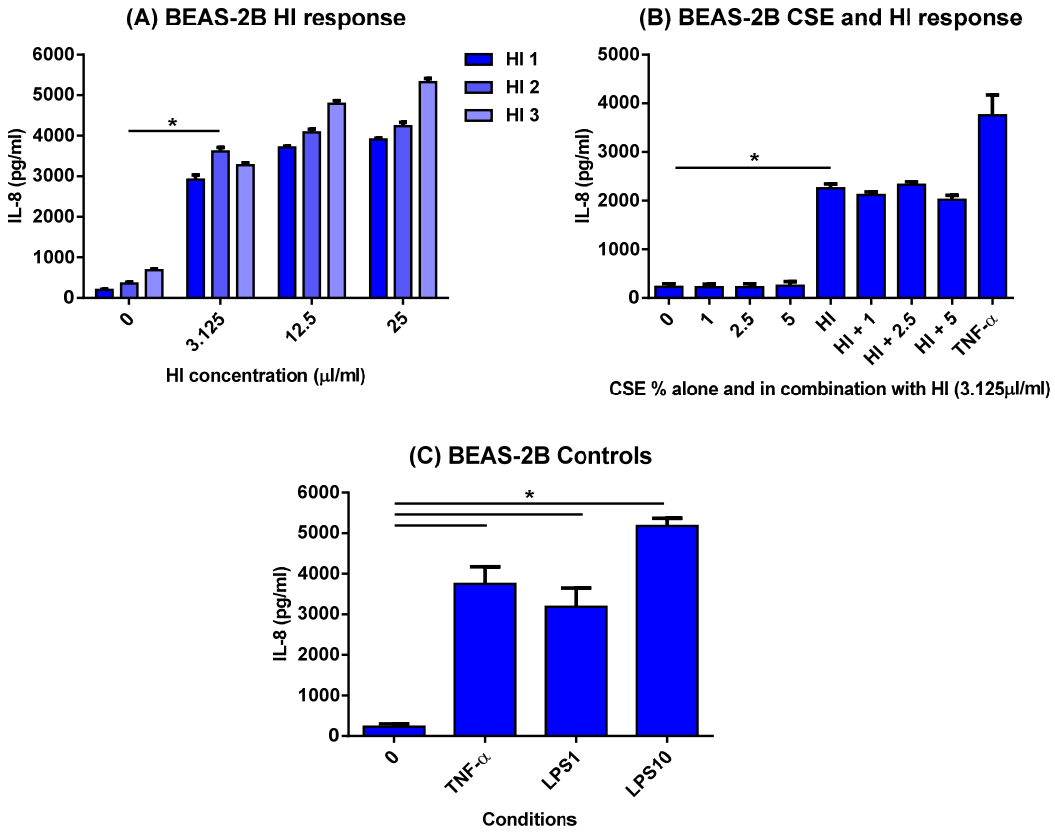


Figure 36 BEAS-2B responses to HI bacterial whole cell lysates

Cells were treated in triplicate with HI (0-25ul/ml) ± CSE (0-5%). TNF-α (20ng/ml) and LPS (1µg/ml and 10µg/ml) were used as positive controls. After 24 hours media was harvested for analysis of IL-8 levels by ELISA. (A) IL-8 levels increased in response to increasing doses of HI treatment (n=3, $p<0.05$, paired T tests). (B) Combined CSE/HI treatments had no further effect on IL-8 levels compared to either treatment alone (n=3, $p>0.05$, paired T tests). (C) BEAS-2B demonstrated a positive response to TNF-α and LPS (at both doses tested) (n=3, $p<0.05$, paired T tests). Data displayed as mean (± SEM) (*$p<0.05$).

6.3.3 Investigation of the effect of corticosteroid treatment on TNF-α induced IL-8 release from BEAS-2B cells

There are a number of different corticosteroids used both clinically and in research but all have the same basis for mechanism of anti-inflammatory action. In our laboratory, Dexamethasone has previously been used successfully to suppress *in-vitro* models of inflammation. In addition, to consider more clinically relevant steroid preparations, and to ensure that there was not an issue with metabolism to an active compound in our model system, both Beclomethasone dipropionate (BDP) and 17-Beclomethasone monopropionate (17-BMP, the active metabolite of BDP) were used (the 17-BMP was kindly supplied by Chiesi for the project). BDP is commonly used in an inhaled corticosteroid preparation used for the treatment of COPD.

The corticosteroid preparations required solubilisation in dimethyl sulfoxide (DMSO) and this impacted on the highest concentrations which could be used (1mM steroid stock solutions in DMSO, highest final concentration of DMSO in culture media 0.1%, therefore highest steroid dose tested 1µM, see methods section for details). Initial experiments were performed in BEAS-2B cells to assess the effectiveness of the corticosteroid treatment in suppression of TNF-α induced IL-8 release. The dose range chosen was based on the stock solutions available as described above, previous literature showing potent effects of Dexamethasone at 1µM concentration (Kwon et al., 1994), and from details provided by Chiesi with respect to 17-BMP activity (subnanomolar EC50 to induce leucine zipper mRNA expression in airway smooth muscle cells). In terms of relevance to the *in-vivo* situation, use of the lowest dose possible for inhibition in the primary cell culture model was planned.

Corticosteroid pre-treatment significantly reduced TNF-α induced IL-8 release from BEAS-2B cells

Increased levels of IL-8 were detected in culture media following TNF-α stimulation alone as seen in the previous results (Dexamethasone experiment baseline IL-8 253±12 pg/ml; TNF-α IL-8 2414±21 pg/ml) (Figure 37). A degree of suppression of baseline IL-8 release was noted with DMSO or 17-BMP alone at 4 hours (17-BMP baseline IL-8 115±11 pg/ml; DMSO 88±2 pg/ml; 17-BMP 10^{-6}M 94±5 pg/ml), and for DMSO and

either steroid preparation at the 24 hour time point (Dexamethasone baseline IL-8 253±12 pg/ml; DMSO 172±3 pg/ml; Dexamethasone 10^{-6}M 120±5 pg/ml). The response to TNF-α was suppressed by all concentrations of steroid tested using 4 hour pre-treatment (Figure 37A and C): Dexamethasone results - TNF-α alone IL-8 release 2414±21 pg/ml; with Dexamethasone 10^{-15}M 2002±72 pg/ml; 17-BMP results - TNF-α alone IL-8 release 2675±16 pg/ml; with 17-BMP 10^{-15}M 2298±103 pg/ml. The same pattern of result was observed with 24 hour steroid pre-treatment (Figure 37B and D), with more marked suppression of IL-8 levels observed at this time point: Dexamethasone 10^{-15}M 968±190 pg/ml; 17-BMP 10^{-15}M 1116±7 pg/ml.

Based on this initial data and more effective 24 hour pre-treatment in terms of suppression of TNF-α induced IL-8 release, a further assessment of a wider range of corticosteroid concentrations was performed at this time point (see Figure 38). In addition the dose response to BDP was tested in this experiment to assess if the response would match that of the metabolite, 17-BMP. The same pattern of results was observed with suppression of TNF-α induced IL-8 release at all doses of steroid treatment tested. The dotted line represents a 50% reduction in TNF-α induced IL-8 release (total mean release 1740 pg/ml, 50% of mean value = 870 pg/ml). This was observed at a concentration of 10^{-9}M Dexamethasone (mean IL-8 870±68 pg/ml) (Figure 38A). At 17-BMP 10^{-9}M concentration, mean IL-8 levels were 920±84 pg/ml (47% reduction) (Figure 38B). At BDP 10^{-9}M concentration, mean IL-8 levels were 945±23 pg/ml (46% reduction) (Figure 38C).

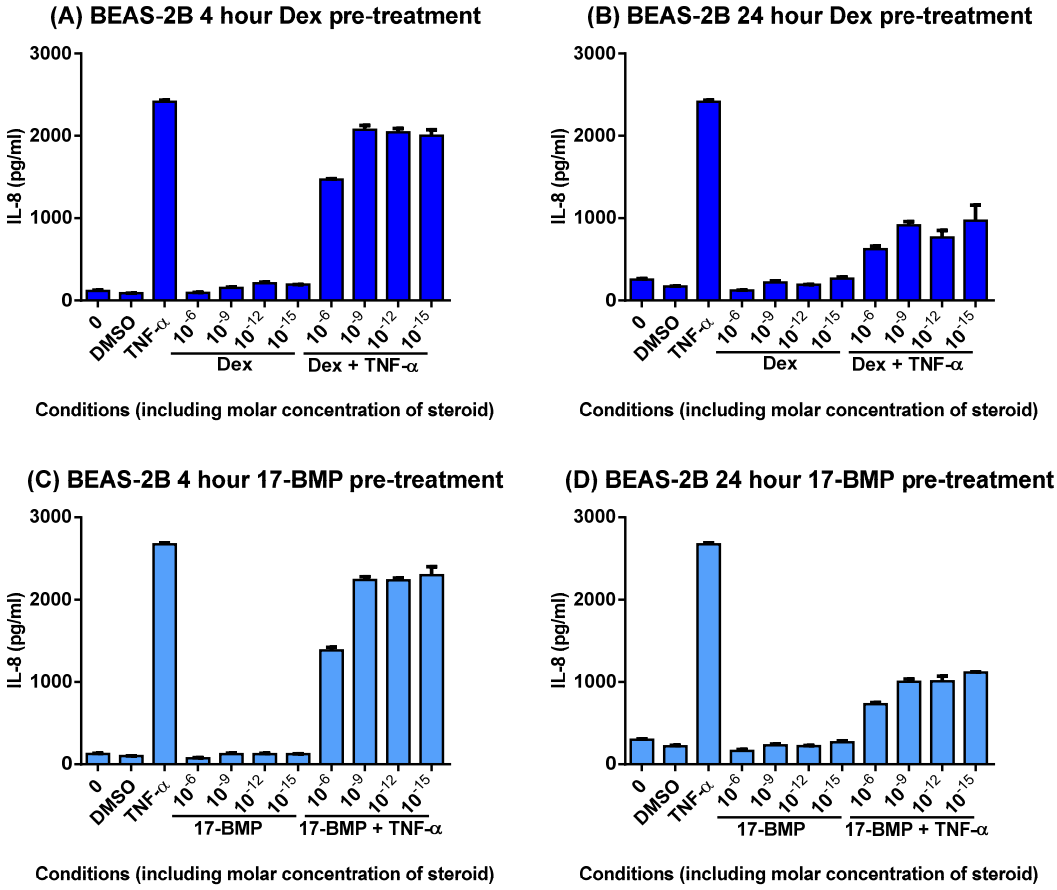


Figure 37 Initial dose response experiments to assess the effect of *in-vitro* corticosteroid treatment on TNF-α induced IL-8 release from BEAS-2B cells
Cells were treated in triplicate with normal media, media containing 0.1% DMSO, and media containing increasing concentrations of Dexamethasone (Dex) or 17-BMP for 4 or 24 hours, prior to the addition of TNF-α (20ng/ml), for a further 24 hours. Media was harvested for analysis of IL-8 levels by ELISA. Treatment with either Dexamethasone (A, B), or 17-BMP (C, D) reduced IL-8 levels, with a more pronounced effect after 24 hours pre-treatment (each n=1 in triplicate). Data displayed are mean values (± SEM).

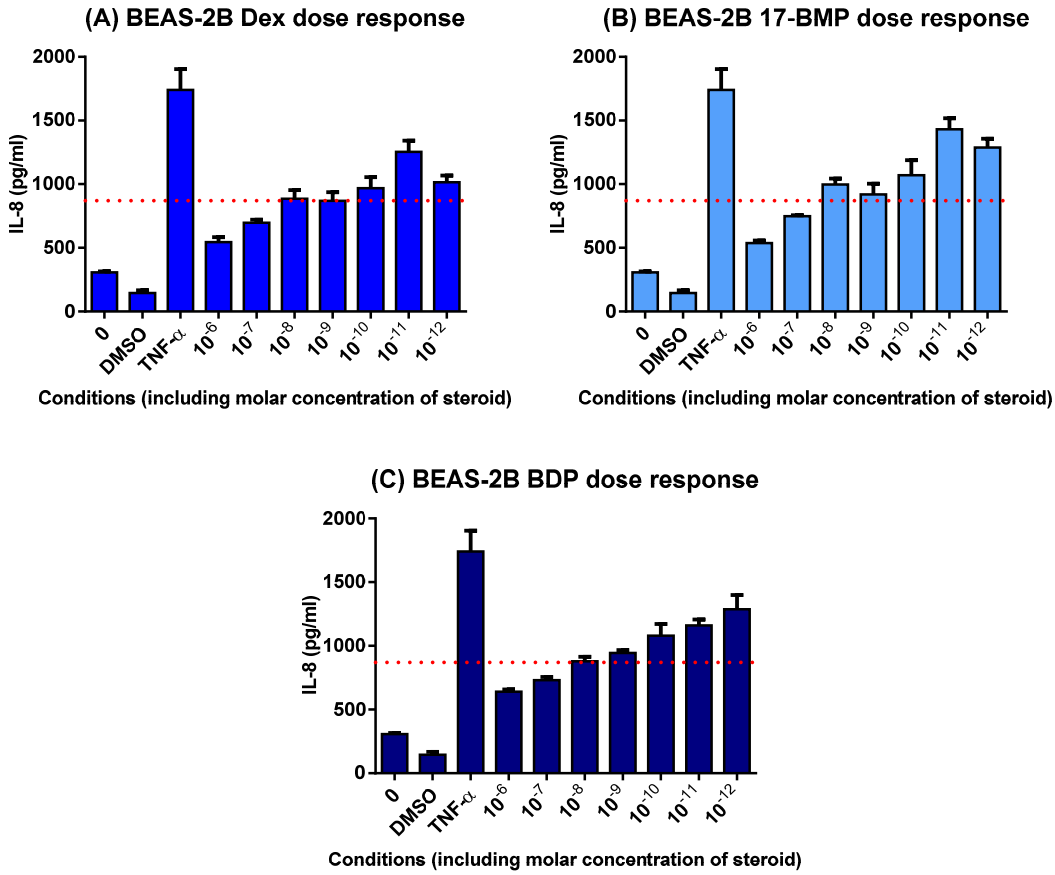


Figure 38 Extended dose response experiments to assess the effect of *in-vitro* corticosteroid treatment on TNF-α induced IL-8 release from BEAS-2B cells

Cells were treated in triplicate with normal media, media containing 0.1% DMSO, and media containing increasing concentrations of Dexamethasone (Dex), 17-BMP, or BDP for 24 hours, prior to the addition of TNF-α (20ng/ml), for a further 24 hours. Media was harvested for analysis of IL-8 levels by ELISA. Treatment with either (A) Dexamethasone, (B) 17-BMP, or (C) BDP all reduced IL-8 levels (each n=1 in triplicate). Data displayed are mean values (± SEM). The dotted line represents a 50% reduction in TNF-α induced IL-8 release (50% of total mean IL-8 1740 pg/ml = 870 pg/ml).

6.4 Setting up the primary airway epithelial cell culture model

Having assessed cell treatments in the airway epithelial cell lines, variability was noted in cellular responses making it essential to evaluate the effects in primary airway epithelial cells prior to a more detailed assessment of specific stimulation treatments. Therefore further work was performed to assess primary cell viability following CSE or HI treatment, cell proliferation following CSE treatment, and IL-8 release in response to HI. Primary cells were treated at P2; this passage was chosen as a compromise between adequate cell volume for experimental work, and to minimise loss of the *in-vivo* cell phenotype due to ongoing passage and culture.

As described under cell characterisation work (Chapter 5), all initial cell cultures were set up in SAGM media. Due to issues with NAEC growth, the culture media was subsequently changed to BEGM, and this also highlighted consideration of the most appropriate media type for LAEC culture. LAEC were therefore cultured in SAGM and BEGM for the initial viability and proliferation experiments, and also for the HI dose response work. The data displayed for the viability and proliferation results is from LAEC cultured in BEGM only; no significant difference was seen compared to cells cultured in SAGM (data not shown). The data displayed for the HI dose response work is included for LAEC cultured in BEGM and SAGM; no statistically significant differences were seen. On the basis of aiming to create the optimal cell culture conditions for each cell type, LAEC were subsequently all cultured in BEGM (as media developed for bronchial airway epithelial cell culture, as opposed to SAGM developed for SAEC culture).

In addition to this, control experiments were performed using complete and incomplete media, to ensure that a more marked response to the cell treatments was not being missed due to high baseline mediator release. Complete media was made up as described in the methods section. Incomplete media was made up using only Insulin, Transferrin, and Gentamicin/Amphotericin from the SingleQuot kits, with the addition of Penicillin/Streptomycin.

6.4.1 Effect of cigarette smoke extract and Haemophilus influenzae whole cell lysate on viability and proliferation in primary airway epithelial cells

As for the cell lines tested, no effect of CSE concentrations (up to 10%) or HI (up to 25µl/ml) was observed on cell viability (Figure 39). Cellular proliferation however, significantly decreased with increasing concentrations of CSE in all cell types tested (Figure 40).

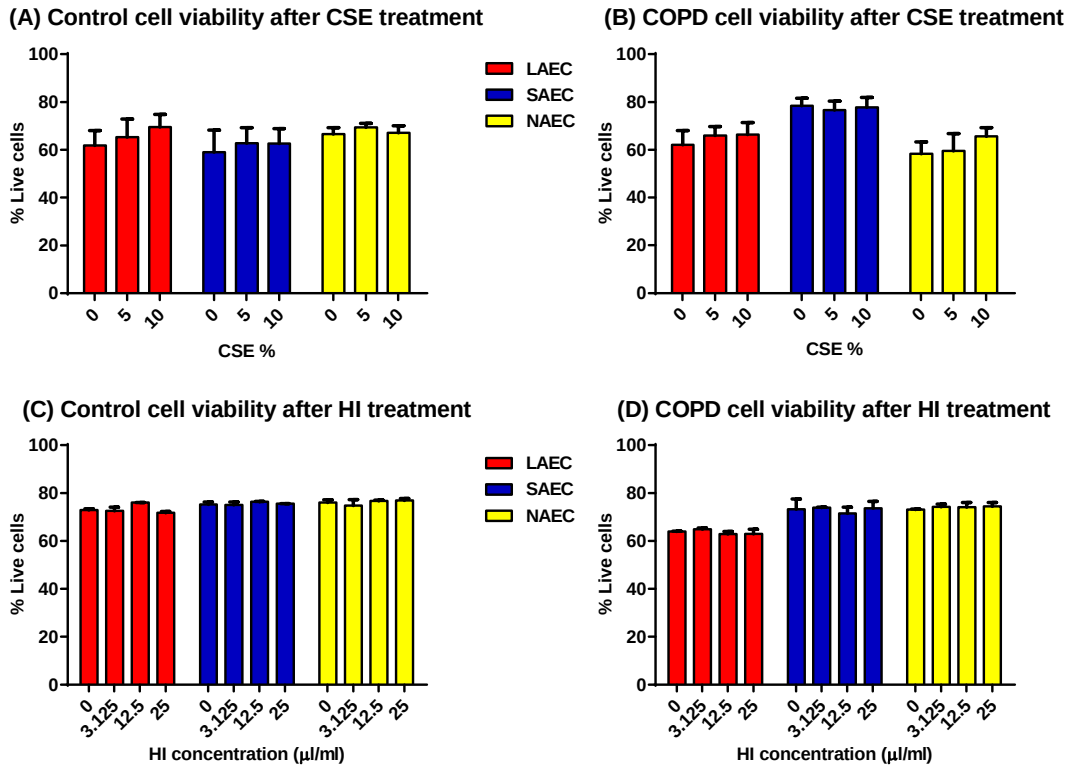


Figure 39 Primary airway epithelial cell viability following CSE or HI treatment
Cells were treated in triplicate with increasing concentrations of CSE (0-10%) or HI (0-25µl/ml) for 24 hours and viability analysed by propidium iodide uptake on flow cytometry. No change in cell viability was observed ($p>0.05$, unpaired T tests of raw data). (A) Data from n=3 SAEC, n=2 LAEC/NAEC. (B) Data from n=3 SAEC/NAEC, n=2 LAEC. (C) and (D) Data shown are the results from n=1 donor cells, using 2 different HI preparations (from the same strain). All data are displayed as mean values (± SEM).

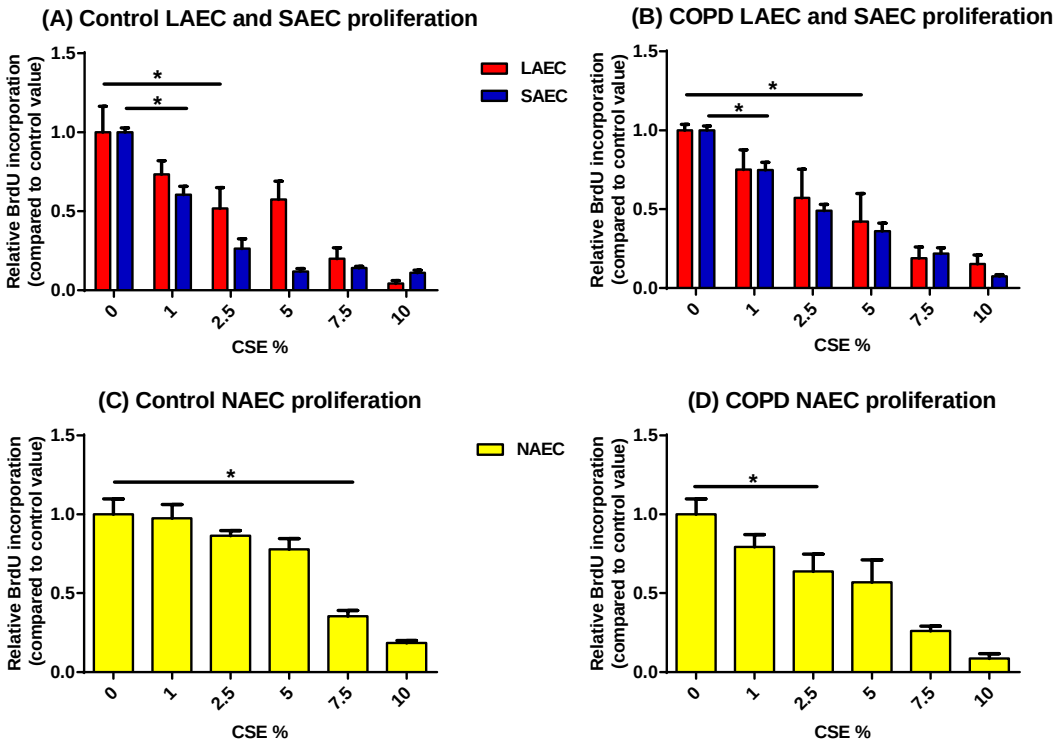


Figure 40 Primary airway epithelial cell proliferation after CSE treatment
Cells were treated in triplicate with increasing concentration of CSE (0-10%) and the effect on proliferation assessed by BrdU incorporation (cells cultured for 24 hours in media containing CSE, following by 24 hours of BrdU labelling time). Displayed data show the mean results (± SEM) from 2 sets of donor cells of each type tested, expressed as values relative to the control sample mean BrdU incorporation. In all cell types tested a reduction in proliferation was observed with increasing CSE concentration (*$p<0.05$, unpaired T tests of raw data).

6.4.2 Effect of Haemophilus influenzae whole cell lysate on IL-8 release from primary airway epithelial cells

Primary airway epithelial cell culture data are presented as values relative to the mean of "no treatment control" results for each cell type and donor; the data have been displayed and analysed in this format to normalise for baseline variability in mediator release between primary cells, and to allow comparison between paired donor samples (*i.e.* LAEC compared to SAEC). Experiments were performed using the same standardised method to minimise differences between cell counts in different treatment wells. Cells were seeded into plates at the same density and allowed to grow to approximately 90% confluence prior to application of treatments. The plates were all checked under the microscope to ensure that the visible cell growth was comparable across the culture plates used for each experiment. Cell counts were performed for control wells only, to allow calculation of baseline mediator release for each cell type. This data is shown in each results section (Table 32 and Table 33).

In healthy control cells no significant increase in IL-8 levels was observed in response to any of the HI doses tested (see Figure 41). A trend towards an increase in IL-8 levels was observed in SAEC with HI 25µl/ml (mean relative IL-8 1.6±0.5 compared to control, n=3, p=0.1). There was no change compared to baseline in either LAEC (mean relative IL-8 1.1±0.1 compared to control, n=3, p>0.05) or NAEC (1.1±0.1 compared to control, n=3, p>0.05). An increase in IL-8 levels was observed for all cell types in response to TNF-α treatment (LAEC mean relative IL-8 3.0±0.3, n=3, p<0.05; SAEC mean relative IL-8 4.2±1.1, n=3, p=0.1; NAEC mean relative IL-8 4.5±0.7, n=3, p<0.05; all compared to control values). The response to LPS was less marked (LAEC mean relative IL-8 1.5±0.4, SAEC mean relative IL-8 2.4±1.0, NAEC mean relative IL-8 1.9±0.4; all compared to control, n=3, p>0.05).

In COPD cells there was a significant increase in IL-8 levels in SAEC treated with HI 3.125µl/ml (mean relative IL-8 1.9±0.2 compared to control, n=3, p<0.05) (Figure 42A). No significant change in IL-8 levels was seen with HI doses up to 25µl/ml in LAEC (1.2±0.2 compared to control, n=3, p>0.05) or NAEC (1.1±0.1 compared to control, n=3, p>0.05) (Figure 42A and C). At HI 25µl/ml a difference was noted between LAEC

and SAEC in relative IL-8 levels but this did not meet statistical significance (mean LAEC relative IL-8 1.2±0.2 compared to mean SAEC relative IL-8 2.5±0.1, n=3, p=0.057). As for healthy control cells an increase in IL-8 levels was observed for all cell types in response to TNF-α (LAEC mean relative IL-8 3.8±0.8, n=3, p>0.05; SAEC mean relative IL-8 6.2±0.7, n=3, p<0.05; NAEC mean relative IL-8 3.5±1.1, n=3, p>0.05). There was no significant change in IL-8 levels in response to LPS at 10μg/ml (LAEC mean relative IL-8 2.8±0.7; SAEC mean relative IL-8 4.8±1.1; NAEC mean relative IL-8 1.7±0.2; all compared to control, n=3, p>0.05).

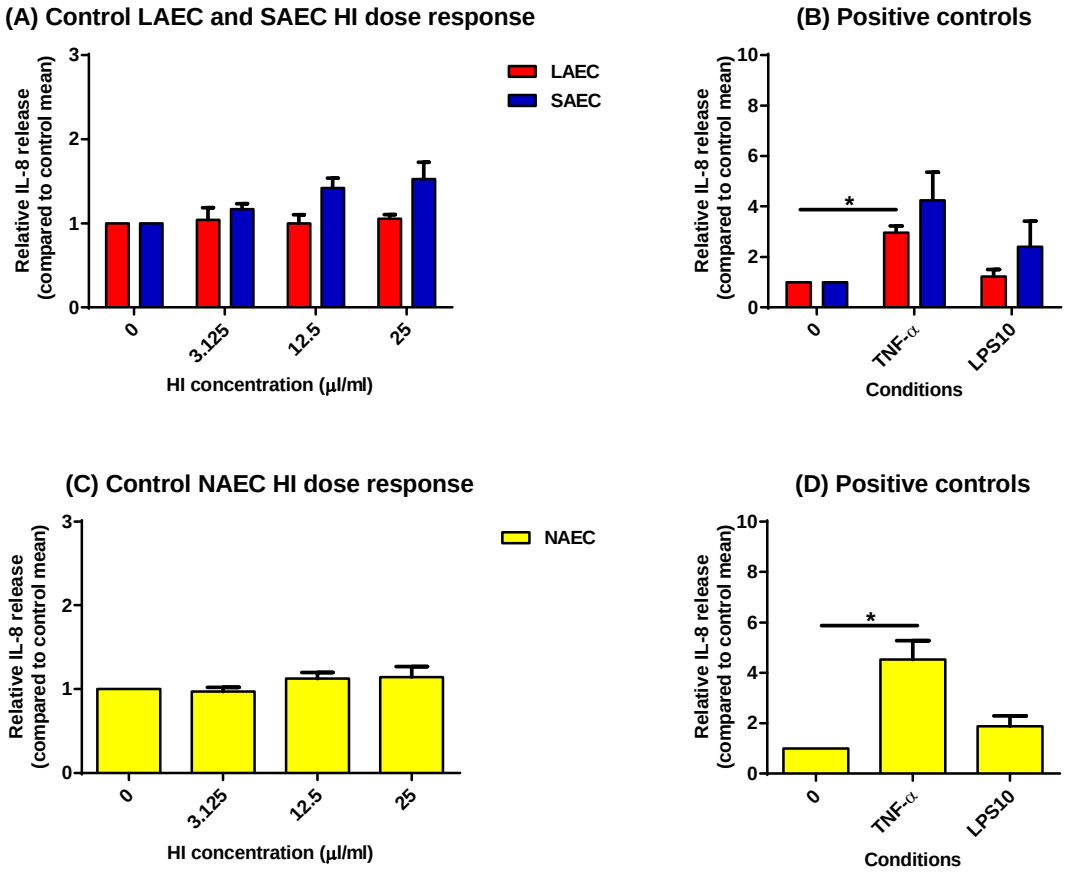


Figure 41 Healthy control cell responses to HI

Cells were treated in triplicate with increasing concentrations of HI (0-25µl/ml), TNF-α (20ng/ml) or LPS (10µg/ml) for 24 hours. Media was harvested and analysed for IL-8 levels by ELISA. Data shown are the mean results from n=3 complete control donor cell sets (*$p<0.05$, paired T tests). Data are shown as values relative to the mean control IL-8 release, to allow for baseline variability between primary cell baseline mediator release (see Table 31). A trend towards increased IL-8 release was observed in SAEC (A) but no change was noted in LAEC (A) or NAEC (C) in response to increasing HI concentration. All cells responded to TNF-α with an increase in IL-8 release but the response to LPS was minimal (B, D). Baseline mean IL-8 levels detected are shown in Table 31.

Cell type	Baseline IL-8 mean (±SEM)	Calculated mean IL-8 per 100,000 cells (±SEM)
LAEC	3791 pg/ml (915)	2268 pg (484)
SAEC	2672 pg/ml (724)	2146 pg (777)
NAEC	2194 pg/ml (432)	1311 pg (503)

Table 32 Baseline healthy control cell IL-8 levels

Cell counts were performed for individual "no treatment control" wells. The baseline IL-8 release was then used to calculate a "mean IL-8 level" per 100,000 cells for comparison between different cell types (n=3 control donor cell sets as above).

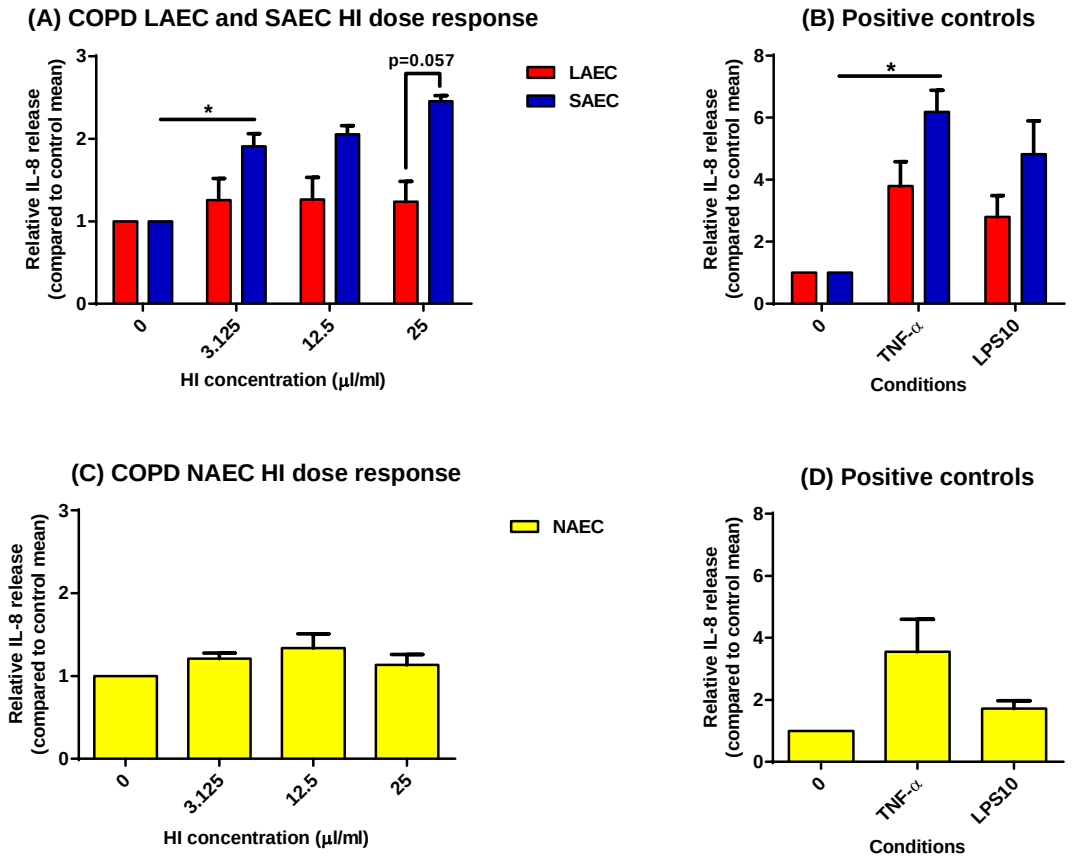


Figure 42 COPD cell responses to HI

Cells were treated in triplicate with increasing concentrations of HI (0-25µl/ml), TNF-α (20ng/ml) or LPS (10µg/ml) for 24 hours. Media was harvested and analysed for IL-8 levels by ELISA. Data shown are the mean results from n=3 complete COPD donor cell sets (*p<0.05, paired T tests). Data are shown as values relative to the mean control IL-8 release, to allow for baseline variability between primary cell baseline mediator release (see Table 32). Increased IL-8 release was observed in SAEC (A) but no change was noted in LAEC (A) or NAEC (C) in response to increasing HI concentration. All cells responded to TNF-α with an increase in IL-8 release but the response to LPS was less marked (B, D). Baseline mean IL-8 levels detected are shown in Table 32.

Cell type	Baseline IL-8 mean (±SEM)	Calculated mean IL-8 per 100,000 cells (±SEM)
LAEC	2910 pg/ml (91)	2502 pg (1343)
SAEC	2119 pg/ml (468)	1154 pg (227)
NAEC	3787 pg/ml (1023)	3543 pg (778)

Table 33 Baseline COPD cell IL-8 levels

Cell counts were performed for individual "no treatment control" wells. The baseline IL-8 release was then used to calculate a "mean IL-8 level" per 100,000 cells for comparison between different cell types (n=3 COPD donor cell sets as above).

6.4.3 Choice of cell culture conditions and media appropriate to cell type for further assessment of primary airway epithelial cell responses

As described LAEC were initially cultured in both SAGM and BEGM. Figure 43 shows the results of LAEC responses to HI, TNF-α and LPS for cells cultured in these different media types. There was a trend towards increased IL-8 release from LAEC cultured in SAGM. However, there was no statistically significant difference in IL-8 levels following HI treatment at any of the doses tested in either healthy control (LAEC BEGM mean IL-8 following HI 25µl/ml 1.1±0.1 compared to LAEC SAGM mean IL-8 2.0±0.4, n=3, p=0.1), or COPD cells (LAEC BEGM mean IL-8 following HI 25µl/ml 1.2±0.2 compared to LAEC SAGM mean IL-8 2.0±0.5, n=3, p=0.3). There was also no difference in TNF-α induced IL-8 release (healthy control LAEC BEGM mean IL-8 3.0±0.3 compared to LAEC SAGM mean IL-8 6.4±1.8, n=3, p=0.2). Figure 44 demonstrates that the pattern of cell responses observed in primary cell stimulation experiments was the same using either "complete" or "incomplete" cell culture media.

Overall, on the basis of the above experiments, and details provided in Chapter 5 regarding the LAEC cell growth characteristics in SAGM, further stimulation experiments were all performed using "complete media" with SAEC cultured in SAGM, and LAEC and NAEC cultured in BEGM. This was performed with the aim to achieve the optimal culture conditions for each cell type.

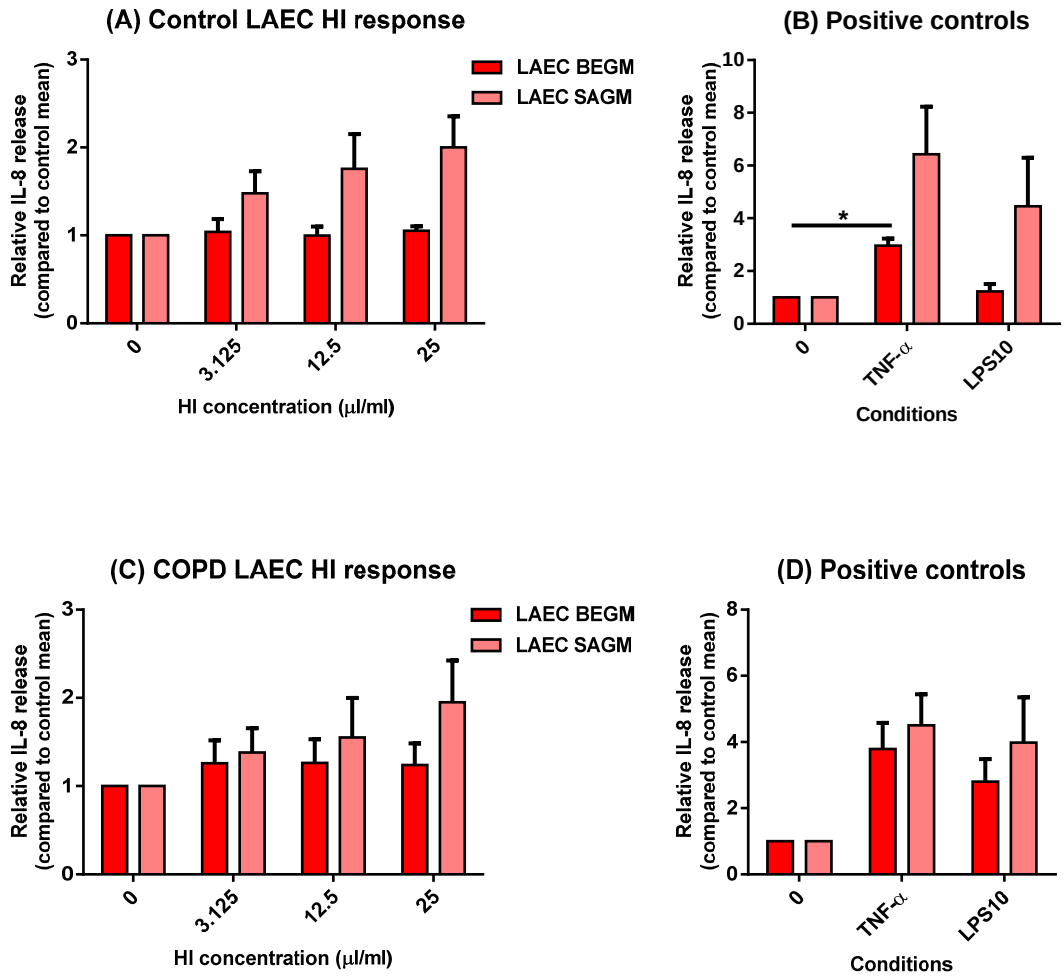


Figure 43 Review of LAEC cultured in different media types and IL-8 release in response to HI

LAEC were cultured from initial seeding in either BEGM (LAEC BEGM) or SAGM (LAEC SAGM) media supplemented as described in the methods section. Cells were treated in triplicate with HI (0-25µl/ml), TNF-α (20ng/ml) or LPS (10µg/ml) for 24 hours. Media was harvested and analysed for IL-8 levels by ELISA. Data shown are the mean results from n=3 complete donor cell sets (values relative to the mean control IL-8 release, to allow for baseline variability between primary cell baseline mediator release, see Table 34). No significant difference was observed in IL-8 release between cells cultured in different media types.

Group	Cell type	Baseline IL-8 mean (±SEM)	Calculated IL-8 release per 100,000 cells
Control	LAEC BEGM	3791 pg/ml (915)	2268 pg (484)
	LAEC SAGM	1926 pg/ml (531)	1455 pg (306)
COPD	LAEC BEGM	2910 pg/ml (91)	2502 pg (1343)
	LAEC SAGM	3037 pg/ml (914)	1672 pg (529)

Table 34 Baseline IL-8 levels in LAEC cultured in different media types

Cell counts were performed for individual "no treatment control" wells. The baseline mean IL-8 release was then used to calculate a "mean IL-8 level" per 100,000 cells for comparison between different cell types (n=3 donor cell sets as above).

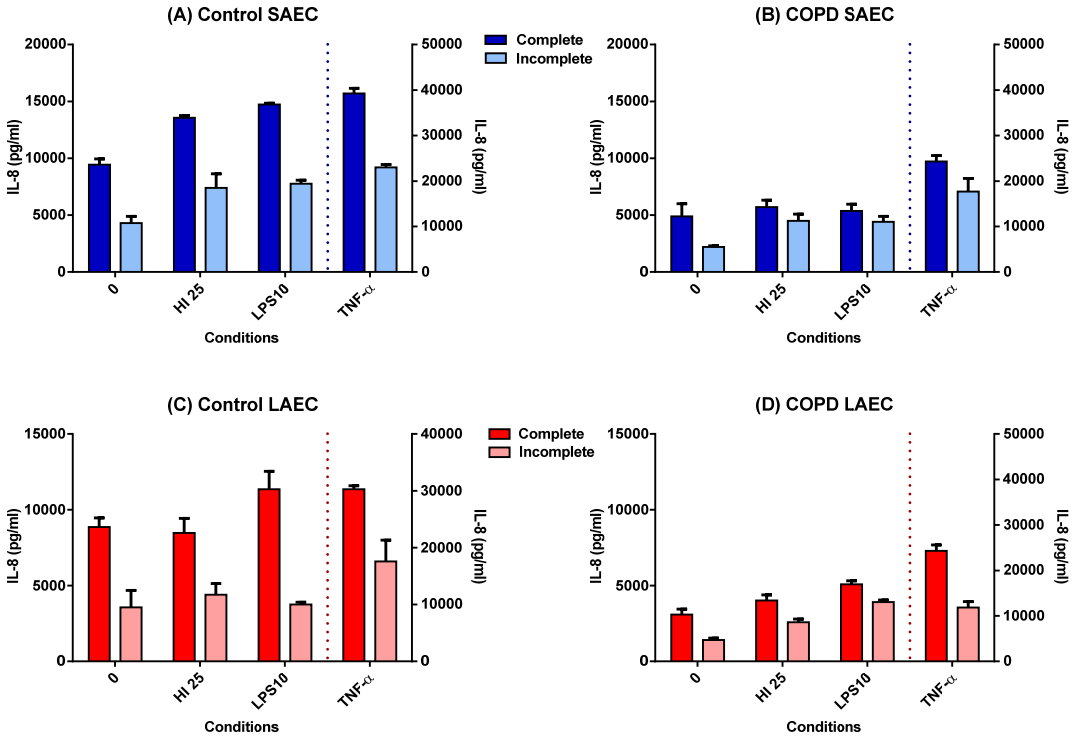


Figure 44 Effect of culture media supplementation on observed cellular responses
Cells were cultured in complete (containing all SingleQuot supplements as described in methods), or incomplete (containing only insulin, transferrin and gentamicin-amphotericin supplements) media for 24 hours prior to treatment with fresh media of the same type containing HI (25µl/ml), TNF-α (20ng/ml) or LPS (10µg/ml) for a further 24 hours. Media was then harvested for analysis of IL-8 levels by ELISA. Data shown are results from n=1 control and n=1 COPD donor, with cells treated in triplicate. Both baseline and treated IL-8 levels were higher in complete media, but the pattern of results was the same for both media types.

6.5 Effect of cigarette smoke extract and *Haemophilus influenzae* whole cell lysate on IL-8 release from primary airway epithelial cells from healthy control and COPD donors

Taking the results from the previous section forwards, specific cell treatments were selected for further investigation in a larger number of paired sets of healthy control and COPD primary airway epithelial cells. As previously all cells were treated at P2. The following data show results of cell stimulation work with CSE 0-5% alone and in combination with HI 25µl/ml. TNF-α (20ng/ml) was used as a positive control. The effect of these treatments was analysed by IL-8 release by ELISA. As described above in section 6.4.2, the following data are again presented as values relative to the mean of "no treatment control" results for each cell type and donor. Cell counts were performed for control wells only, to allow calculation of baseline mediator release for each cell type. This data is shown separately in results section 6.8.

The cells used for this work (and later IL-6 and IL-1β analysis) were from healthy control donors 5-10, and COPD donors 10 and 13-17. These study subjects are highlighted in the tables of demographic and clinical information in Chapter 5 (Table 31 and Table 31). A summary of the relevant details for discussion is provided in Tables 35 and 36. The possibility of a carryover effect of clinical ICS treatment on the results obtained is difficult to assess due to the small numbers included (4 on ICS, 2 not on ICS), and other possible confounding variables including smoking status; both subjects not on ICS were smokers, and there were no smokers in the ICS group.

Subject No	Age	Gender	Smoking status	FEV1% pred
5	44	M	Never	117
6	57	M	Never	126
7	42	M	Never	115
8	55	F	Never	86
9	54	M	Ex	90
10	70	M	Ex	108

Table 35 Data for healthy control donors for *in-vitro* cell culture experiments

Subject No	Age	Gender	Smoking status	FEV1% pred	GOLD	ICS use
10	57	M	Ex	46	Severe	Y
13	66	F	Ex	38	Severe	Y
14	48	M	Current	72	Moderate	N
15	62	M	Ex	81	Mild	Y
16	47	M	Current	96	Mild	N
17	65	F	Ex	73	Moderate	Y

Table 36 Data for COPD donors for *in-vitro* cell culture experiments

Levels of IL-8 were greater in SAEC than LAEC or NAEC, from both healthy control and COPD donors, in response to cigarette smoke extract and Haemophilus influenzae whole cell lysate stimulation

Figure 45 and Figure 46 show IL-8 levels detected by ELISA for healthy control and COPD donor cell sets treated with CSE and HI respectively. An increase in IL-8 levels following treatment with TNF-α was seen in all cell types tested as a positive control.

In healthy control cells (see Figure 45) the response to CSE 1-5% treatment alone was minimal with a significant increase only noted on treatment of SAEC with 5% CSE (IL-8 mean 1.4±0.05 relative to control, n=6, $p<0.05$). CSE alone had no significant effect on IL-8 levels detected from LAEC (IL-8 mean 1.3±0.1 relative to control, n=6, $p>0.05$), or NAEC (IL-8 mean 1.0±0.04 relative to control, n=4, $p>0.05$). HI treatment (25µl/ml) alone increased IL-8 release compared to baseline in both LAEC and SAEC, but no effect was observed in NAEC (LAEC mean IL-8 1.3±0.1, n=6, $p<0.05$; SAEC mean IL-8 2.0±0.5, n=6, $p=0.08$; NAEC mean IL-8 0.9±0.03, n=4, $p>0.05$). Combined CSE and HI treatment had the greatest effect observed with increased IL-8 release on stimulation with HI 25µl/ml and 5% CSE: LAEC mean IL-8 1.7±0.3, n=6, $p=0.07$; SAEC mean IL-8 2.7±0.5, n=6, $p<0.05$; NAEC mean IL-8 1.3±0.1, n=4, $p=0.08$).

On comparing LAEC and SAEC cell IL-8 levels, a significant difference was noted between healthy control cell samples (paired from each donor) as shown in Figure 45E. On treatment with HI (25µl/ml) and 5% CSE IL-8 levels were significantly higher in SAEC compared to LAEC cultures (n=6 pairs, $p<0.05$).

In COPD cells (see Figure 46) a similar pattern of results was observed as for the healthy control cells. Minimal effect of CSE was again observed in SAEC only (following 5% CSE treatment mean IL-8 1.4±0.3 relative to control, n=6, $p>0.05$). An increase response to HI alone was also only observed in SAEC (mean IL-8 1.7±0.3 relative to control, n=6, $p=0.07$) and not LAEC or NAEC (LAEC mean IL-8 1.1±0.1, n=6, $p>0.05$; NAEC mean IL-8 1.0±0.1, n=4, $p>0.05$). Combined CSE (5%) and HI treatment had no significant effect in LAEC (mean IL-8 1.3±0.2 relative to control, n=6, $p>0.05$). A small increase was noted in NAEC (mean IL-8 1.2±0.03, n=4, $p<0.05$). A more marked effect

was observed in SAEC again (mean IL-8 2.1±0.5, n=6, p=0.06). Overall IL-8 release in response to treatments was greater from SAEC compared to LAEC (Figure 46E) but this did not meet statistical significance (5% CSE+HI treatment, n=6 pairs, p=0.1).

In addition to analysis of the results using paired T tests of mean values from n=6 pairs of LAEC and SAEC cells from the same individual donors, Bland and Altman plots have been used to further assess the trends observed (see Figure 47). Bland and Altman describe a method to demonstrate "agreement" between repeated measurements (Bland and Altman, 1986). The average of two measurements is plotted against the difference between the measurements in a graphical display. This can be used to illustrate differences between paired samples *e.g.* as described by Ward *et al* where plots of results from two bronchoscopy procedures are displayed showing the difference in values between the procedures against the mean results (Ward et al., 1995). In further assessing the data from the current study, this method can also be used to assess results from paired large and small airway samples. Rather than displaying individual data points around a mean value, without reference to pairing of results, this graphical display instead allows consideration of the relationship between results of samples from each donor, and gives a better illustration of the trend in the sample results than a p value alone *i.e.* if there is no difference ("agreement"), a positive or negative difference in values between paired samples.

The data presented show an individual data point for each control or COPD donor, plotted as the average of relative LAEC and SAEC IL-8 levels, against the difference between the LAEC and SAEC from that donor. This demonstrates that in control cells a negative difference was always observed, *i.e.* IL-8 levels from LAEC were lower than from SAEC. In COPD cells there was either no difference, or a negative difference between LAEC and SAEC for each donor, *i.e.* IL-8 levels from COPD LAEC were either the same as, or lower than those observed from COPD SAEC. A negative difference was observed in both COPD donors with severe disease.

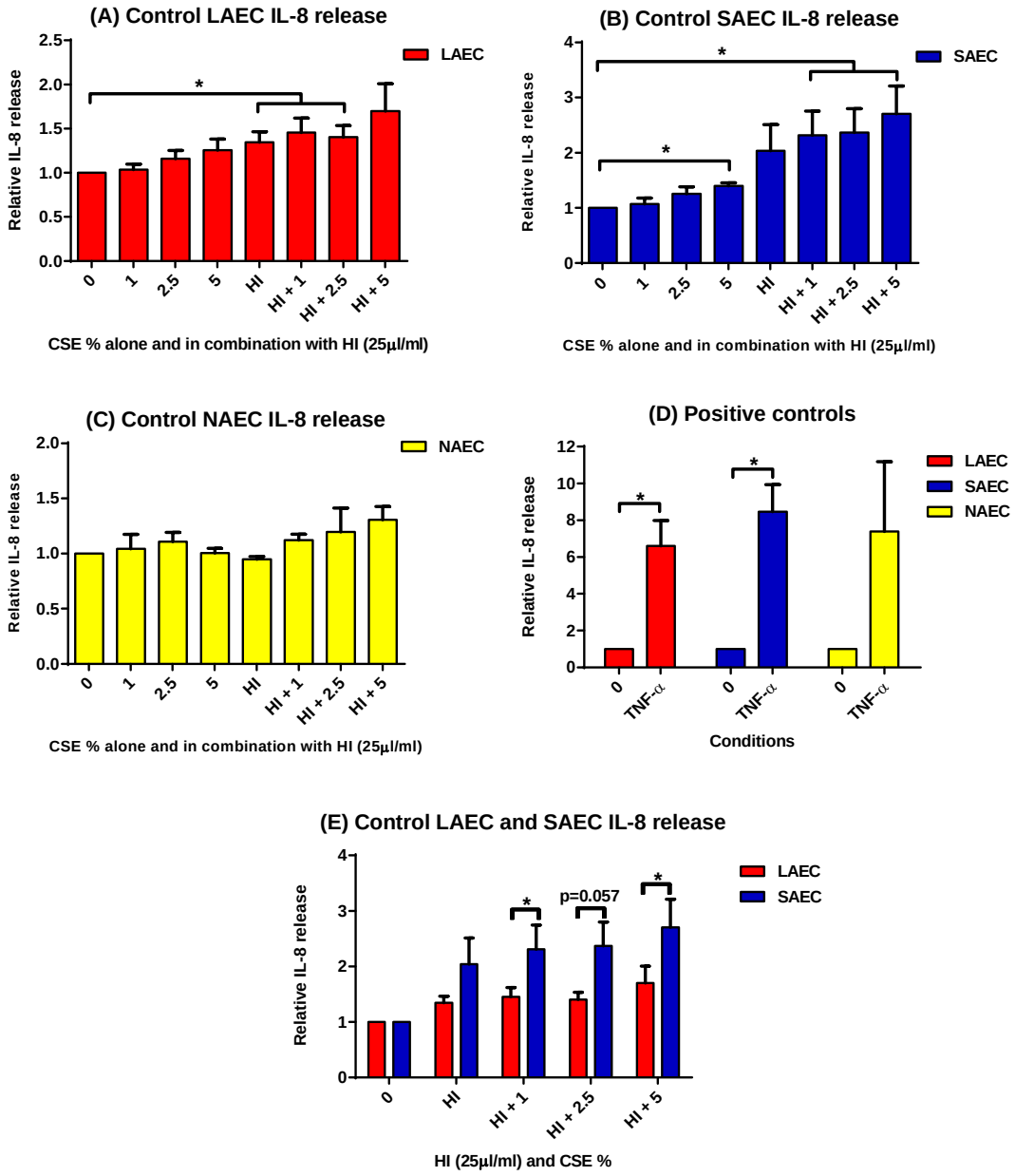


Figure 45 Healthy control cell IL-8 release in response to CSE and HI
Cells were treated in triplicate with CSE (0-5%) ± HI (25µl/ml), or TNF-α (20ng/ml) for 24 hours. Media was harvested and analysed for IL-8 levels by ELISA. Data shown are the mean results from n=6 (LAEC, SAEC) or n=4 (NAEC) complete control donor cell sets (*p<0.05, paired T tests). Data are shown as values relative to the mean "no treatment" control IL-8 release (± SEM). An increase in IL-8 release was observed in both LAEC (A) and SAEC (B) in response to combined CSE and HI, with a greater effect observed in SAEC (E). No significant response was observed in NAEC (C) but all cell types responded to TNF-α (D).

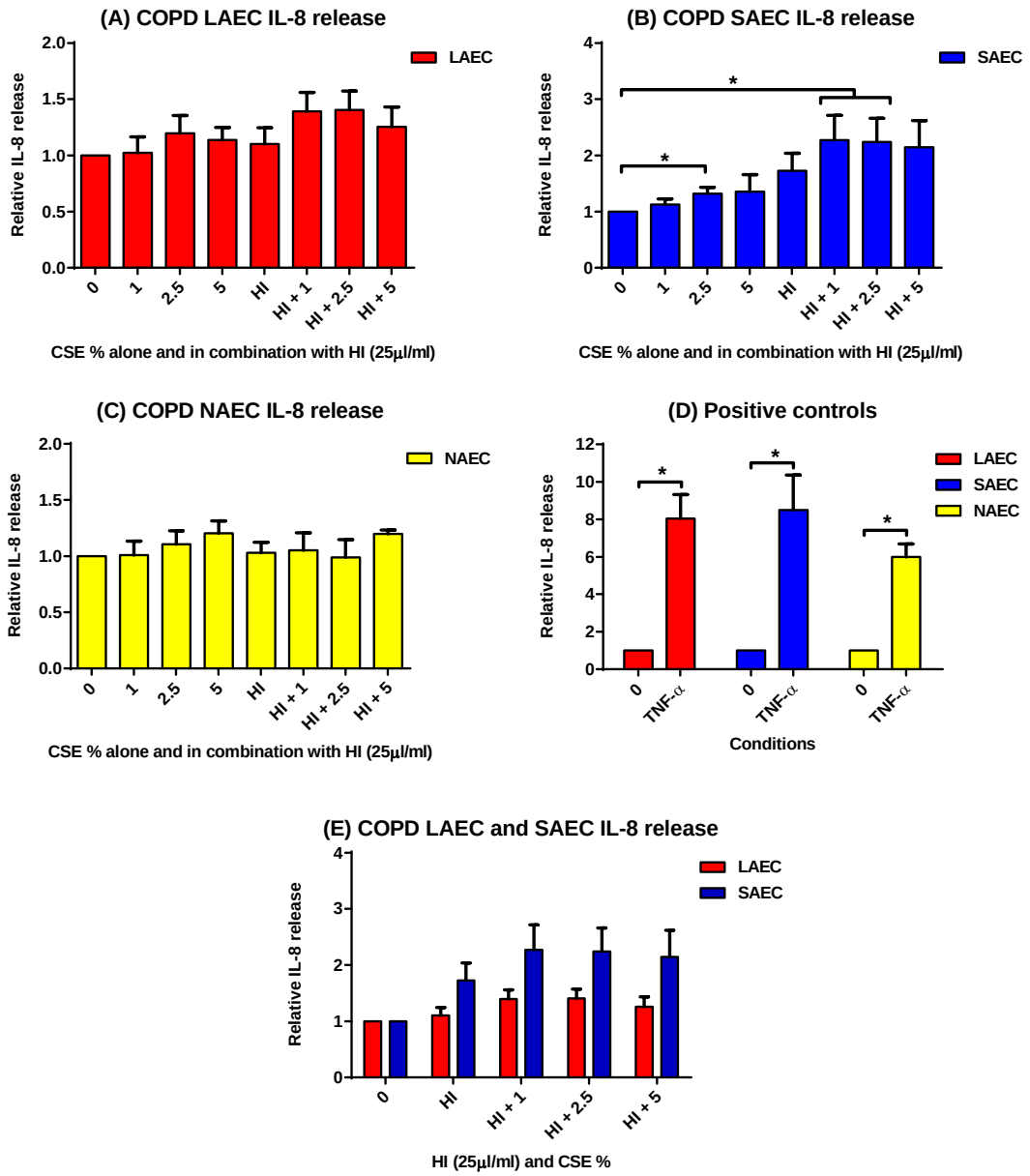


Figure 46 COPD cell IL-8 release in response to CSE and HI

Cells were treated in triplicate with CSE (0-5%) ± HI (25µl/ml), or TNF-α (20ng/ml) for 24 hours. Media was harvested and analysed for IL-8 levels by ELISA. Data shown are the mean results from n=6 (LAEC, SAEC) or n=4 (NAEC) complete control donor cell sets (*$p<0.05$, paired T tests). Data are shown as values relative to the mean "no treatment" control IL-8 release (± SEM). An increase in IL-8 release was observed in SAEC (B) in response to combined CSE and HI, but not LAEC (A) or NAEC (C). All cell types responded to TNF-α (D). IL-8 release was greater in SAEC compared to LAEC (E) for all treatments tested but this did not meet statistical significance.

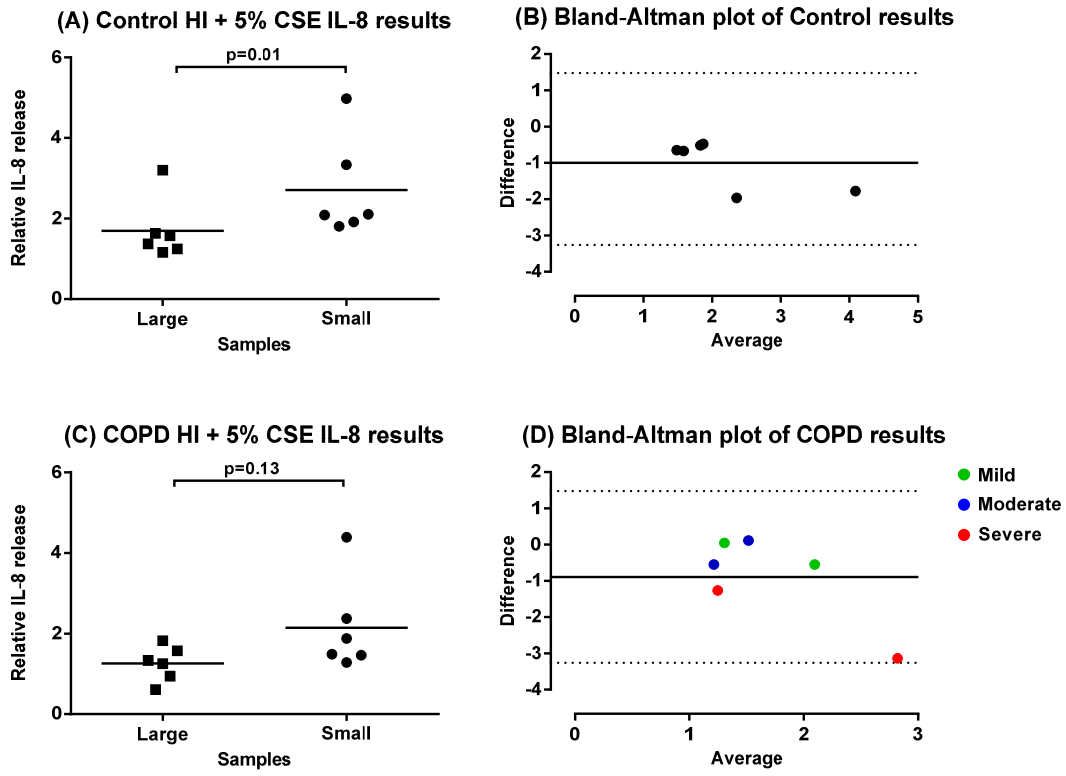


Figure 47 Bland and Altman plot review of the effect of HI + 5% CSE on LAEC and SAEC IL-8

Data are presented in graphs (A) and (C) as the individual mean relative IL-8 release compared to control values for each healthy control or COPD donor (with a line at the overall mean for the whole data set).

This data has been used to generate the Bland and Altman plots shown in (B) and (D). The solid line represents the mean of the differences between LAEC and SAEC samples. The dotted lines represent two standard deviations of the differences between samples (95% limits of agreement). Each donor is represented by a single data point which plots the average IL-8 value from LAEC and SAEC results against the difference between these values. In control cells a negative difference was observed *i.e.* higher levels of IL-8 were seen in SAEC samples. In COPD cells, either no difference or a negative difference was also observed.

6.6 Effect of cigarette smoke extract and *Haemophilus influenzae* whole cell lysate on other pro-inflammatory cytokine release from primary large and small airway epithelial cells

Following review of the IL-8 results above, to determine if differences between LAEC and SAEC responses to CSE and HI would be observed for other pro-inflammatory mediators, further analysis was performed on these samples using an MSD Tissue Culture 4-plex assay to detect IL-6, IL-1β, TNF-α and IFN-γ. This platform was chosen as a method able to sensitively detect low levels of these mediators, with particular interest in focussed on IL-6, IL-1β and TNF-α. Plates with IFN-γ were chosen not specifically to investigate this mediator, but instead of plates with further IL-8 analysis potential: high levels of IL-8 in the samples could affect analysis of other mediators in a 4-plex plate if neat samples were used. IL-6, IL-1β and TNF-α were chosen as pro-inflammatory mediators of interest in COPD with previous research showing evidence of increased levels *in-vivo* and detection of these mediators in *in-vitro* airway epithelial cell culture (as discussed in Chapter 1, and reviewed in (Barnes, 2009) and (Thorley and Tetley, 2007). In the current study results from BAL also showed differences in the levels of these cytokines between control and COPD samples.

The use of the 4-plex plate chosen allowed use of neat samples with more accurate analysis. Table 37 shows the lower limits of detection for each of the mediators. TNF-α and IFN-γ levels were below the levels of detection for all samples tested other than healthy control donor 3 SAEC (data not shown). Levels of IL-8, IL-6 and IL-1β were also noted to be higher in samples from this donor.

Cytokine	Lower limit of detection (mean ± SEM)
IL-6	0.6 ± 0.1 pg/ml
IL-1β	1.7 ± 0.9 pg/ml
TNF-α	7.2 ± 1.1 pg/ml
IFN-γ	11.8 ± 2.8 pg/ml

Table 37 Values for lower limit of detection of MSD 4-plex Tissue Culture plates
Values shown are the mean values reported over the 8 plates used for this analysis.

As for the IL-8 levels reported above, the following data are presented as values relative to the mean of the "no treatment control" results. Baseline mediator levels adjusted for cell count are discussed in section 6.8.

6.6.1 Effect of cigarette smoke extract and Haemophilus influenzae whole cell lysate on LAEC and SAEC IL-6

Figure 48 and Figure 50 show the results for healthy control and COPD donor cell sets treated with CSE and HI. IL-6 levels were above detection for all samples tested except in control 4 SAEC where the no treatment control, 1% CSE and 2.5% CSE sample levels were below the LLD. However, IL-6 was detected in samples from SAEC from this donor treated with 5% CSE, HI alone, and HI in combination with CSE. This data was not combined with the remaining healthy control donor results for IL-6 for the analysis displayed; use of values relative to control meant that there was a significant increase in SAEC treatments compared to control which skewed the data. The raw data results for this control donor are shown separately in Figure 49. Of note on including the LAEC relative data and using a value of 1 as no change to control for all SAEC results for this donor, this did not significantly change the results observed on comparing paired LAEC and SAEC healthy control cell results shown in Figure 48C.

Combined cigarette smoke extract and Haemophilus influenzae whole cell lysate increased IL-6 release from both LAEC and SAEC, with the greatest effect observed in healthy control and COPD SAEC

In healthy control cells (see Figure 48) increased IL-6 was seen following treatment with 5% CSE in SAEC (mean IL-6 1.5±0.2 relative to control, n=5, $p<0.05$) which was less marked in LAEC (mean IL-6 1.2±0.1, n=6, $p>0.05$). The same pattern was observed for HI treatment alone: SAEC mean IL-6 1.4±0.2, n=5, $p>0.05$; LAEC mean IL-6 1.2±0.1, n=6, $p>0.05$. An increase in IL-6 was seen in samples from both cell types on combined treatment with 5% CSE and HI (LAEC mean IL-6 1.8±0.3, n=6, $p=0.06$; SAEC mean IL-6 2.7±0.8, n=5, $p=0.07$). A greater increase in IL-6 release following treatment with combined CSE and HI was observed in SAEC compared to LAEC, with a significant difference observed in relative IL-6 release on treatment with 1-2.5% CSE combined with HI (n=5 pairs, $p<0.05$).

In COPD cells (see Figure 50) overall a similar pattern of results was observed. Increased IL-6 was observed in SAEC following treatment with 5% CSE (1.4±0.3, n=6, p>0.05) or HI alone (1.6±0.4, n=6, p>0.05), but no change was noted in LAEC compared to baseline. Combined 5% CSE and HI increased IL-6 in both LAEC (mean IL-6 1.5±0.4, n=6, p>0.05) and SAEC (mean IL-6 2.5±0.6, n=6, p=0.06). Overall the increase in IL-6 levels following treatment was greater in SAEC compared to LAEC but this did not meet statistical significance (n=6, p=0.3).

As for the IL-8 results, further review of this IL-6 data is shown in Bland and Altman plots (Figure 51). Although the results for combined 5% CSE and HI treatment did not meet statistical significance for a difference between paired LAEC and SAEC sample, these plots demonstrate that in the 5 pairs of control cells analysed, a negative difference was always observed, *i.e.* IL-6 levels from LAEC are lower than from SAEC. In COPD cells, a negative difference was observed in 4 out of the 6 paired samples, and no difference in another sample set *i.e.* IL-6 levels from COPD LAEC were mostly either the same as, or lower than those observed from COPD SAEC. A positive difference was observed in one COPD sample set demonstrating higher IL-6 levels from LAEC than from SAEC in this donor.

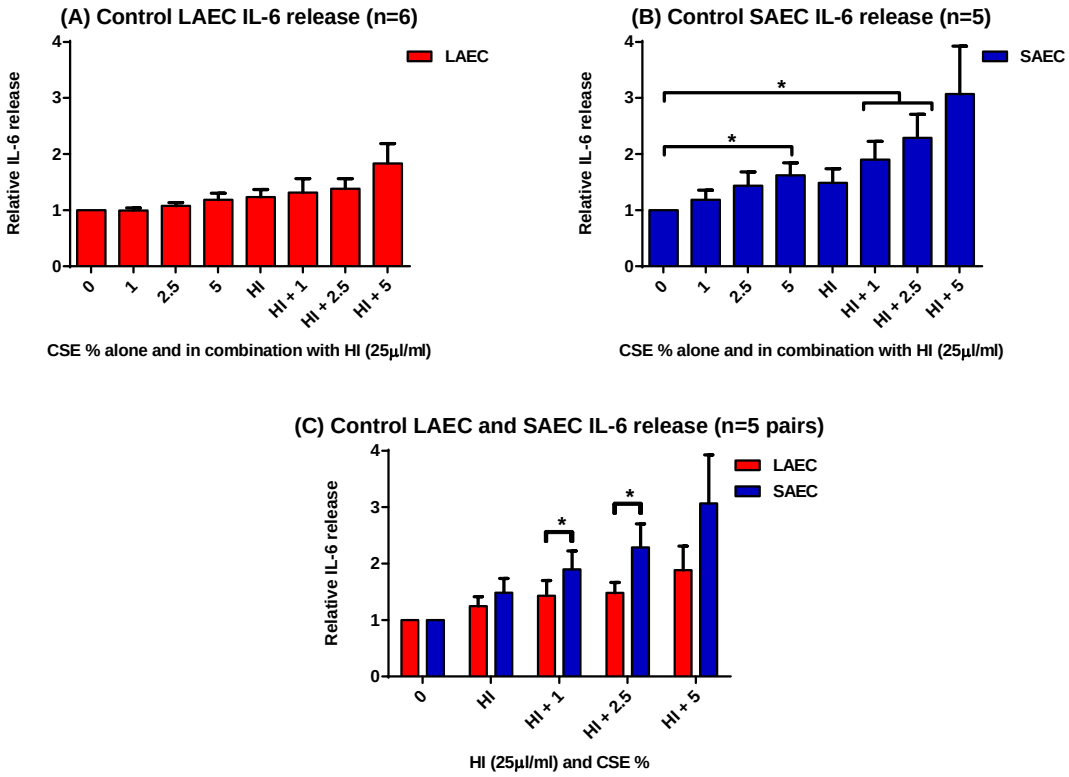


Figure 48 Healthy control cell IL-6 release in response to CSE and HI
Cells were treated in triplicate with CSE (0-5%) ± HI (25µl/ml) for 24 hours. Media was harvested and analysed for IL-6 levels using the MSD assay. Data shown are the mean results from (A) n=6 LAEC, (B) n=5 SAEC, and (C) n=5 paired donor cell sets (*p<0.05, paired T tests). Data are shown as values relative to the mean control IL-6 release (± SEM). A significant increase in IL-6 was observed in SAEC (B) in response to 5% CSE, and combined HI and 1-2.5% CSE treatments. No significant increase was observed in LAEC (A), and IL-6 release was greater in SAEC compared to LAEC (C).

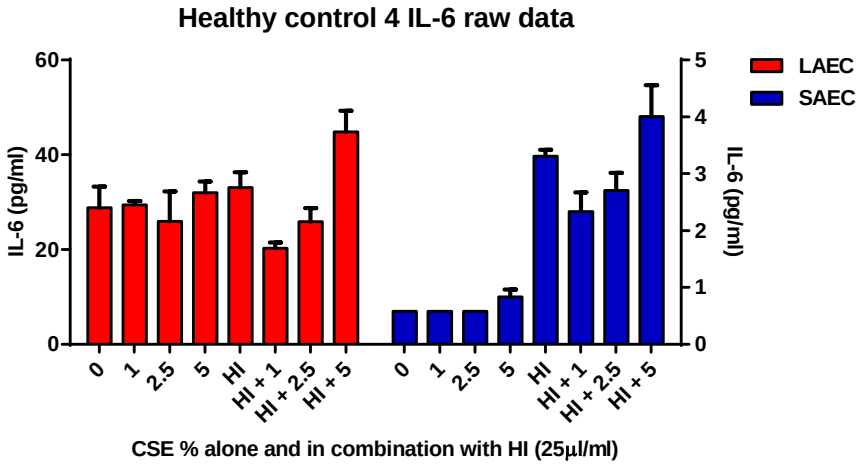


Figure 49 Healthy control donor 4 LAEC and SAEC IL-6 raw data
Cells were treated in triplicate with CSE (0-5%) ± HI (25µl/ml) for 24 hours. Media was harvested and analysed for IL-6 using the MSD assay. Data shown are the mean results (± SEM) from n=1 control donor cells as raw data. IL-6 was undetected in 0, 1% and 2.5% CSE treatments in SAEC; the baseline values shown for these treatments are the LLD of IL-6 for the MSD plate on which this donor set of samples was analysed (0.58 pg/ml).

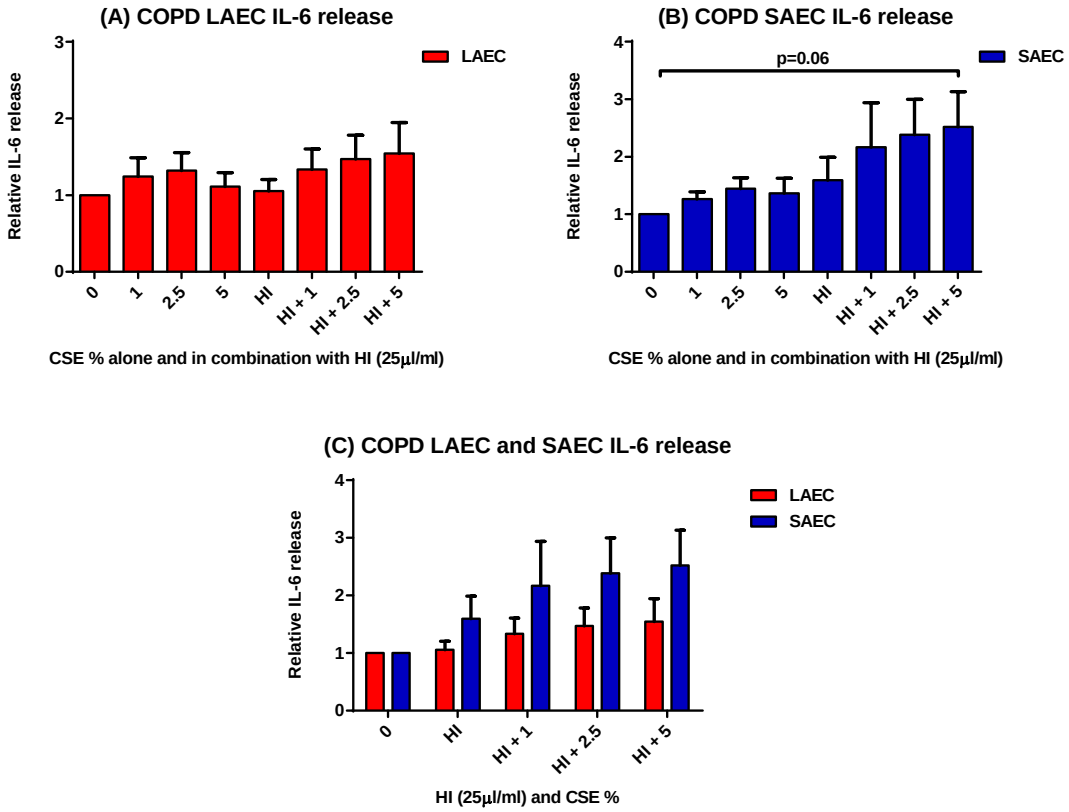


Figure 50 COPD cell IL-6 release in response to CSE and HI

Cells were treated in triplicate with CSE (0-5%) ± HI (25µl/ml) for 24 hours. Media was harvested and analysed for IL-6 levels using the MSD assay. Data shown are the mean results from (A) n=6 LAEC, (B) n=6 SAEC, and (C) n=6 paired donor cell sets (*p<0.05, paired T tests). Data are shown as values relative to the mean control IL-6 release (± SEM). An increase in IL-6 was observed in SAEC (B) in response to combined CSE and HI treatments. No effect was observed in LAEC (A). Following combined HI and CSE treatment IL-6 levels were greater in SAEC compared to LAEC (C).

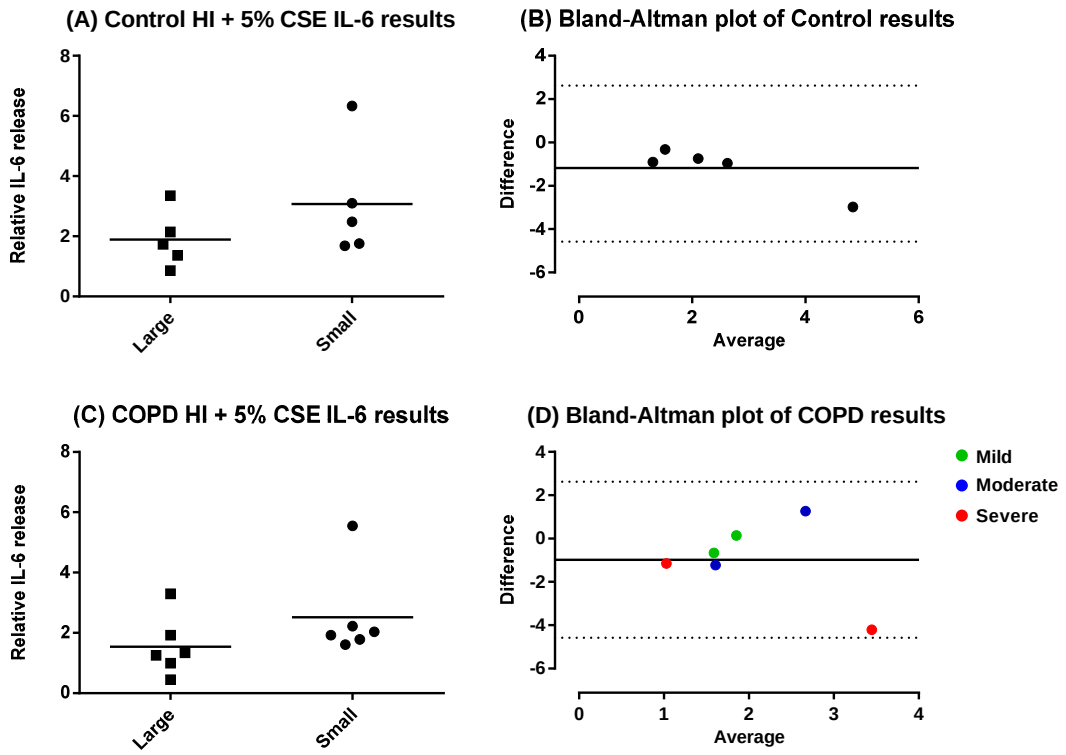


Figure 51 Bland and Altman plot review of the effect of HI and 5% CSE on LAEC and SAEC IL-6

Data are presented in graphs (A) and (C) as the individual mean relative IL-6 release compared to control values for each healthy control or COPD donor (with a line at the overall mean for the whole data set).

This data has been used to generate the Bland and Altman plots shown in (B) and (D). The solid line represents the mean of the differences between LAEC and SAEC samples. The dotted lines represent two standard deviations of the differences between samples (95% limits of agreement). Each donor is represented by a single data point which plots the average IL-6 value from LAEC and SAEC results against the difference between these values. In control cells a negative difference was observed *i.e.* higher levels of IL-6 were seen in SAEC samples. In COPD cells, this effect was also observed for 4 samples. One COPD donor set showed no difference between LAEC and SAEC, and a further set showed higher IL-6 levels from LAEC than SAEC.

6.6.2 Effect of cigarette smoke extract and Haemophilus influenzae whole cell lysate on LAEC and SAEC IL-1β

IL-1β levels were above the lower limit of detection in 5 control LAEC, 3 control SAEC, 4 COPD LAEC and 4 COPD SAEC (details in Table 38). In the figures which follow data is presented for all samples with detectable levels for each cell type. Paired LAEC and SAEC results are also presented for those samples where IL-1β was detected in both LAEC and SAEC. Where no IL-1β was detected it was possible to substitute a value of 1 as no change to control value, to allow further comparison of results between LAEC and SAEC; this did not significantly alter the results observed in Figure 52C and Figure 53C (data not shown). However, it is possible that there were changes in undetectable levels of IL-1β with the treatment used - this approach to analysis would not take this into account.

Donor	LAEC	SAEC		Donor	LAEC	SAEC
Control 1	+	-		COPD1	+	+
Control 2	-	-		COPD2	+	+
Control 3	+	+		COPD3	+	+
Control 4	+	-		COPD4	-	-
Control 5	+	+		COPD5	+	-
Control 6	+	+		COPD6	-	+

Table 38 IL-1β levels detectable using MSD 4-plex assay in cell culture media samples

An increase in IL-1β was observed following cigarette smoke extract treatment in SAEC but not LAEC

In healthy control cells (Figure 52) no treatment had any significant effect on IL-1β in LAEC samples. In SAEC an increase in IL-1β was observed following 5% CSE treatment (mean IL-1β 1.6±0.2, n=3, p=0.1). A similar change was noted in samples following treatment with combined 5% CSE and HI (mean IL-1β 1.6±0.2, n=3, p=0.09). No effect of HI alone was observed in SAEC; it is possible that the effect observed on combined treatment was only due to the CSE present. In comparing LAEC and SAEC, a significant difference in IL-1β levels was observed on comparing levels following 5% CSE (n=3 pairs, $p<0.05$). No other significant differences were noted.

In COPD samples (Figure 53) the same trends were observed in IL-1β levels with no change following treatment in LAEC, and an increase in IL-1β in SAEC following 5% CSE treatment either alone (mean IL-1β 1.6±0.5, n=4, $p>0.05$) or in combination with HI (mean IL-1β 1.9±0.7, n=4, $p>0.05$). However, no significant difference between paired LAEC and SAEC samples was observed.

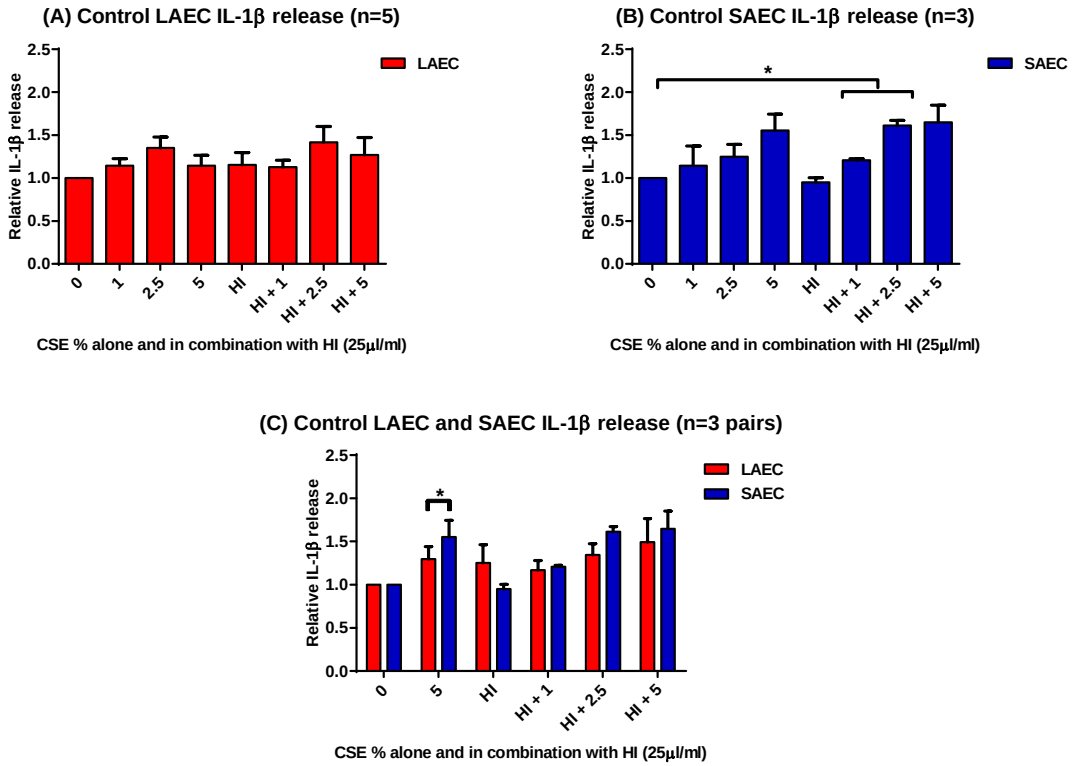


Figure 52 Healthy control cell IL-1β release in response to CSE and HI

Cells were treated in triplicate with CSE (0-5%) ± HI (25µl/ml) for 24 hours. Media was harvested and analysed for IL-1β levels using the MSD assay. Data shown are the mean results from (A) n=5 LAEC, (B) n=4 SAEC, and (C) n=3 paired donor cell sets (*$p<0.05$, paired T tests). Data are shown as values relative to the mean control IL-1β release (± SEM). An increase in IL-1β was observed in SAEC (B) in response to 5% CSE, and combined HI and CSE treatments. No significant increase was observed in LAEC (A). IL-1β levels were significantly greater in SAEC samples following 5% CSE treatment, compared to paired LAEC samples (C).

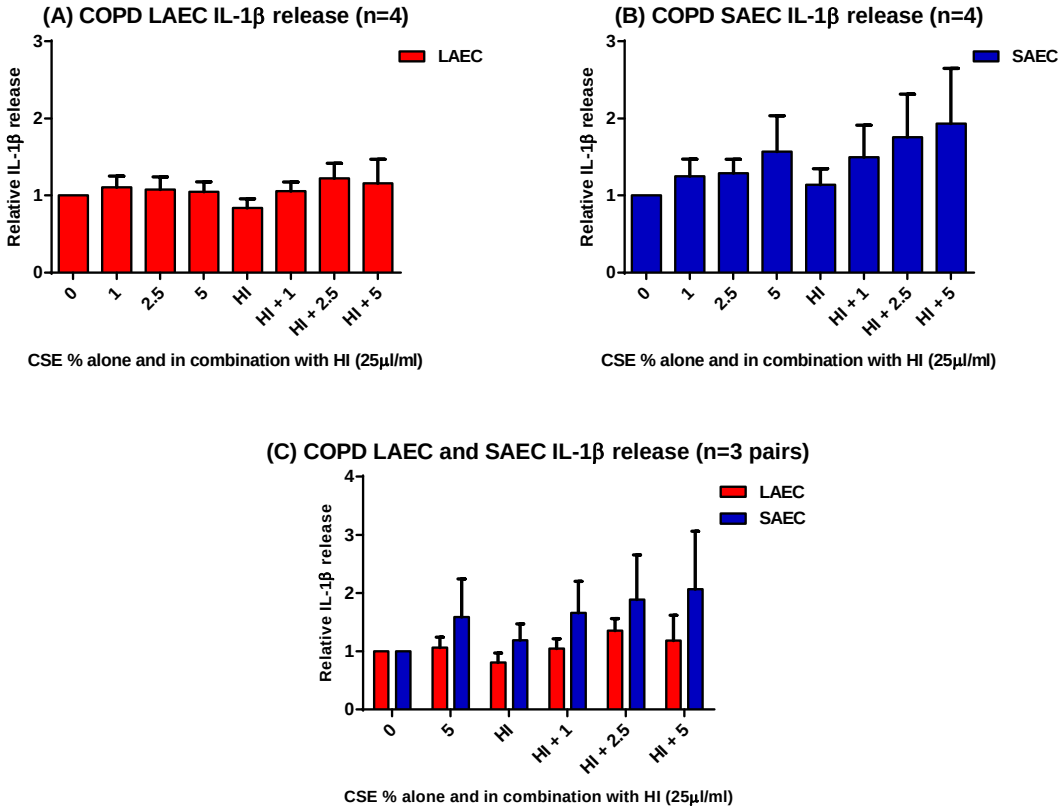


Figure 53 COPD cell IL-1β release in response to CSE and HI

Cells were treated in triplicate with CSE (0-5%) ± HI (25µl/ml) for 24 hours. Media was harvested and analysed for IL-1β levels using the MSD assay. Data shown are the mean results from (A) n=4 LAEC, (B) n=4 SAEC, and (C) n=3 paired donor cell sets (*$p<0.05$, paired T tests). Data are shown as values relative to the mean control IL-1β release (± SEM). An increase in IL-1β was observed in SAEC (B) in response to 5% CSE, and combined HI and CSE treatments. No increase was observed in LAEC (A). IL-1β levels were overall greater in SAEC samples compared to paired LAEC samples (C).

6.7 Review of the effect of cigarette smoke extract and *Haemophilus influenzae* whole cell lysate treatments on cytokine release from LAEC and SAEC

On review of the mediator release in response to the cell treatments tested, the most significant results were for combined CSE and HI treatments with increased IL-8 and IL-6 release. Further analysis of these results shows that the observed mediator release was at least additive of the effects of either 5% CSE or HI treatment alone, with the actual release greater than this in some instances (Figure 54).

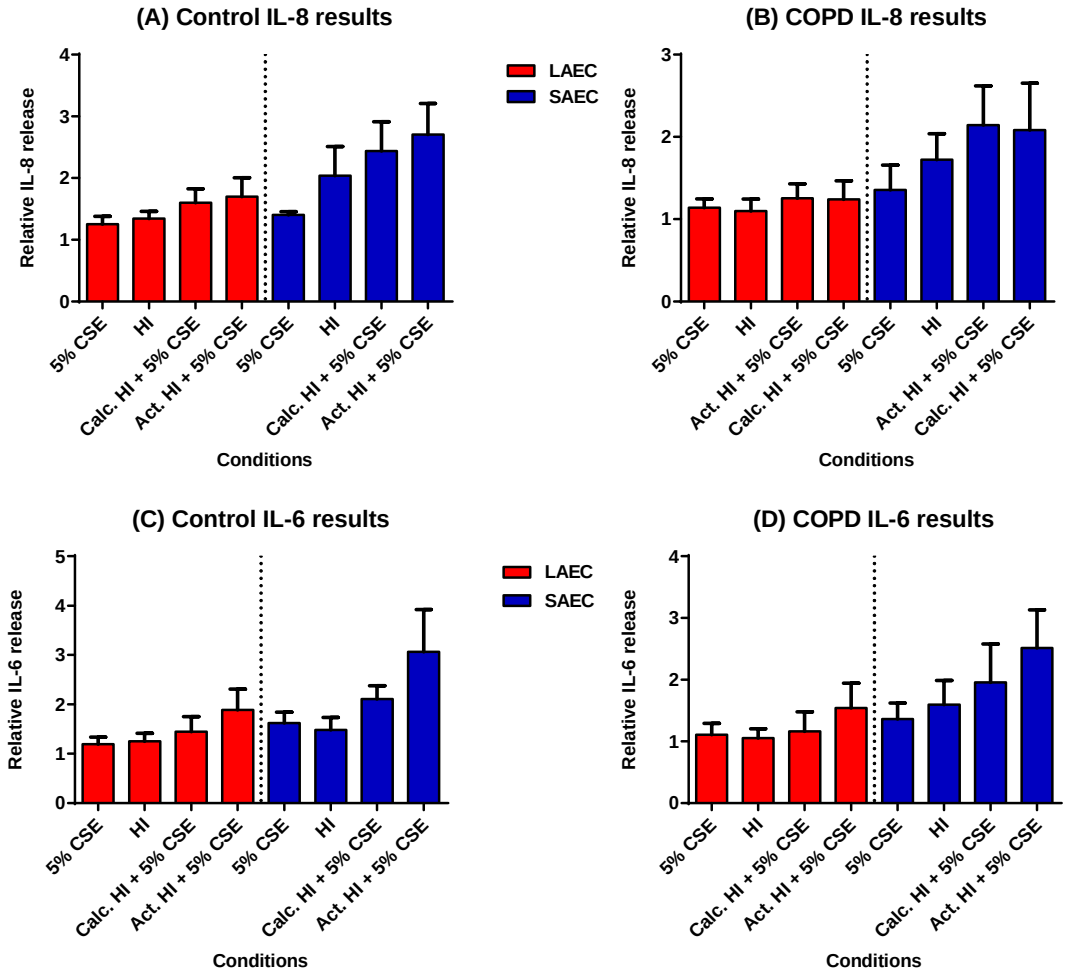


Figure 54 The effect of combined CSE and HI on LAEC and SAEC IL-8 and IL-6 release
Data are displayed for n=6 control (A, C) and n=6 COPD (B, D) donor cell sets, as mean (± SEM). The calculated HI + CSE% was worked out from the addition of individual 5% CSE and HI treatment effects above the relative mean value of 1. At least the same as an additive effect was observed for both IL-8 (A, B), and IL-6 (C, D), in response to HI combined with 5% CSE in both LAEC and SAEC.

6.8 Baseline mediator levels in primary airway epithelial cells in culture

In addition to considering the effect of CSE and HI treatments on levels of IL-8, IL-6 and IL-1β, it was important to assess if there was any difference in baseline mediator release, both between LAEC and SAEC, and between healthy control cells and COPD cells (see Figure 55). Baseline levels of each of these mediators was analysed in 3 "no treatment" control wells for each experiment. To ensure that differences in cell number present did not affect the results observed cell counts were performed for each well, and the baseline mediator release was adjusted for this as described (Table 39).

Overall baseline mediator levels were higher in SAEC from both control and COPD donors, but there was no significant difference between cell types, or between healthy and COPD cells, with variability in results between primary cell sets observed.

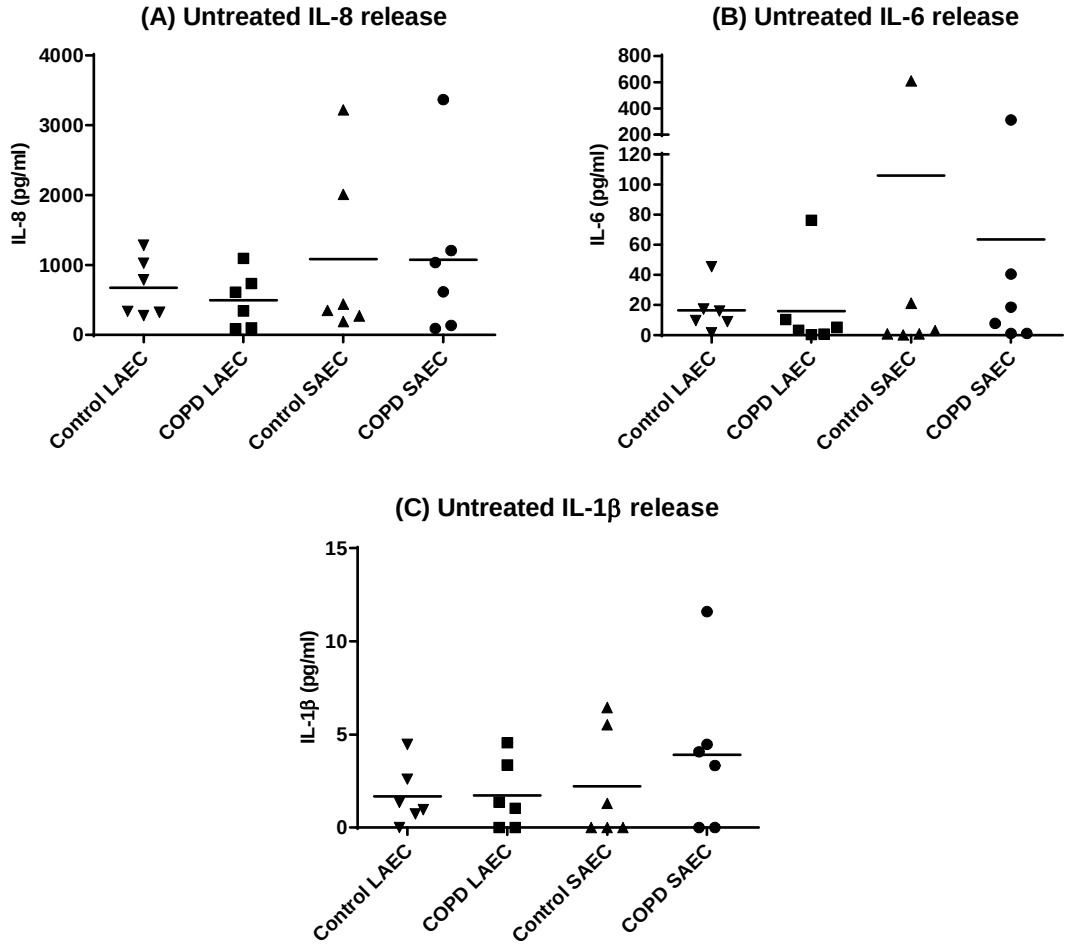


Figure 55 Baseline mediator release adjusted for cell counts
Data presented are the levels of IL-8 (A), IL-6 (B) and IL-1β (C) detected by ELISA (IL-8) and MSD assay (IL-6 and IL-1β) for cells cultured in triplicate as "no treatment control" wells. The data have been adjusted for cell count with a mean value displayed for each cell type from each donor set. The lines indicate the mean of the sample results (see Table 36). No significant differences were observed between baseline mediator release from LAEC and SAEC in either group (paired T tests, n=6, p>0.05). No significant difference was detected between control and COPD cells (unpaired T tests, n=6, p>0.05).

Cell type	IL-8	IL-6	IL-1β
Control LAEC	673 (173)	16 (6)	1.7 (0.7)
COPD LAEC	495 (161)	16 (12)	1.7 (0.8)
Control SAEC	1082 (511)	106 (101)	2.2 (1.2)
COPD SAEC	1076 (494)	64 (50)	3.9 (1.7)

Table 39 Mean baseline mediator release adjusted for cell counts
Cell counts were performed for the 3 "no treatment control" wells from each experiment. For each well the cell count and mediator release were used to calculate the baseline mediator release per 100,000 cells. The data are expressed as mean values from n=6 donor cell sets with all data expressed as mean pg/ml ± SEM.

6.9 Effect of corticosteroid pre-treatment on primary airway epithelial cell responses

On the basis of the results observed in BEAS-2B cells, with suppression of TNF-α induced IL-8 release following 24 hour pre-treatment with either 1nM Dexamethasone or 17-BMP, these treatment conditions were used to investigate if the cellular responses to CSE and HI observed could be suppressed in primary airway epithelial cells.

Figures 56-59 show the results from these experiments with analysis of IL-8 levels in cell culture media detected by ELISA. Despite the significant effects observed in BEAS-2B cells, no effect of either corticosteroid treatment was observed, in either control (Figure 56) or COPD cells (Figure 58) under the conditions used. In addition, no effect on suppression of TNF-α induced IL-8 release was observed (Figure 57 and Figure 59).

Having reviewed the response to 1nM corticosteroids in the culture model, the possibility of requirement for a higher dose of corticosteroid for suppression was raised. Therefore some further dose response work was performed in primary cells as previously for BEAS-2B cells, using TNF-α induced IL-8 release. It was only possible to complete this for n=2 sets of control and n=2 sets of COPD LAEC and SAEC cells. This work was performed using cells from healthy control subjects 6 (control 1) and 10 (control 2), and COPD subjects 13 (COPD1) and 17 (COPD2). The demographic details for these participants is summarised in Table 35 in section 6.5. Of note both COPD donors were on ICS treatment. Figures 60 and 61 show the results of these experiments respectively.

Following healthy control LAEC 24 hour pre-treatment there was a trend towards reduced IL-8 with 10^{-6}M Dexamethasone (control 1 mean IL-8 with TNF-α 2.7±0.2 decreased to 2.4±0.1 with 10^{-6}M Dexamethasone; control 2 mean IL-8 with TNF-α 1.9±0.1 decreased to 1.4±0.1 with 10^{-6}M Dexamethasone) (see Figure 60). A similar trend was seen in control 1 LAEC following 10^{-6}M 17-BMP treatment (mean IL-8 2.7±0.2 decreased to 2.4±0.04), but not in control 2 LAEC (with significant variability noted overall in results from these cells). Only minimal change was noted in control 1

SAEC following treatment with 10^{-6}M 17-BMP (mean IL-8 with TNF-α 5.2±0.1 decreased to 4.8±0.2).

In COPD LAEC reduced IL-8 was observed following 10^{-6}M 17-BMP treatment with a marked response in COPD donor 2 cells (COPD 1 mean IL-8 with TNF-α 2.2±0.1 decreased to 2.1±0.1; COPD 2 mean IL-8 with TNF-α 2.7±0.7 decreased to 1.7±0.1) (see Figure 61). No effect was observed with Dexamethasone pre-treatment. In COPD SAEC only minimal change was noted in COPD 2 following treatment with either Dexamethasone or 17-BMP (*e.g.* mean IL-8 with TNF-α 2.4±0.04 decreased to 2.1±0.1 with Dexamethasone).

Overall, the marked effect of 10^{-6}M Dexamethasone or 17-BMP treatment observed in BEAS-2B cells was not replicated in the primary cells tested (see Figure 62).

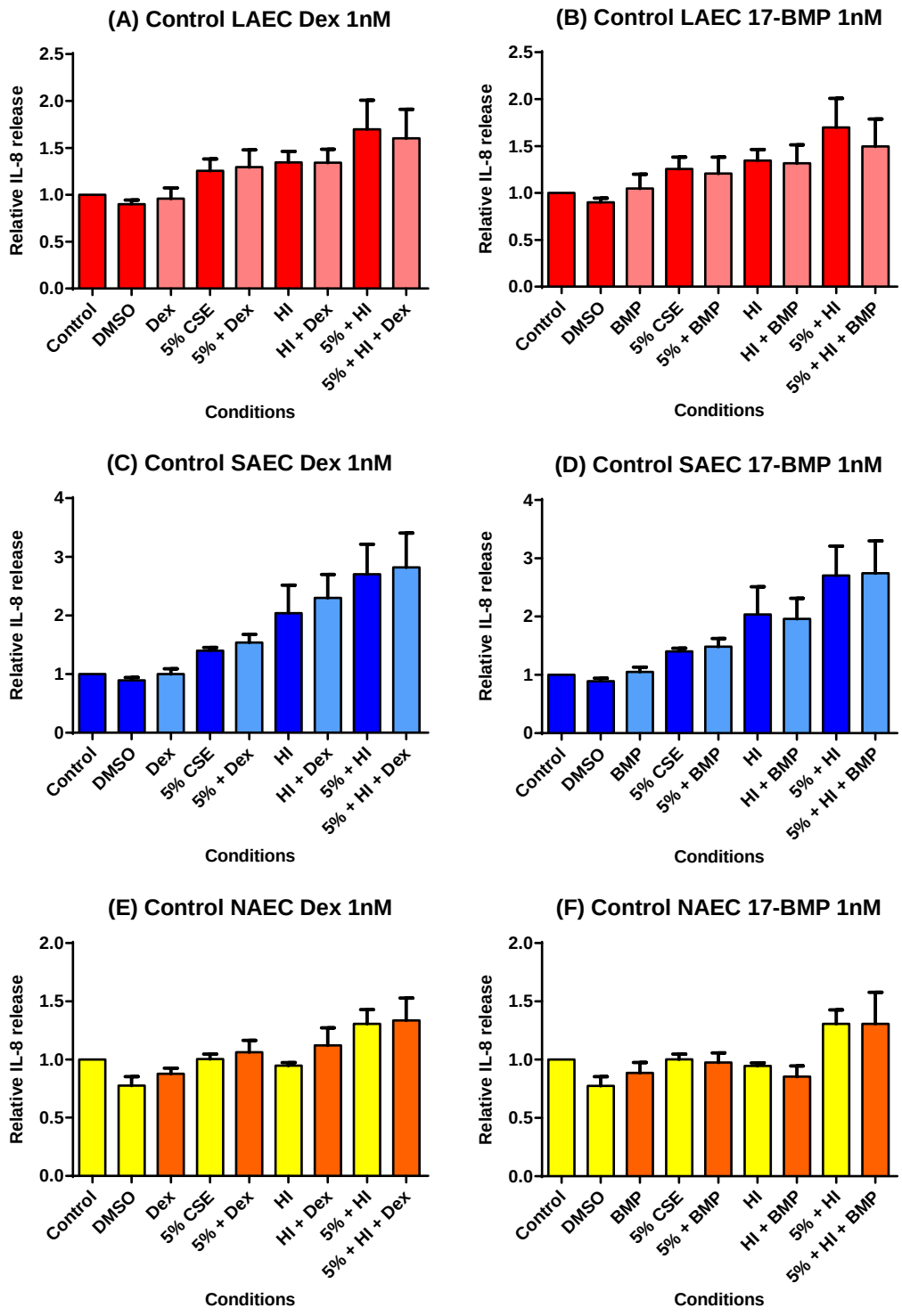


Figure 56 Healthy control cell corticosteroid pre-treatment results
Cells were treated in triplicate with normal media, media containing 0.1% DMSO, media with 1nM Dexamethasone (Dex), or media with 1nM 17-BMP for 24 hours, prior to the addition of CSE (to make final concentration 5% CSE), HI (to make final concentration 25µl/ml), or both CSE and HI combined. After a further 24 hours media was harvested and IL-8 levels measured by ELISA. Data are displayed as mean values relative to baseline (± SEM). In control LAEC (A, B, n=6), SAEC (C, D, n=6) and NAEC (E, F, n=4) no effect of either corticosteroid pre-treatment was observed (all $p>0.05$).

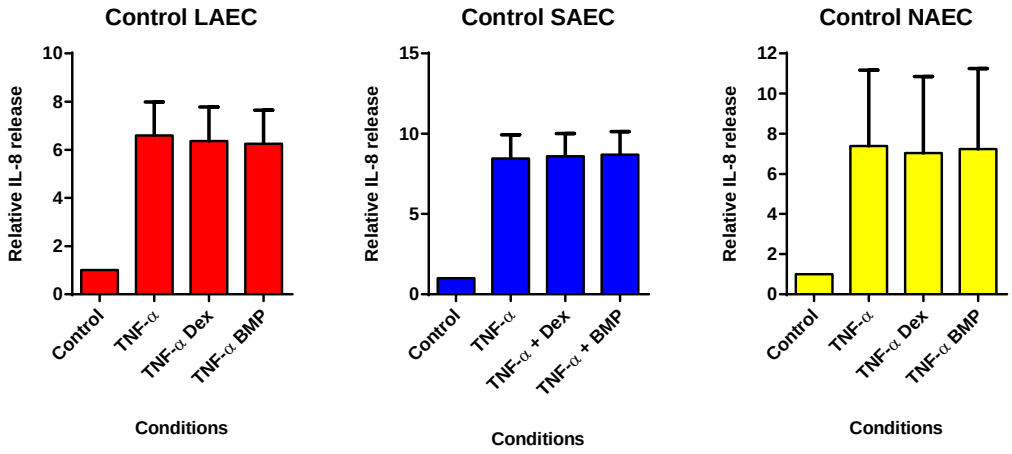


Figure 57 Healthy control cell corticosteroid pre-treatment results - TNF-α controls
Cells were treated in triplicate with normal media, media containing 0.1% DMSO, media with 1nM Dexamethasone (Dex), or media with 1nM 17-BMP for 24 hours, prior to the addition of TNF (to make final concentration 20ng/ml). After a further 24 hours media was harvested and IL-8 levels measured by ELISA. Data are displayed as mean values relative to baseline (± SEM). In control LAEC (n=6), SAEC (n=6) and NAEC (n=4) no significant effect of either corticosteroid pre-treatment was observed (all $p>0.05$).

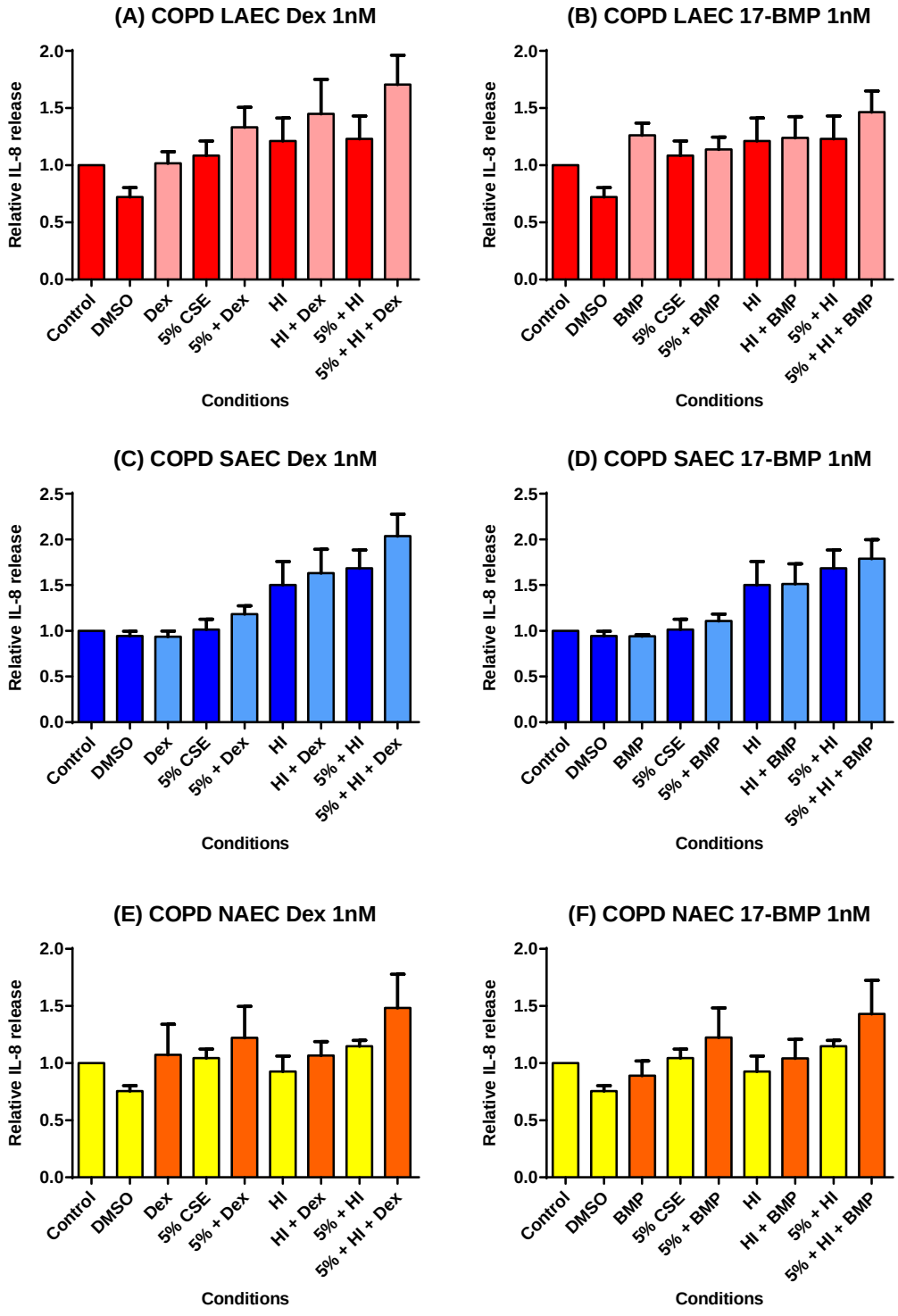


Figure 58 COPD cell corticosteroid pre-treatment results

Cells were treated in triplicate with normal media, media containing 0.1% DMSO, media with 1nM Dexamethasone (Dex), or media with 1nM 17-BMP for 24 hours, prior to the addition of CSE (to make final concentration 5% CSE), HI (to make final concentration 25µl/ml), or both CSE and HI combined. After a further 24 hours media was harvested and IL-8 levels measured by ELISA. Data are displayed as mean values relative to baseline (± SEM). In COPD LAEC (A, B, n=6), SAEC (C, D, n=6) and NAEC (E, F, n=4) no effect of either corticosteroid pre-treatment was observed (all $p>0.05$).

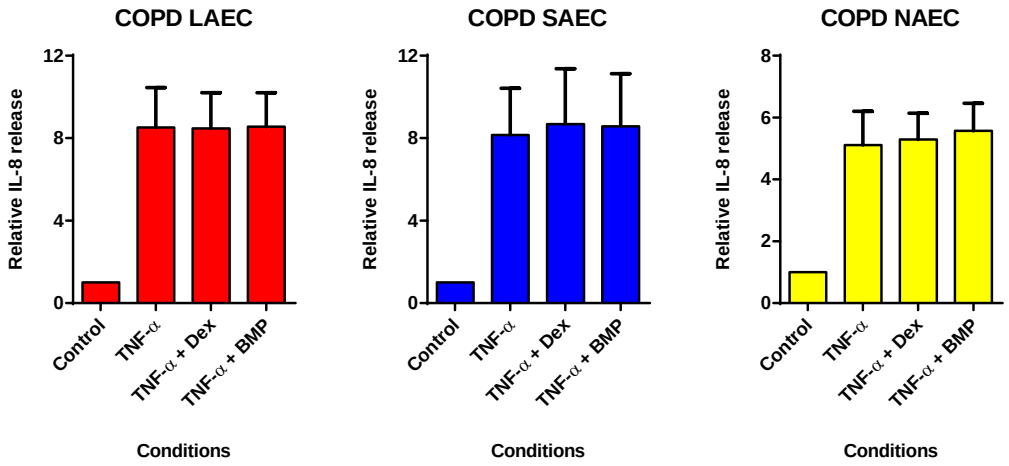


Figure 59 COPD cell corticosteroid pre-treatment results - TNF-α controls
Cells were treated in triplicate with normal media, media containing 0.1% DMSO, media with 1nM Dexamethasone (Dex), or media with 1nM 17-BMP for 24 hours, prior to the addition of TNF (to make final concentration 20ng/ml). After a further 24 hours media was harvested and IL-8 levels measured by ELISA. Data are displayed as mean values relative to baseline (± SEM). In control LAEC (n=6), SAEC (n=6) and NAEC (n=4) no significant effect of either corticosteroid pre-treatment was observed (all $p>0.05$).

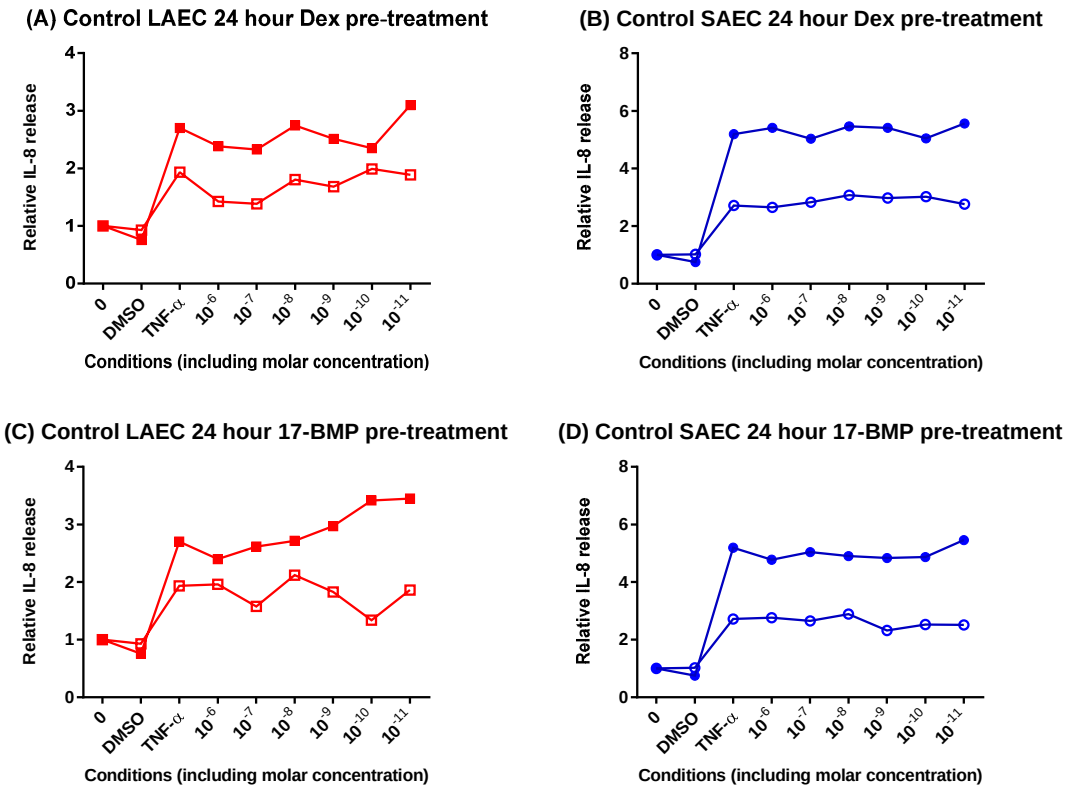


Figure 60 Dose response experiments to assess the effect of *in-vitro* corticosteroid treatment on TNF-α induced IL-8 release from healthy control LAEC and SAEC

Cells were treated in triplicate with normal media, media containing 0.1% DMSO, and media containing increasing concentrations of Dexamethasone (Dex) or 17-BMP for 24 hours, prior to the addition of TNF-α (20ng/ml), for a further 24 hours. Media was harvested for analysis of IL-8 levels by ELISA. Displayed data show mean results (expressed as values relative to the "no treatment control" baseline) of cells from 2 healthy control donors (control 1 solid symbols, control 2 clear symbols). Treatment with Dex 10^{-6}M reduced IL-8 levels in both sets of LAEC (A), and 10^{-6}M 17-BMP reduced IL-8 levels in only control 1 LAEC (C). Minimal effect was in SAEC from control 1 only following 10^{-6}M 17-BMP (B, D).

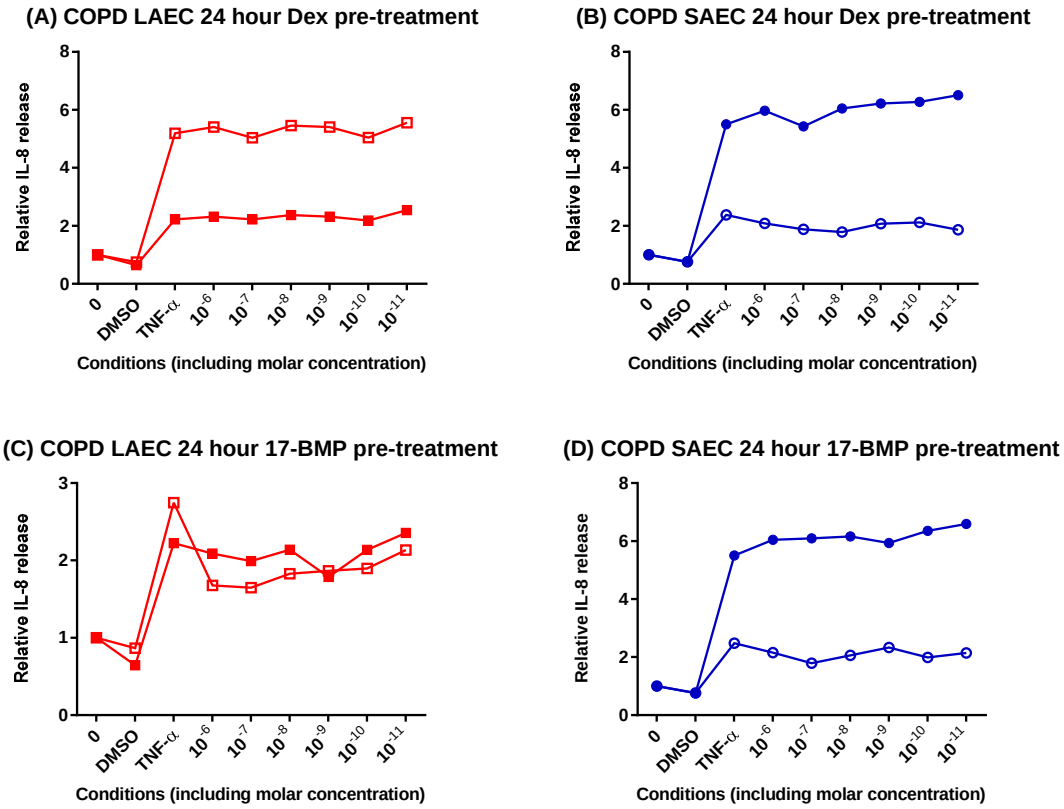


Figure 61 Dose response experiments to assess the effect of *in-vitro* corticosteroid treatment on TNF-α induced IL-8 release from COPD LAEC and SAEC

Cells were treated in triplicate with normal media, media containing 0.1% DMSO, and media containing increasing concentrations of Dexamethasone (Dex) or 17-BMP for 24 hours, prior to the addition of TNF-α (20ng/ml), for a further 24 hours. Media was harvested for analysis of IL-8 levels by ELISA. Displayed data show mean results (expressed as values relative to the "no treatment control" baseline) of cells from 2 COPD cell donors (COPD 1 solid symbols, COPD 2 clear symbols). Treatment with 10^{-6}M 17-BMP reduced IL-8 levels in both sets of LAEC but this effect was much more marked in cells from COPD donor 2. No inhibitory effect was observed following pre-treatment with Dex in LAEC. Only minimal change in IL-8 levels in was observed in SAEC following pre-treatment with either Dex or 17-BMP at 10^{-6}M concentration.

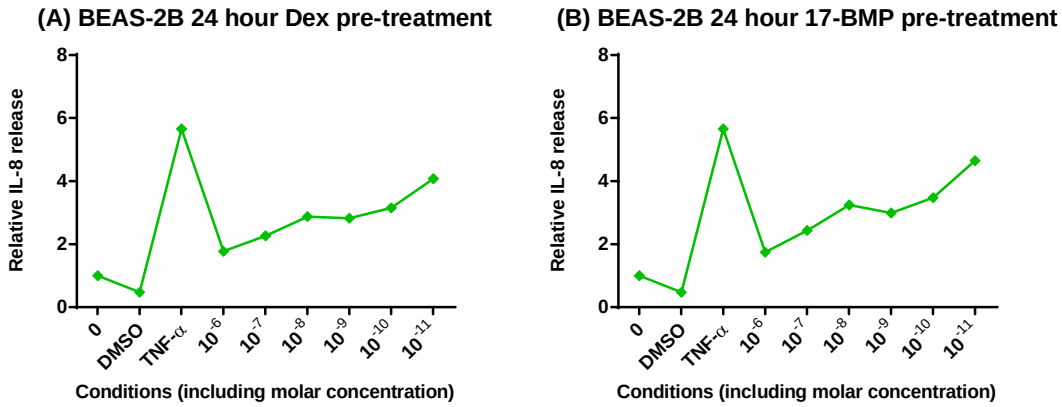


Figure 62 Review of the effect of corticosteroid treatment on TNF-α induced IL-8 release from BEAS-2B cells

Data from previous figures (see section 6.3.3) expressed as values relative to the "no treatment control" baseline for comparison to primary airway epithelial cell data.

6.10 Discussion

6.10.1 Summary of results presented

The aim of this work was to develop an *in-vitro* cell culture model to assess the effect of cigarette smoke and bacterial pathogens on innate immune responses in airway epithelial cells. Cell lines were initially used to establish experimental parameters for the cell treatments to be used, with cigarette smoke extract used to model cigarette smoke exposure, and bacterial whole cell lysates used to model the presence of common respiratory pathogens. Cell viability and proliferation were taken into account, with particular note of the effect of CSE on cellular proliferation, even at low concentrations (1-2.5%). Later cell treatments were however, only performed when cells had already reached approximately 90% confluence as an epithelial monolayer. HBE cells showed an increase in IL-8 with increasing CSE concentration, but no change in IL-8 was observed in response to any of the bacterial whole cell lysates tested. An increase in IL-8 was observed however following treatment with TNF-α and LPS (though with a dose of 10µg/ml LPS). In contrast, in BEAS-2B cells, no effect of CSE (up to 5%) was observed on IL-8 release, but a significant increase in IL-8 was observed after all doses of HI tested. In both cell lines combined CSE and HI treatments were performed; however no additional effect was observed compared to the treatments alone *i.e.* the CSE effect was observed in HBE cells and the HI effect was observed in BEAS-2B cells.

Taking these observations forward into primary cells the same effect of CSE on cellular proliferation was noted. An increase in IL-8 was observed in COPD SAEC with all doses of HI tested, with a similar trend noted in control SAEC at a higher dose (25µl/ml). The higher dose was therefore applied to a larger number of cell sets, alone, and in combination with CSE (up to 5%) for further investigation of the hypothesis proposed. Appropriate cell culture conditions were considered, including effect of culture media type and supplementation with growth factors.

Overall, in both healthy control and COPD primary airway epithelial cells, CSE alone had minimal effect, with an increase in IL-8, IL-6 and IL-1β observed at CSE 5% in SAEC but not in LAEC. The response to HI alone was also greater in SAEC with an increase in

IL-8 and IL-6 observed, with some change in control LAEC cytokine levels also noted. Following combined treatment, a response to CSE and HI was observed in both LAEC and SAEC, with an increase in IL-8 and IL-6. This effect was greater in SAEC, and a significant difference was observed between LAEC and SAEC in control cells. The same trend was observed in COPD cells but this did not meet statistical significance. NAEC were only assessed for IL-8 with no significant change in with the treatments tested in this cell type. All cells responded to TNF-α with increase in IL-8.

Corticosteroid treatment was shown to suppress TNF-α induced IL-8 release in BEAS-2B cells. However, at a 1nM concentration which had been effective in suppression in the BEAS-2B model, neither Dexamethasone, nor 17-BMP suppressed the primary airway epithelial cell IL-8 release in response to CSE and HI. Only a minimal effect of higher doses up to 1μM was shown in primary LAEC and SAEC on investigation of the effect on TNF-α induced IL-8 release in these cells.

6.10.2 Cell line findings

The cell line work performed informed assessment of appropriate cell treatments to induce an immune response. In addition this work allows comparison to previous research in this area and to the findings observed in primary cells. It was interesting to see differences in response between different airway epithelial cell lines. Previous research in HBE cells has also demonstrated an increase in IL-8 release on CSE treatment in this cell line. Pace *et al* showed that CSE treatment also increased LPS binding and TLR4 expression (Pace et al., 2008); combined CSE and LPS (1μg/ml) treatment further increased IL-8 release via an ERK intra-cellular signalling pathway. Mortaz *et al* demonstrated a dose-dependent release of IL-8 from HBE in response to CSE; this effect was reduced by blocking TLR4 or TLR9, and decreased TLR surface expression was observed following CSE treatment (Mortaz et al., 2011).

In contrast to the CSE effect observed, the HBE cells did not respond to the bacterial whole cell lysate preparations used. Of note HBE cells were cultured in media supplemented with fetal bovine serum which could increase baseline IL-8 levels (Becker et al., 2000). It is possible that these high resting levels could have masked cellular responses to HI; but as above a positive response was noted to other

treatments investigated, including LPS. Contamination of the cell culture with LPS is another possibility, which could lead to desensitisation of the cells to stimuli (Becker et al., 2000).

HBE cells have been shown to express TLRs 1-6 and TLR9 (Greene et al., 2005). It was expected that this cell line would therefore be able to respond to the bacterial PAMPs present in the whole cell lysate preparations. As described in the methods section, these were prepared by sonication, DNase treatment (to breakdown bacterial DNA) and Proteinase K digestion (which was then deactivated by boiling). This method was used as an established technique in our group for preparation of lysates from other bacteria including *Burkholderia* and *Pseudomonas* (De Soyza et al., 2004); both digested and undigested lysates of *Burkholderia cepacia* have been shown to induce cellular responses (De Soyza et al., 2004). It is possible that the lysate processing affected the integrity of TLR ligands present; this could be assessed through comparison of treatment with digested and undigested lysate preparation. However, the lysates did induce the expected response in both THP-1 and BEAS-2B cells.

It is possible that the HBE cells did not respond to the lysate preparations due to lower overall concentrations of PAMPs present, *e.g.* Lipo-oligosaccharide (LOS) in the HI preparation. An increased concentration of LPS has been shown to be required for airway epithelial cell activation (1-5µg/ml), as opposed to monocytic cell activation (10ng/ml); this may be due to TLR4 co-receptor expression, with low levels of MD2 expression (Jia et al., 2004) and CD14 (Becker et al., 2000) reported previously. In addition, an intra-cellular localisation of TLR4 has been shown which may also affect this (Guillot et al, 2004).

In contrast to the HBE cell line response to CSE, no effect of CSE treatment was observed in BEAS-2B cells. This was reported previously BEAS-2B cells treated with CSE (Laan et al., 2004); however only short 15 minute CSE exposures were performed in this study. CSE alone suppressed basal IL-8 levels at 2 hours and no significant effect was observed at 6 or 18 hours. A further study using BEAS-2B cells reported an increase in IL-8 levels after 24 hours treatment; however higher concentrations of CSE

were used in this study from 12.5-50% CSE (Hudy et al., 2010). The reasons for different behaviour in the BEAS-2B cell line compared to the HBE cell line in the same model are not entirely clear. BEAS-2B cells were cultured in BEGM media which is serum-free. The CSE preparations for each cell type were made up using the culture media used for the particular cell type: this could have impacted on the potential pro-inflammatory effects observed. The same effects of CSE on cellular proliferation were seen in both cell lines however.

A significant increase in IL-8 was observed in BEAS-2B cells treated with either LPS or HI. This cell line has previously been used to investigate LPS responses in airway epithelial cells with observation of similar effects using LPS or NTHi (live or heat killed). Guillot *et al* showed that LPS induced IL-8 and IL-6 release from BEAS-2B cells, with no reported effect on TLR4 expression (Guillot et al., 2004). TLR3 has been shown to be involved in BEAS-2B and PBEC cell responses to NTHi, with loss of the stimulatory effect observed through blocking this TLR or use of siRNA to silence expression (Teng et al., 2010). NTHi has been shown to upregulate TLR2 expression in PBECs, with lipoprotein activation of NFκB via TLR2 reported (Shuto et al., 2002).

No additional effect was seen on combined treatment with HI and CSE in BEAS-2B cells. Laan *et al* reported that CSE pre-treatment inhibited LPS-induced IL-8 release from BEAS-2B cells (Laan et al., 2004). A negative effect of CSE on cellular responses has also been reported in A549 cells treated with CSE and heat killed NTHi, in which a reduction in IL-8 release was observed with decreased IL-8 mRNA expression (Kulkarni et al., 2010). This is in contrast to the work by Hudy *et al* which showed at least an additive effect of CSE treatment with rhinovirus treatment, on increasing IL-8 levels, mediated through mRNA stabilisation (Hudy et al., 2010).

In the current study CSE treatment did not appear to have any negative impact on the BEAS-2B response to HI. As demonstrated by the previous research outlined above, NTHi may induce pro-inflammatory cellular responses through a number of different mechanisms including via activation of TLRs 2, 3 and 4. CSE has been shown to have a negative impact on LPS-induced IL-8 release in this cell line, but not necessarily the

other pathways involved in the immune response to NTHi which could have been activated in this culture model through the use of whole cell lysate preparations. The timing of treatments and assessment of mediator release, and the cell stimulation methods used are also important to consider; Laan *et al* observed a reduction in LPS-induced IL-8 release at 2 and 6 hours following CSE exposure for 15 minutes, but this was not sustained at 18 hours (Laan et al., 2004). However, multiple CSE exposures (15 minutes every 2 hours) did result in a more sustained inhibitory effect, with a duration of up to 10 hours reported.

6.10.3 Primary cell findings

The data presented here show that only primary SAEC responded to 5% CSE, but not LAEC or NAEC. This effect was observed in both healthy control and COPD cells, though it was less marked in COPD cells. Previous research has demonstrated the responsiveness of SAEC to CSE; Kode *et al* tested primary cells and a number of different cell lines and shown an increase in IL-8 and IL-6 release from primary SAEC following CSE treatment which was not observed in the cell lines tested including A549 cells (Kode et al., 2006). Moretto *et al* also demonstrated this response in primary SAEC (Moretto et al., 2009). Other work has shown cytokine release from primary LAEC in response to CSE, in healthy bronchial airway epithelial cells in submerged (Nadigel et al., 2013), or ALI (Comer et al., 2013) cell culture models, with increased cytokine release. However, this effect was not replicated in COPD cells in these studies (with a reduction in cytokines reported in the ALI model).

An effect of CSE on cytokine release was not observed from LAEC in the current study but was observed from both healthy and COPD SAEC. As for the cell lines, different culture media was used for these individual cell types in the model developed; however, both SAGM and BEGM are serum-free, with supplementation with the same growth factors. The only difference in supplementation of growth factors was the addition of bovine serum albumin to SAGM and not to BEGM (see details in the materials section 3.2). It is possible that this influenced the resulting CSE preparation and could have impacted on the results observed. However, differences in response to HI stimulation were also observed between the cell types, with an increased response in SAEC, which would not be explained by this. In addition, despite no response to CSE

alone, and lesser response to HI compared to SAEC, LAEC also showed increase in IL-8 and IL-6 levels following treatment with combined CSE and HI, suggesting that the CSE may have had an effect here.

Previous research has also demonstrated differences in baseline cytokine release from unstimulated COPD bronchial epithelial cells compared to those isolated from healthy controls (Schulz et al., 2003, Comer et al., 2012). In the current study no significant difference between either disease and control groups or LAEC versus SAEC was observed. This could be due to loss of this effect in submerged cell culture, and the findings may also have been affected by smaller sample numbers with variability between donor cell sets.

In the present study, no significant change in IL-8 release was observed from NAEC with either CSE or HI stimulation alone, or following combined treatment. This was the same for both control and COPD cells, which however did responded positively to TNF-α stimulation. This assessment was more limited than that for LAEC and SAEC in terms of additional cytokines analysed; however, in the context of the current model and data available from this, NAEC could not be used as a substitute to investigate either LAEC or SAEC responses. Higher baseline release of pro-inflammatory mediators from NAEC compared to paired LAEC has been reported previously, but with correlation between cell types in response to cell stimulation with TNF-α and IL-1β observed; on this basis it was suggested that NAEC could be used as a surrogate for LAEC responses, though no patients with COPD were studied (McDougall et al., 2008). In the context of COPD research, further work in this area showed a correlation between COPD NAEC and LAEC responses with IL-8 release following *Pseudomonas aeruginosa* (PA) LPS exposure (Comer et al., 2012). However, this was not observed for CSE treatment (5% CSE stimulatory in NAEC at 24 hours but reduced IL-8 in LAEC at 24 hours), or on assessment of IL-6 release. This group also found therefore that NAEC could not model LAEC.

Comer *et al* have also reported work comparing healthy controls, smokers without evidence of airflow limitation and COPD patients, using LAEC cultures at ALI. In their

model CSE increased baseline IL-6 and IL-8 levels, and increased the response to PA LPS in control cells and those from smokers without airflow limitation (Comer et al., 2013). However, CSE reduced the LPS response in COPD cells, with associated reduction in TLR4 expression, MAPK and NFKB activation found in cells from this group but not the control groups. The same model of CSE preparation was used as in the current study (1 cigarette and 25 ml culture media). In contrast to the results described however, in the current study no effect of CSE was observed in LAEC, and in both LAEC from controls and COPD patients, an increase in mediator release was seen following combined treatment with CSE and HI. As for the cell line results discussed, the differences in results reported could relate to cell activation by a mixture of ligands as opposed to LPS alone, and also potential differences in using an ALI model.

6.10.4 Reflection on results observed compared to the original study hypothesis

To date, no published research has reported investigation of differences in small compared to large airway epithelial cell innate immune responses in COPD. The hypothesis for the current study proposed that in health, the small airways may mount a more potent immune response to pathogen exposure than the large or nasal airway epithelium. The results of the novel *in-vitro* cell culture model developed clearly support this. It was proposed that this effect may be blunted by cigarette smoke. In contrast to this an increase in IL-8 and IL-6 was observed in both LAEC and SAEC in response to combined CSE and HI, and this was more marked in SAEC. This response was at least additive, and in some cases more than this suggesting the possibility of a synergistic effect of combined treatment. A suppressive effect of CSE had originally been hypothesised based on previous research in the literature which has been discussed above. In considering the results observed, the use of bacterial whole cell lysate rather than a single TLR ligand may again be important to consider in this context. CSE has been shown to have an inhibitory effect on TLR4 (Comer et al., 2013), and has also been shown to reduce TLR5 expression in differentiated primary LAEC in culture (Wang et al., 2012). However, effects on other TLRs and studies using SAEC have not been reported. It is possible that in the current model, with the use of CSE and the presence of multiple bacterial PAMPs, that a number of different pathways

could have been affected, but with the overall result of stimulated cell mediator release.

In established COPD a dampened response was proposed *i.e.* a less potent response in the small airway epithelium. However, the same pattern of results was observed in both COPD and control cells, though the difference between LAEC and SAEC responses was less marked in COPD cells. This could reflect an effect of the disease process as suggested in the original hypothesis. However, it would appear more likely to be influenced by greater variability in the COPD cells used and small sample size. The COPD cells were from patients with a range of disease severity, and included current and ex-smokers. It would be interesting to analyse responses in a larger number of paired LAEC and SAEC from patients at each different stage of COPD, to determine if cells in milder disease have a phenotype closer to control cells and if this changes with an increase in disease severity, or if it is maintained throughout. In addition to the differences in clinical phenotypic features in the COPD subject donors, the impact of disease heterogeneity within the individual patients must also be considered. Samples were taken from different airway levels for comparison. However, with differences in the pattern of the disease throughout the airways and lungs (as discussed in Chapter 1), the cells sampled may have been more or less affected by the disease process and if from "healthier" airways this may have reduced any potential differences between disease and control findings. Previous research in cells isolated from patients with asthma and healthy controls was also unable to find any difference in cellular responses between health and disease (Woodman et al., 2013). It was suggested that other factors present in the microenvironment of the airway epithelium may contribute to differences observed *in-vivo*.

Although an increase in mediator levels or "release" was observed in the current study, this could have been due to a number of factors, from effects on gene expression or mRNA stability, to stored mediator release; further investigation is required to elucidate the mechanisms underlying the results shown as discussed below.

6.10.5 Review of corticosteroid treatment effects

In the current study, corticosteroid pre-treatment was effective in suppression of cell line responses but this was not found in the primary cell experiments. Previous research has shown that *in-vitro* steroid treatment can reduce inflammatory responses from airway epithelial cells in culture. Dexamethasone (1µM for 24 hours) suppressed both baseline and TNF-α stimulated IL-8 from primary bronchial epithelial cells (Kwon et al., 1994). Reduction of TNF-α induced (50ng/ml) RANTES and soluble ICAM-1 has been reported in PBECs, following treatment with Fluticasone (10^{-4}-10^{-6}M, both mediators) and BDP (10^{-4}M only RANTES) (Wang et al., 1997). TNF-α (10ng/ml) induced GM-CSF and IL-8 release was reduced by 40% following treatment with 10^{-8}M Budesonide in normal PBECs (Korn et al., 2001).

A dose dependent inhibition of TNF-α (10ng/ml) induced IL-8 release was reported with 2 hour Budesonide pre-treatment (1-100nM) in PBECs from healthy controls, smoking controls and COPD and asthma patients (Heijink et al., 2014). PBECs from asthma and COPD patients were however noted to be less responsive to the inhibitory effect of Budesonide, in particular with less effect on GM-CSF release. In contrast, in a study comparing nasal and bronchial epithelial cells from healthy controls and asthma patients, stimulated cells from both asthma patients and healthy controls were resistant to treatment with Prednisolone (10µM) (Woodman et al., 2013). However, suppression of the inflammatory response was achieved with use of an IKK2 inhibitor. The lack of inhibition observed in the current study in primary airway epithelial cells may relate to the cytokine response investigated, with IL-8 only analysed; it is possible that suppression of other mediators could have occurred, *e.g.* suppression of the GM-CSF response noted to be more significant in COPD and asthma cells in previous studies (Heijink et al., 2014). However, the previous study in asthma and control cells by Woodman *et al*, reported similar findings in terms of steroid responsiveness, but found an effect of an IKK inhibitor, active on the NFκB signalling pathway (Woodman et al., 2013). In COPD, steroid resistance has been suggested to be related to a number of mechanisms, including the effects of oxidative stress on histone deacetylase (HDAC) 2 activity; corticosteroids effect the recruitment of HDAC 2 to inflammatory genes, to reduce acetylation and therefore gene expression. Oxidative stress can result in

reduced HDAC2 expression and activity, leading to loss of this anti-inflammatory action (Barnes, 2013). Potential mechanisms of steroid resistance have not been investigated in the current study; effects of oxidative stress and HDAC 2 expression in cells in culture could be considered. In moving forwards using the current model assessment of a wider range of inflammatory mediators and inhibitors in a larger sample number could be informative. In addition, the potential for a carryover effect of clinical ICS treatment should be considered in any COPD cell samples analysed.

6.10.6 Review of the in-vitro cell culture model used

Cigarette smoke extract (CSE)

CSE was used to model the effects of cigarette smoke *in-vitro*. This type of preparation contains a combination of water soluble particulate and gaseous phases of whole cigarette smoke (Clunes et al., 2008). Active volatile and semi-volatile components of this may be lost soon after preparation (Rennard, 2004); preparations were used within 30 minutes in all of the experiments described to minimise this. The exact method by which cigarette smoke extract is made (*i.e.* number and type of cigarettes used, volume of media, final concentration applied to cells), the duration of treatment and time points at which cell responses are tested may all impact on the results observed. This makes comparison between research studies difficult. However, the CSE preparation method used was an established method in our laboratory, and the same effect on cellular responses was observed in cell lines between multiple preparations and experiments, to allow comparisons between the different cell types studied within this model. This method has also been described by other groups, including assessment of optical density of different preparations with no significant differences between preparations observed (Comer et al., 2014). Considering other possible methods to expose cells to cigarette smoke *in-vitro*, even use of whole cigarette smoke is not without problems including consideration of the dose of smoke applied to the cells (Thorne and Adamson, 2013); additionally the systems used for whole smoke exposure are likely to be much less widely available than the current model of CSE used. *In-vivo* the exposure generated from smoking is also highly variable, and chronic exposure is involved in the development of COPD which cannot

be replicated in cells in culture. Overall, methods of smoke exposure are used as a tool to facilitate *in-vitro* investigation of the biological processes which may be involved in smoking-related lung disease, and are accepted as such (Rennard, 2004, Shapiro, 2004).

Bacterial whole cell lysate

Bacterial whole cell lysates were used in this study to allow cell exposure to a range of bacterial PAMPs. In particular non-typeable *Haemophilus influenzae* (NTHi) was chosen as a common pathogen in COPD exacerbations and also present in the lung microbiome. Use of whole cell lysates may be more reflective of bacterial presence *in-vivo*, with exposure to a number of different stimuli with potential for signalling through multiple TLR pathways, than exposure to a single TLR ligand. The airway epithelium may be able to react directly to certain stimuli *e.g.* bacterial wall components. *In-vivo* exposure to other PAMPs may first require uptake and processing of bacterial components by other innate immune cells *e.g.* macrophages or dendritic cells as antigen presenting cells. The HI whole cell lysate should contain PAMPs including lipoproteins (TLR2 ligand), LOS (TLR4 ligand) and bacterial DNA (TLR9 ligand - unmethylated CpG DNA) (as per details in Chapter 1).

Other studies have described the use of whole bacterial cell lysates (Shuto et al., 2001), heat-killed bacteria (Jia et al., 2004), and live strains (Kulkarni et al., 2010). Consideration of a co-culture model with airway epithelium, innate immune cells with the ability to phagocytose and present bacterial PAMPs *e.g.* AM, and whole live/dead bacteria may allow a more representative modelling of *in-vivo* pathogen exposure.

Use of multiple potential PAMPs may make the analysis of resultant cell responses more difficult. In order to delineate the mechanisms involved in the responses observed, use of individual ligands may be required. This could include LPS/LOS isolation from relevant bacterial strains from which whole cell lysates could also be tested (De Soyza et al., 2004). In addition, inhibition of particular TLR ligands within the lysates could be considered, for example using Polymixin B to inhibit LPS-induced TLR4 activation.

Analysis of pro-inflammatory mediators

The current study investigated levels of IL-8, IL-6, IL-1β and TNF-α as pro-inflammatory mediators in the *in-vitro* cell culture model described. Of note TNF-α was below the level of detection of the assay used for all samples tested other than from one healthy control donor (see Section 6.6). This cytokine has been detected from PBECs in previous research (Profita et al., 2003); the LLD of the assay used was quoted as <0.18pg/ml (compared to 7.2pg/ml in the current study). Levels ranging from 17pg/ml in control cell cultures, up to 165pg/ml in COPD cell cultures were reported. Work using primary type II alveolar epithelial cells however reported no detectable basal TNF-α release (LLD of assay 15.6pg/ml), though this was detectable after LPS stimulation (Thorley et al., 2007). Potential differences in cell culture conditions, sample harvest and storage, and the assay used may have affected the results of the current study. To further assess TNF-α levels use of a more sensitive assay could be investigated, or methods to concentrate the protein in the culture supernatant tried as discussed for BAL samples in Chapter 4.

In the context of this small pilot study it was not feasible to measure a wider range of mediators with the time and resources available. This is an area where the current model could be developed further, and alternative methods of assessing the "read out" from the model system considered. Additional cytokines and chemokines could be investigated; based on previous research in this area discussed in sections 1.9.1 and 1.9.2 these could include: GM-CSF (Hellermann et al., 2002), MCP-1 (Fuke et al., 2004), GRO-α (Schulz et al., 2004), and ICAM-1 (Takizawa et al., 2000). To address which TLRs may be involved in the findings described in the current model, individual TLR ligands could be used to compare to results observed with bacterial whole cell lysate. Use of TLR blocking antibodies or inhibitors in cultured cells could also be used to aid this investigation, with the potential to inhibit changes in cytokine levels (Mortaz et al., 2011, Nadigel et al., 2013).

Consideration of alternative methods

Alternative methods of analysing the immune response could also be implemented. TLR mRNA expression was measured at baseline in cultured epithelial cells; changes in

expression levels following CSE and/or HI treatment could be investigated. With potential for investigation of other important immune function genes, gene array could be used to assess a wider panel of mRNA expression in cultured cells at baseline and following treatment. This approach could allow an overview of potentially important mediators and receptors in the responses observed and this could then be further investigated at the protein level, similar to the approach used by Wang *et al* with investigation of TLR5 in SAEC (Wang et al., 2012). Expression of TLRs and important co-receptor molecules could be assessed at the protein level using western blot, or using immunocytochemistry to assess expression qualitatively, with particular interest in cellular localisation of TLR receptors (Guillot et al., 2004). Investigation of downstream TLR signalling pathways could follow on from this; activation can be measured by detection of phosphorylated protein kinases involved by western blot (*e.g.* p38, JNK, ERK, see Figure 2, Chapter 1) (Comer et al., 2013, Nadigel et al., 2013). Activation of NFκB can also be investigated using assays to analyse nuclear translocation based on p65 subunit levels in nuclear extracts (Comer et al., 2013, Moretto et al., 2009). Use of inhibitors to block the action of specific kinases can help to confirm which pathways are involved based on reduction in cytokine levels or complete loss of treatment effect (Nadigel et al., 2013).

The results presented here reflect cell responses from cells which have been cultured submerged under a layer of nutrient media. These cells are known to become de-differentiated compared to the broader population of ciliated and non-ciliated cells which are initially seeded from brushings collection (de Jong et al., 1994). Increasingly air-liquid interface (ALI) cell cultures are used to try to better represent the make-up of the airway epithelium *in-vivo*. Analysis of gene expression profiles in freshly isolated brushed airway epithelial cells, cells in submerged culture, and cells cultured at ALI have been performed (Pezzulo et al., 2011). This work has demonstrated that cells at ALI most closely reflect the "transcriptome" of the airway epithelium *in-vivo*. Previous work using PBECs in both submerged and ALI models have shown that although both baseline and stimulated cell TLR expression and mediator release may be of greater magnitude in cells at ALI, the same pattern of results was observed in cells in submerged culture (Comer et al., 2013, Wang et al., 2012). These models included

CSE, LPS and flagellin treatments. The current submerged culture model could be reviewed using ALI cultures to determine if the same effects are observed.

In considering relevance to the *in-vivo* disease process, further investigation could also use freshly brushed cells from large and small airways, and also biopsy samples, to analyse expression of mediators and receptors as close to the disease microenvironment as possible, *e.g.* using gene array, ICC or IHC. This would reduce the effect of *in-vitro* culture and help to direct research appropriately to what may be important in patients rather than in cells removed from the body. Endobronchial biopsies may be used from large airway but a comparable sample from small airways is not possible via bronchoscopy sampling; transbronchial lung biopsies can be performed but these sample lung parenchyma rather than the distal airways. Use of lung biopsy samples from explanted tissue could be considered, as described by Isajevs *et al* who investigated HDAC 2 and NFκB expression in large and small airways (Isajevs et al., 2011).

Chapter 7. Final discussion

Having discussed the results presented in each of the relevant chapters this final discussion will be used to review the study as a whole, consider the significance of the findings, and the direction in which this work could be taken in the future.

In this study, the role of bacterial presence causing airway inflammation in COPD was assessed using culture and culture-independent techniques to investigate the lung microbiome. Overall lower bacterial diversity was observed in COPD compared to control samples. In addition, a potential relationship between the richness and diversity of the bacteria identified in COPD samples and disease severity was identified. Potentially pathogenic bacteria were more commonly isolated from COPD samples. These results add to the increasing reports in the literature describing differences in the lung microbiome between health and disease. The potential impact of a change in the microbiome in the development and progression of COPD is of particular interest. This could be influenced by smoking, airway epithelial damage, and subsequent treatment including antibiotics and corticosteroids for exacerbations. Study of COPD disease phenotypes which may be associated with disease progression are an important target for new treatments. If we can better understand how the lung microbiome is maintained in harmony with the airway epithelium and innate immunity in health, and how this may change in disease, we may be able to find ways of restoring this balance to affect the natural course of the disease process.

The findings reported in the COPD samples in the current study however, did not translate into differences in inflammatory indices in BAL. The approach used was limited by the sample size, the semiquantitative nature of the method used for bacterial DNA analysis and also sampling from a single site in each subject. In planning future research in this area these elements of study design need to be taken into account. Future studies could involve increased numbers of patients from mild, moderate and severe disease categories, current and ex-smokers, in addition to controls. Concurrent methods of bacterial analysis including that applied here alongside a quantitative technique, and further measurements of inflammation would

potentially provide more informative results. In assessing the interplay between the microbiome and COPD pathogenesis, identifying a clinically detectable change in the microbiome which precedes progression of the disease and could be altered to affect patient outcomes would be of greatest significance. In order to investigate this, studies of the microbiome and possible associated inflammatory change, in different parts of the airway using different sampling techniques (*e.g.* BAL vs PSB) could advise which is the most relevant and accessible compartment to sample. Assessment of this longitudinally over time may then provide the best avenue for further exploration in this area.

Changes observed in the small and large airways in COPD, and the increasingly recognised relevance of respiratory pathogens in disease pathogenesis, were applied to an *in-vitro* cell culture model to investigate potentially relevant cellular responses. In support of the proposed hypothesis, the cell culture model developed in this study demonstrated an increased response from small compared to large airway epithelial cells exposed to bacterial PAMPs *in-vitro*. CSE appeared to have a synergistic effect with the bacterial whole cell lysate used in inducing greater release of inflammatory cytokines. The pattern of results observed was the same in cells from both COPD and control donors. This work was limited however, by small sample numbers, of particular relevance in the COPD results, where disease heterogeneity may have affected the variability observed.

This comparison of small and large airway epithelial cell responses is novel work which warrants further investigation to confirm and explore the findings described. Initially as for the microbiome work, this could involve investigation of larger numbers of paired cell sets from patients in different categories of disease severity compared to controls, to determine if changes in the small airway responses are observed between health and disease development. In order to demonstrate that this is the case *in-vivo*, with the loss of the influence of the local microenvironment and potential for change in cell phenotypes in *ex-vivo* cell cultures, first analysis of either freshly brushed airway epithelial cells or lung biopsies may be most informative. ALI cultures could also be developed, and consideration of co-culture models with other immune cell types

introduced to try to more accurately model the *in-vivo* disease process. Further mechanistic work would subsequently be required to understand the differences observed, with consideration of investigation of TLR expression and activation, and associated cell signalling pathways.

In addition to the above findings, limited work in the current study suggested resistance to the anti-inflammatory effects of corticosteroid treatment in both small and large airway epithelial cells in culture. Confirmation and further investigation of steroid responsiveness in ongoing *in-vitro* cell culture work could help to clarify this. Importantly *in-vitro* culture models can be used in *ex-vivo* analysis of drug targeting and testing. On the basis of the differences observed between small and large airway epithelial cells in the current study, further investigation and development of models focussing on SAEC may be of relevance in the advancement of novel therapeutic strategies for the treatment of COPD.

Much of the discussion of methods and results from the current study has highlighted the difficulties of investigating such a heterogeneous and complex disease process. In taking COPD research forwards, accurate phenotyping of patients including investment in new methods of assessing small airways disease, is required. Ongoing projects, such as those described above, are needed to try to understand the key factors involved in disease progression despite smoking cessation. If this can be achieved, the future could hold the possibility of the application of personalised medicine in this field. Patients could be treated depending on the relative contribution of small airways disease and features of the individual lung microbiome, with the translation of research findings into patient benefit.

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