

The University of Nottingham

The effect of cigarette smoke extract on macrophages

Abeer Sharaf

School of Life Sciences

Supervisors:

Dr. Ian Todd, Dr. Lucy Fairclough, Dr. Paddy Tighe

October 2018

Abstract

Chronic obstructive pulmonary disease (COPD) is a progressive respiratory disorder which causes permanent airway obstruction, and is one of the leading causes of mortality and morbidity around the world. It is characterised by irreversible obstruction of the airways, the development of chronic infections, exaggerated inflammatory responses leading to bronchitis and emphysema, and a progressive decline in respiratory function. Whilst many risk factors contribute to disease occurrence, cases commonly occur due to oxidative insults, particularly cigarette smoke, which can trigger an immune response and cause inflammation. 15-20 % of smokers are diagnosed with COPD, and the mechanisms underlying the development of COPD need to be investigated further.

Macrophages play a crucial role in the establishment of the immune response to pathogens and irritants within the body, and they play an important role in the progress and outcome of many diseases, including COPD. Current evidence suggests that irritants activate epithelial cells and macrophages (especially alveolar macrophages), which recruit other inflammatory cells to the lungs.

Some studies have suggested macrophages can cause a deleterious effect in COPD due to an increased number of alveolar macrophages with an M2 profile, which can result in increased tissue damage and fibrosis (Vlahos & Bozinovski 2014). As the relative roles of M1 and M2 macrophages are not fully understood in terms of COPD progression and severity, macrophages may be involved in both fighting and inducing the disease (Vlahos & Bozinovski 2014).

The hypothesis of this thesis was that studying the effect of cigarette smoke on macrophage function may promote understanding of the importance of macrophages in the pathogenesis of COPD. The initial experiments with the THP-1 cell line showed that these cells were not a suitable choice for investigating how macrophages are affected by cigarette smoke extract

(CSE) or nicotine, therefore, peripheral blood mononuclear cells (PBMCs) were chosen as a replacement. This thesis has established a procedure to generate macrophages from cluster of differentiation-14 ($CD14^+$) monocytes isolated by positive selection from PBMCs and stimulated to mature using macrophage colony stimulating factor (M-CSF). It has also shown the novel use of hexamethyldisilane (HMDS) for coating tissue culture plates, which facilitated the recovery of high numbers of cells with no effect on the expression of cell surface markers.

The investigation into the effects of different combinations of CSE, nicotine, polyinosinic-polycytidylic acid (Poly-IC) and lipopolysaccharide (LPS) on the expression of CD cell surface markers showed there was significant suppression of CD14 expression by nicotine with and without Poly-IC, and significant suppression of CD206 after treatment with nicotine only, Poly-IC, or LPS, when compared to the controls.

To further investigate the effects of these treatments on macrophages, the expression of markers that are key regulators of macrophage function and associate with either the M1 or the M2 macrophage phenotype were investigated in monocyte-derived macrophages by reverse-phase protein microarray (RPPA). The initial analysis of the RPPA data for the individual signalling molecules did not show any clearly significant changes associated with CSE or nicotine treatment, therefore further analysis was carried out following subtraction of all the relevant blank values from the corresponding values for the signalling molecules expression.

The results from this chapter show that RPPA detected differences in the markers selected: there were significant differences after 2 h treatment, with mainly overall down-regulation of the signalling molecules by treatment with CSE. Lesser effects were observed after 24 or 48 hours of treatment, suggesting some re-equilibration of the signalling molecule expression following the acute changes. However, the effects of nicotine on global expression of the signalling molecules was less consistent than that of CSE.

To complement the RPPA results, changes in the expression of interleukin-10 (IL-10) and tumour necrosis factor alpha (TNF-α) were investigated by ELISA. The results showed mainly suppression of IL-10 production by nicotine, whereas both CSE and nicotine enhanced production of TNF-α.

Overall, the most consistent findings from the RPPA data and the ELISA data are that CSE induced overall down-regulation of the signalling molecules at 2 hours, and that CSE up-regulated TNF-α production at 24 and 48 hours. Since the majority of the signalling molecules analysed were M2-related, and TNF-α is predominantly produced by M1 macrophages, these findings support the idea that CSE suppresses M2 function and favours M1 activation. Although the effects of CSE on the signalling molecules ameliorated by 24 hours of treatment, the early effects observed at 2 hours of treatment might trigger the intra-cellular processes resulting in TNF-α production at 24-48 hours.

With respect to cytokine production, nicotine again up-regulated TNF-α production in certain conditions, and down-regulated IL-10 production. As with CSE, this is also consistent with nicotine promoting a general shift towards M1 function.

This study indicates that CSE predominantly promotes M1 macrophage function. However, some of the results with CSE, and still more so with nicotine, also provide evidence for the promotion of M2 function in some conditions. These in vitro observations are consistent with in vivo findings that smoking differentially promotes M1 and M2 macrophage function in different lung compartments, or the generation of macrophages with a mixed M1/M2 phenotype.

Declaration

The work presented in this thesis was done between 2013 and the end of 2018 in the School of Life Sciences, Queen Medical Centre, University of Nottingham, UK. Apart from the help and advice mentioned in the Acknowledgments, the work described is totally my own. This thesis has not been previously submitted for any other degrees.

Abeer Sharaf

October 2018

Acknowledgments

First, I would like to thank Allah for all his gifts that surround me and make me the person I am today.

I would like to express my sincere gratitude to my supervisors Dr. Ian Todd, Dr. Paddy Tighe and Dr. Lucy Fairclough for their patience, encouragement and advice that they have provided throughout our supervisory meetings. I have been extremely lucky to have supervisors who cared so much about my work, and who responded to my questions and queries so promptly during this challenging and interesting project. Without them, this study would not have been completed, and I hope that the work I have done has reached their expectations.

I am also grateful to Dr. Luisa Martinez-Pomares and Dr. Sonali Singh from the School of Life Sciences, University of Nottingham, for providing me with protocols and supplying me with some reagents. In addition, I would like to thank Dr. David Onion in the Flow Cytometry Facility for his endless patience in teaching me the basics of flow cytometry – his help is appreciated and was really useful in this study.

I would also like to acknowledge the Saudi Ministry of Health who sponsored my scholarship, the Saudi Ministry of Higher Education, and the Saudi Cultural Bureau for all their support and cooperation.

I am greatly indebted to Dr. Hawazen Ismail, the head of T.B. Department at King Fahad General Hospital in Jeddah, who encouraged me to do my PhD in the UK as she did. I hope I have achieved what she wanted me to.

A big thanks to Dr. Nuha Al Khattabi, a previous Saudi PhD student in the research group, whose help and cooperation when I joined the group was invaluable in enabling me to stand on my feet quickly.

A special thanks to Dr. Ola Negm and Dr. Omar Ahmad who made their experience available to me. Their friendship and support are highly appreciated.

I would also like to thank Mr. Paul Radford who trained me in the tissue culture lab and Mr. Colin Nicholson who was always happy to give me a hand during the lab work. Many thanks to my lab mates in the Mechanisms, Biomarkers and Therapy of Inflammation (MBTI) Research Group for their help and explanations that kept me focused during these four years.

To all my colleagues, past and present, in the Saudi Ministry of Health, in King Abdul-Aziz University and in the School of Life Sciences in the University of Nottingham, in general – thank you for your ready help and camaraderie.

I am forever in debt to my parents for all their supplications, moral support, and the wonderful encouragement during my PhD. My thanks also extend to my siblings and their families.

It is with fond memories that I would like to thank my oldest uncle (Hussain Sabir), who sadly passed away during my PhD study; his emotional support and supplication during my study were greatly received and are sorrowfully missed, I wish he were still alive today to witness this joyous moment.

Last but not least, words cannot express my feelings and emotions towards my loyal husband (Waleed), who is always beside me, raising my belief and confidence in myself and helped me to make my dream come true. Thank you for your patience, and for sacrificing your money and life; I truly appreciate it. In addition, big hugs, thanks and love to my children (Mishary, Ghufran, Bader and Nouran), who showed patience throughout my study period and encouraged me to have a big name and be always at the top of my career ladder.

I hope I made all of you proud.

Abeer

Conferences and meetings attended

This work was presented in poster sessions in the following meetings:

- Nottingham and Leicester Joint Respiratory Day, Nottingham, 6th May 2015.
- The 9th Saudi Society Conference at the UK, Birmingham, 30-31 January 2016.
- International symposium, Development of tissue- and pathogen-specific cellular innate immunology- basic and translation aspects, Freiburg, Germany, 27 – 29 September 2017.
- BSI 2017 Congress, Brighton 4-7 December 2017.

Table of contents

Abstract ii
Declaration v
Acknowledgments vi
Conferences and meetings attended viii
Table of contents ix
List of figures xiv
List of tables xvii
Abbreviations xix
1. Introduction 1
1.1. General introduction 1
1.2. The History of COPD 3
1.3. Pathogenesis of COPD 3
1.3.1. The structure of the lungs 3
1.3.1.1. Importance of surfactants 4
1.3.2. Pulmonary function testing 5
1.4. COPD classification and severity 6
1.5. Diseases classified as COPD 7
1.5.1. Chronic bronchitis 7
1.5.2. Emphysema 8
1.6. Risk factors involved in the development of COPD 9
1.6.1. Working environment 9
1.6.2. Age and gender 10
1.6.3. Alpha-1 antitrypsin 10
1.6.4. Other genetic risk factors associated with COPD 11
1.6.5. Smoking cigarettes 12
1.7. The effect of COPD in the lungs 13
1.7.1. Inflammation 15
1.8. The role of cytokines in COPD 15
1.9. Epithelial cell co-ordinated response 19
1.9.1. Epithelial cells 19

1.9.2. Smooth muscle.....22
1.9.2.1. Smooth muscle in health and disease.....22
1.10. Macrophage co-ordinated response 23
1.10.1. The importance of macrophages.....23
1.10.2. Maintaining homeostasis of AMs and their activation in lung tissues 29
1.10.3. Role of AMs in innate host defence.....29
1.10.4. The role macrophages as antigen presenting cells.....31
1.10.5. Macrophage activation.....31
1.10.6. Downstream events of AMs activation.....36
1.11. Macrophages and pattern recognition receptors 40
1.11.1.1. The role of PRRs in COPD.....44
1.12. Involvement of other immune cells in COPD.....46
1.12.1. Neutrophils.....46
1.12.2. T cells.....46
1.12.3. Natural Killer Cells (NKC).....47
1.12.4. Dendritic cells (DCs) 48
1.12.5. B cells.....49
1.13. Management and treatment of COPD.....50
1.14. Modelling macrophages and COPD 52
1.14.1. Animal models of COPD.....52
1.14.2. An *in vitro* cell line model 53
1.14.3. Investigating the role of macrophages in COPD using Peripheral Blood Mononuclear Cells 53
1.14.4. Activation of AMs in model systems.....55
1.15. The effects of cigarette smoke extract (CSE) on macrophages 57
1.15.1. Contents of cigarette smoke extract.....61
1.15.2. Effect of cigarette smoke on immune function and other lung diseases 64
1.15.3. Effects of nicotine 66
1.16. Conclusions.....67
1.17. Aims and objectives.....68

2. Materials and methods.....70
2.1. Materials 70
2.2. Methods.....73
2.2.1. THP-1 cell culture.....73
2.2.2. Stimulating THP-1 cells with PMA.....73
2.2.3. Isolation of PBMC 74
2.2.4. Cell counting.....74
2.2.5. $CD14^+$ Monocyte magnetic separation 74
2.2.6. Checking the purity of $CD14^+$ monocytes 75
2.2.7. Staining for flow cytometry analysis 75
2.2.8. Flow cytometry 76
2.2.9. Coating culture plates with HMDS.....76
2.2.10. Generation of MΦs from $CD14^+$ monocytes 76
2.2.11. Cigarette smoke extract (CSE) preparation 77
2.2.12. Cigarette smoke stimulation 78
2.2.13. Preparation of Cell Lysates after stimulating MΦ with CSE and Nicotine to be prepared for time-course Reverse-Phase Protein Microarray (RPPA) experiments 78
2.2.14. Bicinchoninic acid (BCA) assay for protein concentration determination 81
2.2.15. Microarray set up 82
2.2.15.1. Denaturation of the protein lysate samples ready for printing....82
2.2.15.2. Printing the samples onto the slides.....83
2.2.15.3. Blocking the slide for microarray 83
2.2.15.4. Application of primary and secondary antibodies to the slide....84
2.2.16. Analysing microarray data.....85
2.2.17. ELISA of IL-10 and TNF-α expression.....86
2.2.18. Statistical analysis.....86
3. Investigation of the suitability of macrophages from the THP-1 cell line for use in studying COPD 88
3.1. Introduction.....88
3.2. Materials and methods 89

3.2.1. Staining for flow cytometry analysis 89
3.3. Results 89
3.3.1. Phenotyping THP-1 cells 89
3.3.2. Stimulation of THP-1 cell line 95
3.4. Discussion 104
3.4.1. Conclusions 106
4. Investigating the suitability of peripheral blood mononuclear cells as a model to study COPD 107
4.1. Introduction 107
4.1.1. Investigating the role of macrophages in COPD using Peripheral Blood Mononuclear Cells 107
4.2. Methods 109
4.2.1. Coating culture plate with Hexamethyldisilazane (HMDS) 109
4.3. Results 110
4.3.1. Checking the purity of $CD14^-$ cells 110
4.3.2. Checking the purity of $CD14^+$ cells 112
4.3.3. Culturing $CD14^+$ cells in different concentration of Hexamethyldisilazane (HMDS) 114
4.3.4. Phenotyping of macrophages on day 8 123
4.3.5. Median fluorescence intensity (MFI) and statistical analysis 130
4.4. Discussion 136
4.4.1. Conclusions 140
5. Analysis of differences in cell surface markers by RPPA 141
5.1. Introduction 141
5.2. Results 144
5.2.1. Analysis of RPPA after 2 hours incubation 144
5.2.2. Analysis of RPPA after 24 hours incubation 150
5.2.3. Analysis of RPPA after 48 hours incubation 155
5.2.4. Summary of RPPA results 161
5.2.5. ELISA of IL-10 and TNF-α 163
5.2.6. Analysis of ELISA results after 24 h incubation 164
5.2.7. Analysis of ELISA results after 48 h incubation 168

5.2.8. Summary of ELISA results 173
5.3. Discussion 175
5.3.1. Conclusions 179
6. General discussion 180
6.1. Discussion 180
6.1.1. Conclusions 190
6.1.2. Future work 192
7. References 194

List of figures

Figure 1.1: Effect of COPD on alveoli. 1
Figure 1.2: Pathogenesis of COPD. 8
Figure 1.3: Inflammatory and immune cells involved in COPD. 14
Figure 1.4: Scanning electron micrographs of cilia in alveoli showing reduced development. 20
Figure 1.5: Scanning electron micrographs of cilia in alveoli showing reduction in size. 21
Figure 1.6: Nomenclature, spatial organisation and role of the different human macrophage populations. 28
Figure 1.7: Macrophage activation and polarisation. 34
Figure 1.8: AMs in COPD. 61

Figure 2.1: Set up of the apparatus to produce the CSE. 78
Figure 2.2: Timeline for the preparation of cell lysates. 80
Figure 2.3: Summary of the stages of microarray set up. 82
Figure 2.4: Summary of the microarray process of applying antibodies, detection and data analysis. 85

Figure 3.1: Flow cytometric analysis of surface Monocyte CD Markers on THP-1 cells. 91
Figure 3.2: Flow cytometric analysis of surface cells macrophage CD14 and CD44 markers on THP-1 cells. 92
Figure 3.3: Flow cytometric analysis of surface cells macrophage CD11b and CD36 markers on THP-1 cells. 92
Figure 3.4: Mean MFI and percent positive of THP-1 monocyte markers. 93
Figure 3.5: Flow cytometric analysis of surface markers CD69, CD45RA, and CD69 on THP-1 cells. 94
Figure 3.6: Mean MFI and percent positive of THP-1 macrophage markers. 95
Figure 3.7: Flow cytometric analysis of surface monocyte CD markers after stimulation with 50 pg/ml PMA 96

Figure 3.8: Flow cytometric analysis of surface macrophage CD markers stimulating with 50 pg/ml PMA 97
Figure 3.9: Flow cytometric analysis of CD49a expression after increasing PMA stimulation 98
Figure 3.10: Flow cytometric analysis of CD14 expression after increasing PMA stimulation 99
Figure 3.11: Flow cytometric analysis of CD36 expression after increasing PMA stimulation 100
Figure 3.12: Flow cytometric analysis of CD11b expression after increasing PMA stimulation 101
Figure 3.13: Mean MFI of THP-1 macrophage markers after treatment with PMA. 103
Figure 4.1: Testing the purity of CD14 negative cells on day 0 111
Figure 4.2: Testing the purity of CD14 positive cells on day 0 113
Figure 4.3: Macrophages CD markers on day 7, grown on plates not coated with HMDS 115
Figure 4.4: Macrophages CD markers on day 7, grown on plates coated with 5 % HMDS in 70 % ethanol 116
Figure 4.5: Macrophages CD markers on day 7, grown on plates coated with 1 % HMDS in 100 % ethanol 117
Figure 4.6: Macrophages CD markers on day 7, grown on plates coated with 3% HMDS in 100 % ethanol 118
Figure 4.7: Macrophages CD markers on day 7, grown on plates coated with 5 % HMDS in 100 % ethanol 119
Figure 4.8: Macrophages CD markers on day 7, grown on plates coated with 1 % HMDS in 100 % propanol 120
Figure 4.9: Macrophages CD markers on day 7, grown on plates coated with 3 % HMDS in 100 % propanol 121
Figure 4.10: Macrophages CD markers on day 7, grown on plates coated with 5 % HMDS in 100 % propanol 122

Figure 4.11: The number of macrophages collected under the different culture conditions....123
Figure 4.12: CSE extract standard curve. 124
Figure 4.13: Analysis of the controls for expression of all the markers on day 8 after stimulation 125
Figure 4.14: Expression of CD14 measured by flow cytometry for all treatments on day 8 126
Figure 4.15: Expression of CD16 measured by flow cytometry for all treatments on day 8 127
Figure 4.16 Expression of CD206 measured by flow cytometry for all treatments on day 8 128
Figure 4.17: Expression of CD11b measured by flow cytometry for all treatments on day 8 129
Figure 4.18: The MFI of macrophages for all the cell surface markers on day 8, with Poly-IC and LPS only 130
Figure 4.19: The mean MFI of the marker CD14 for all the treatments on day 8....131
Figure 4.20: The mean MFI of the marker CD16 for all the treatments on day 8....132
Figure 4.21: The mean MFI of the marker CD206 for all the treatments on day 8....133
Figure 4.22: The mean MFI of the marker CD11b for all the treatments on day 8....134
Figure 4.23: The mean MFI of CD14, CD16, CD206 and CD11b for all the treatments on day 8 135
Figure 5.1: RPPA of median values for the after 2 h incubation with only Poly-IC or LPS……..146
Figure 5.2: RPPA of median values for all markers after 2 h incubation with CSE +/- Poly-IC and LPS. 148
Figure 5.3: RPPA of median values for all markers after 2 h incubation with nicotine +/- Poly-IC and LPS....150

Figure 5.4: RPPA of median values for the after 24 h incubation with only Poly-IC or LPS.....151
Figure 5.5: RPPA of median values for all markers after 24 h incubation with CSE +/- Poly-IC and LPS. 153
Figure 5.6: RPPA of median values for all markers after 24 h incubation with nicotine +/- Poly-IC and LPS. 155
Figure 5.7: RPPA of median values for all makers after 48 h incubation with only Poly-IC or LPS. 156
Figure 5.8: RPPA of median values for all markers after 48 h incubation with CSE +/- Poly-IC and LPS. 158
Figure 5.9: RPPA of median values for all markers after 48 h incubation with CSE +/- Poly-IC and LPS. 160
Figure 5.10: ELISA standard curves for a) IL-10 and b) TNF-α.....164
Figure 5.11: ELISA of cytokine expression after 24 h incubation of macrophages with or without Poly-IC or LPS. 165
Figure 5.12: ELISA of IL-10 expression after 24 h incubation with all treatment conditions. 166
Figure 5.13: ELISA of TNF-α expression after 24 h incubation with all treatment conditions. 167
Figure 5.14: ELISA of cytokine expression after 48 h incubation of macrophages with or without Poly-IC or LPS. 168
Figure 5.15: ELISA of IL-10 expression after 48 h incubation with all treatment conditions. 170
Figure 5.16: ELISA of TNF-α expression after 48 h incubation with all treatment conditions. 172

List of tables

Table 1.1: Summary of COPD different stages. 6
Table 1.2: The classification of cytokines in COPD.....16
Table 1.3: locations and functions of different tissue macrophages. 26
Table 1.4: Macrophage polarisation: classically activated (M1) and alternatively activated (M2) subset phenotypes. 36

Table 1.5: Characteristics of Human TLR and molecules involved in signalling.....42
Table 1.6: The mediators and cytokines that are released by macrophages when exposed to CSE. 58
Table 1.7: List of hazardous tobacco smoke components with their cancer and non-cancer inhalation risk values.....62

Table 2.1: Buffers used during the generation and phenotyping of macrophages (MΦs) and ELISA……70
Table 2.2: Antibodies used for flow cytometry staining of Macrophages. 72
Table 2.3: Antibodies used for ELISA of Macrophages.....72
Table 2.4: Stimuli used for Macrophages. 73
Table 2.5: Preparation of the BCA standards. 81
Table 2.6: Details of the antibodies used in microarray. 84

Table 3.1: Summary of expression of selected cell surface markers on unstimulated and PMA-stimulated THP-1 cells…….90

Table 5.1: Effects of stimulation of monocyte-derived macrophages with Poly-IC or LPS on the overall expression of the signalling molecules investigated by RPPA, compared to unstimulated macrophages ('Nil')..162

Table 5.2: Effects of CSE and nicotine on the overall expression of signalling molecules investigated by RPPA in monocyte-derived macrophages, either unstimulated or stimulated with Poly-IC or LPS…163

Table 5.3: Effects of stimulation of monocyte-derived macrophages with Poly-IC or LPS on the expression of the cytokines investigated by ELISA, compared to unstimulated macrophages ('Nil')……174

Table 5.4: Effects of CSE and nicotine on the expression of the cytokines investigated by ELISA in monocyte-derived macrophages, either unstimulated or stimulated with Poly-IC or LPS……..175

Abbreviations

AECs	Alveolar epithelial cells
AIM	Apoptosis inhibitor of macrophage
AM	Alveolar macrophages
ANOVA	Analysis of variance
APC	Antigen presenting cells
ATP	Adenosine triphosphate
B.D.	Becton-Dickinson Bioscience Company
BAL	Broncho-alveolar lavage
BALF	Broncho alveolar lavage fluid
Bcl-xL	B-cell lymphoma extra-large
BiP	Binding immunoglobulin protein
bmDCs	Bone marrow-derived DCs
BMP-7	Bone morphogenetic protein 7
cAMP	cyclic adenosine monophosphate
CCR2	Chemokine receptor 2
CD	Cluster of differentiation
Chi3l3	Chitinase-3-like protein-3
CHRNA3	Cholinergic receptor nicotinic alpha 3 subunit
CLE	Central lobular emphysema
CLRs	C -type lectin receptors
CO_2	Carbon dioxide
COPD	Chronic Obstructive Pulmonary Disease
COX-2	Cyclooxygenase-2
CpG	Cytidine-phosphate guanosine

CS	Cigarette smoke
CSE	Cigarette Smoke Extract
CSE-N	The nicotine component of CSE
CSE-O	CSE without nicotine
CXCL1	CXC-chemokine ligand 1
CXCL10	IP- 10 (inducing protein-10)
DAMPs	Danger-associated molecular patterns
DCs	Dendritic cells
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin 1
DMSO	Dimethyl sulfoxide
DN	Double negative
DP	Double positive
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FABP4	Fatty acid binding protein 4
FBS	Foetal bovine serum
FEF	Forced expiratory flow
FEV1	Forced expired vital capacity in 1 second
FITC	Fluorescein isothiocyanate
fluro-1	Fluorescence minus one

FRET	Förster resonance energy transfer
FVC	Forced vital capacity
GAL	GenePix array list
GCs	Glucocorticoids
GIPL	Glycoinositolphospholipid
GM-CSF	Granulocyte-macrophage colony stimulating factor
GOLD	Global Initiative on Obstructive Lung Disease
GROa	Growth regulated oncogene-alpha
H_2O_2	Hydrogen peroxide
HA	Hyaluronic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF	Hypoxia inducible factors
HMDS	Hexamethyldisilane
HMGB1	High-mobility group box 1 protein
HRP	Horseradish peroxidase
HSP	Heat shock proteins
ICs	Immune complexes
IFN-ß	Interferon-beta
IFN-α	Interferon-alpha
IFN-γ	Interferon-gamma
IL	Interleukin
IL1-ra	IL-1 receptor antagonist
iNOS	Nitric oxide synthase
IP- 10	inducing protein-10
iPSCs	Induced pluripotent stem cells

IREB	Iron regulatory binding protein
IRF	Interferon Regulatory Factors
KLF4	Krüppel-like factor 4
LABA	Long acting B2-agonist
LAMA	Long acting muscarinic antagonist
LBP	LPS-binding protein
LDL	Low-density lipoprotein
LGP2	Laboratory of genetics and physiology 2
LIF	Leukocyte inhibitory factor
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LTB4	Leukotriene B4
MAL	MyD88-adaptor-like protein
MAMPs	Microbe-associated molecular patterns
MAO	Monoamine oxidase
MAPK	Mitogen-activated protein kinase
MBL	Mannan-binding lectin
MCMs	Medullary cord macrophages
MCP-1	monocyte chemotactic protein-1
M-CSF	Macrophage colony stimulating factor
Mda5	Melanoma differentiation-associated protein 5
mDCs	Myeloid DCs
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MIP-1α	Macrophage inflammatory protein-1α

miRs	MicroRNAs
MMPs	Matrix-metallo proteinases
MMR	Macrophage mannose receptor
moDCs	Monocyte-derived DCs
MR (CD206)	Mannose receptor
MSDS	β-mercaptoethanol sodium dodecyl sulphate
MSMs	Medullary sinus macrophages
MVV	Maximal voluntary ventilation
MyD88	Myeloid differentiation primary response protein 88
MΦ	Macrophage
nAChRs	Nicotinic acetylcholine receptors
NaF	Sodium fluoride
$NaVO_3$	Sodium metavanadate
NCR	Natural cytotoxicity receptors
NF-κB	Nuclear factor-κB
NHANES	National Health and Nutrition Examination Survey
NK	Natural killer cells
NLRP	NLR family pyrin domain-containing
NLRs	NOD-like receptors
NOD	Nucleotide-binding oligomerisation domain
NOS3	Nitric oxide synthase 3
O_2	Oxygen
p38 MAPKs	P38 MAP kinases
PAMPs	Pathogen-associated molecular patterns
PBA	Phosphate buffered saline

PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate buffered saline
PBS*T	Phosphate buffered saline and Tween 20
PC	Phosphatidylcholine
PCNA	Proliferating cell nuclear antigen
pDCs	Plasmacytoid DCs
PDEs	Phosphodiesterases
PDI	Protein disulphide isomerase
PFT	Pulmonary function testing
PKC	Protein kinase C
PLE	Panlobular emphysema
PMA	Phorbol 12-myristate 13-acetate
Poly-IC	Polyinosinic-polycytidylic acid
PPARγ	Peroxisome proliferator-activated receptor gamma
RAGE	Receptors for advanced glycation end-products
Relm-alpha	Resistin-like molecule-alpha
RIG	Retinoic acid-inducible gene
RIPA	Radioimmunoprecipitation assay buffer
RLRs	RIG-I-like receptors
rMFI	Relative mean fluorescence intensity
RORC2	Retinoic orphan receptor γ t, also known as RORγ t
RORγ t	Retinoic orphan receptor γ t, also known as RORC2
ROS	Reactive oxygen species
RPPA	Reverse-Phase Protein Microarray
SAA	Serum amyloid A

SLPI	Secretory leuko-protease inhibitor
SNP	Single nucleotide polymorphisms
SP	Single positive
SR	Scavenger receptor
SSA	Serum amyloid A
SSMs	Subcapsular sinus macrophages
STAT6	Signal transducer and activator of transcription 6
SuP	Surfactant protein
TAMs	Tumour-associated macrophages
TCRs	T-cell receptors
TGFβ	Transforming growth factor beta
TGM2	Transglutaminase 2
tGPI-mucin	Glycosylphosphatidylinositol-anchored mucin like glycoprotein from *Trypanosoma cruzi* trypomastigotes
Th	T helper cell
Th1	T helper 1
Th17	T helper 17
Th2	T helper 2
THP-1	Human leukaemia monocytic cell line
TICAM1	TIR-domain-containing molecule 1
TICAM2	TRIF-related adaptor molecule
TIR	Toll/IL-1 receptor
TIRAP	TIR-domain-containing adaptor protein
TLR	Toll-like receptor

TNF-α	Tumour Necrosis Factor alpha
TPA	Phorbol 12-myristate 13-acetate
TRAM	TRIF-related adaptor molecule
Treg	T regulatory cells
TRIF	TIR-domain-containing adaptor protein inducing IFN-β
UK	United Kingdom
UPR	Unfolded protein response
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organisation
WR	Working reagent

1. Introduction

1.1. General introduction

Chronic obstructive pulmonary disease (COPD) is a progressive respiratory tract disease which causes a reduction in air flow during breathing (Figure 1.1), and it can result in coughing (with large amounts of mucus), wheezing, shortness of breath, chest tightness, and other symptoms. COPD is characterised by lung abnormalities like chronic bronchitis and/or emphysema (Snider, 1989). The leading cause of COPD is cigarette smoke (CS), but long-term exposure to other lung irritants (air pollution, chemical fumes or dust) can also contribute (Barnes, 2008a).

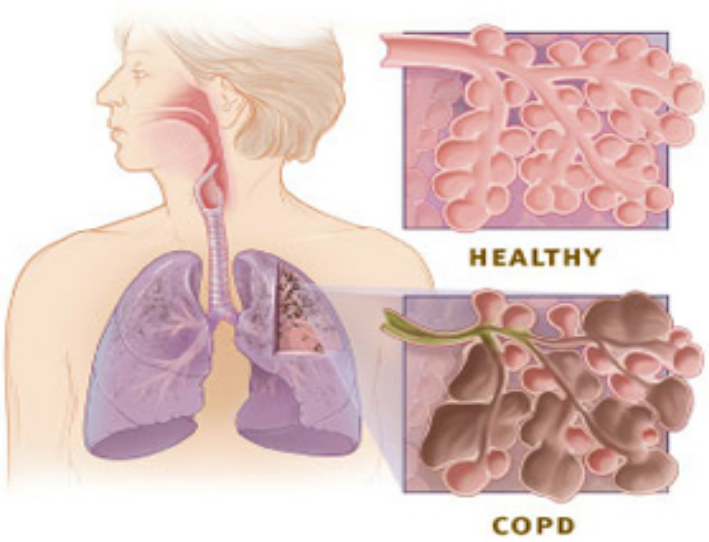


Figure 1.1: Effect of COPD on alveoli.

A schematic representation showing the difference between healthy and diseased alveoli. In healthy airways, the lungs are elastic and bounce back to their original shape after being stretched, which helps to retain the normal structure and to move the air quickly in and out. In people with COPD, the air sacs no longer bounce back to their original shape (brown areas). They can become swollen or thicker than normal and mucus production can increase, all of which can lead to blocked or obstructed airways making it even harder to move air out of the lungs. Diagram reproduced from http://www.nhlbi.nih.gov/health/public/lung/copd/what-is-copd/how-does-copd-affect-breathing.htm.

The impact of developing COPD (such as increased morbidity and mortality, healthcare costs and the degree of pain suffered by patients) are all motives for making this disease a key focus of scientific research interest (Agusti, 2003). COPD is one of the leading causes of chronic morbidity and mortality worldwide and the prevalence is still increasing (Kotz *et al.*, 2014). In the United States, there are around 24 million adults are affected by COPD – diagnosed and undiagnosed), and around 900,000 people in the United Kingdom (UK) have been diagnosed as COPD patients (Shahab *et al.*, 2006), with a total of 64 million people affected worldwide (Rab *et al.*, 2013). The World Health Organisation (WHO) estimated that, in 2012, about 3.1 million people died from COPD across the world (5.6 % of all deaths), and, as the prevalence is increasing, this figure is expected to rise in the future (WHO, 2014).

Both the genetic makeup of an individual and the environmental factors they are exposed to are involved in determining how the disease will progress. For example, cigarette smoke is considered as one of the main causes of COPD, even though only 15-20 % of smokers develop the disease (Joos, Pare and Sandford, 2002). Indeed, many studies have revealed, from comparisons between smokers and non-smokers, that cigarette smoking is the main reason for the progression of the disease (WHO, 2004). It has been shown that lung cells in smokers age faster than in non-smokers (Parkes *et al.*, 2008). Consequently, this might lead to inappropriate stimulation of the immune system, which might lead to even more severe forms of COPD as a result of toxic compounds being released (Taylor, 2010).

Recent research has indicated that the immune system plays an important role in the progression of COPD, with different immune cells being involved to a greater or lesser extent to produce the observed inflammation (Barnes, 2008a; Vlahos and Bozinovski, 2014). Within the lung, macrophages – and more specifically alveolar macrophages (AMs) – are of particular interest as they are an important cellular component of the innate immune system, play a significant role in the identification of microbes, and immunogens, and they help to organise inflammatory responses (Pappas *et al.*, 2013). The macrophages react with soluble proteins that bind microbial products to remove particles and pathogens, which helps to keep the airway tract clear (Pappas *et al.*, 2013). Macrophages can also orchestrate inflammation by releasing chemokines that attract

neutrophils, monocytes and T cells, and also by secreting several proteases (Pappas *et al.*, 2013).

1.2. The History of COPD

Historically, COPD has been known under different names; for example in 1679 the Swiss physician Charles Bonet called it 'voluminous lungs' (Bonet, 1679); in 1769 the Italian anatomist Giovanni Morgagni reported 19 cases of 'turgid', which were later recognised as having typical COPD symptoms (Morgagni and Johnson, 1769); in 1814, bronchitis and chronic bronchitis were identified as disabling health conditions by the British physician Charles Badham, who also introduced the term 'catarrh' (a chronic inflammation of the mucous membrane) (Badham, 1814) and, in 1821, emphysema (which is a component of COPD) was identified by the physician René Laennec (Laennec, 1821). In 1959, a "Ciba Symposium" defined the components for the identification and diagnosis of COPD as it is known today (Ciba Guest Symposium., 1959). However, COPD was still referred to as "chronic airflow obstruction" and "chronic obstructive lung disease" until Dr William Briscoe defined the term COPD at the 9th Aspen Emphysema Conference (Briscoe and Nash, 1965).

Throughout the early 18^{th} and 19^{th} century environmental and genetic factors were thought to be the key factors of the development of this disease, whereas today smoking, combined with other factors, is recognised as one of the primary causes of COPD (Kotz *et al.*, 2014).

1.3. Pathogenesis of COPD

1.3.1. The structure of the lungs

The lungs are organs essential for respiration in mammals and they are located near the backbone on either side of the heart. Their principal function is to transport oxygen into the bloodstream and to release carbon dioxide into the atmosphere, and this is driven by the musculoskeletal system (Maton, 1993). Deoxygenated blood is pumped through the pulmonary artery to the lungs, where oxygen diffuses in and is exchanged for carbon dioxide in the haemoglobin of the erythrocytes. The oxygen-rich blood then returns to the heart via the pulmonary veins (Maton, 1993). Within the lungs, air enters via

bronchi, which progressively subdivide into a system of bronchioles, alveolar sacs and finally alveoli (lung parenchyma), which are wrapped in blood vessels and are where the gas exchange takes place (Mandel and Cockrill, 2013). Each lung is separated into lobes by fissures, with three lobes on the right and two on the left, and these lobes are further divided into segments and then into lobules (Maton, 1993). Each lobe is surrounded by a pleural cavity consisting of two pleurae (parietal and the visceral pleura) with pleural fluid in between the two. The pleural cavity helps to lubricate the lungs and provides surface tension to keep the lung surface in contact with the rib cage (Maton, 1993). Lungs have a tremendous reserve volume when compared to the oxygen exchange requirements when at rest, as only a small portion of the lungs are actually perfused with blood for gas exchange (Mandel and Cockrill, 2013). This excess capacity is one of the reasons that individuals with damage due to smoking, such as COPD (Figure 1.2B), do not have a noticeable decrease in lung function while moving slowly or resting. During exercise, oxygen requirements increase leading to a greater volume of the lungs being used, which allows the body to match its CO_2/O_2 exchange requirements (Mandel and Cockrill, 2013). Destruction of a larger number of alveoli over time leads to emphysema and extreme shortness of breath (Mandel and Cockrill, 2013). Many respiratory illnesses are the result of bacterial or viral infections due to the moist environment, and can lead to inflammation of the lungs (pneumonia) or the pleura (pleurisy) (Mandel and Cockrill, 2013). Diseases of the pulmonary system can be assessed using a spirometer and a pulmonary function test.

1.3.1.1. Importance of surfactants

Within the lungs, surfactants play a key role at the pulmonary air-liquid interface by reducing surface tension, allowing efficient gas exchange, helping maintain the alveoli structural integrity and defence (Bernhard, 2016; Tschernig *et al*., 2016; Akella & Deshpande, 2013). Lung surfactant produced by alveolar type-II cells is made up of a unique combination of proteins and phospholipid, with about 80 % phosphatidylcholine (PC) (made up of palmitoyl-myristoyl-PC, palmitoyl-palmitoleoyl-PC and dipalmitoyl-PC), about 10 % cholesterol, about 10 % anionic phosphatidylglycerol, and 2-5 % surfactant proteins SuP-A to -D (Bernhard, 2016; Akella & Deshpande, 2013). Within the surfactant, lipid-protein interactions play a key role in the structural organization

and function of the surfactant monolayer, as any changes to surfactant biophysical properties or homeostasis can cause insufficiency in the surfactant, which can lead to diseases including chronic lung diseases, lung proteinosis, respiratory distress syndrome and interstitial lung disease (Akella & Deshpande, 2013). Studies have shown that mutations in the genes that regulate surfactant homeostasis can be linked to lung disease (Whitsett *et al*., 2015).

The alveolar type II cells also play an important role after lung injury; they proliferate to restore the epithelium, they clear alveolar fluid and are involved in the innate immune response to inhaled materials (Mason, 2006). In defending the alveoli, the type II cells work with the surfactant film, the alveolar fluid, the type I cells and alveolar macrophages, and they can help regulate lipogenesis through the transcription factor sterol regulatory element binding protein-1c (Mason, 2006).

The surfactant proteins have been shown to play a role in the innate immune response and can act by phagocytosis and help with the clearance of microorganisms and particles (Tschernig *et al*., 2016). SuP A and D are collectins; they are members of a sub-group of C type lectins that are involved in different phagocytosis processes, they act by forming oligomers that have a similar structure to mannose binding lectin, which recognise carbohydrates on bacteria and other pathogens and bind to them (Tschernig *et al*., 2016).

1.3.2. Pulmonary function testing

Pulmonary Function Testing (PFT) is a complete evaluation of the respiratory system including the patient history, a physical examination, chest x-rays, arterial blood gas analysis, and tests of pulmonary function using a spirometer. These tests can help to identify the severity of pulmonary impairment and, in addition, can be used for finding the cause for shortness of breath, assessing the effects of contaminants on lung functions, and progress for disease treatment (Reynolds, 2011).

The Spirometer was invented in 1846 by John Hutchinson to measure the vital lung capacity (Hutchinson, 1846). This device was built upon by Robert Tiffeneau, a French pioneer of respiratory medicine, to create a complete diagnostic instrument for COPD which is still an essential tool for diagnosis today (Tiffeneau, 1947).

During diagnosis, a spirometer takes different measurements: the volume of air that can be breathed out in one second (forced expiratory volume in 1 s or FEV1), the total amount of air breathed out (forced vital capacity or FVC), the volume of air exhaled under forced conditions (forced expiratory flow FEF), forced inspiratory flow rates (FIFs), and the volume of air expired in a specified period during repetitive maximal effort (maximal voluntary ventilation MVV), which are plotted on a spirogram. The process is repeated a few times to get a consistent reading, then the readings are compared with normal measurements (based on age) which can show if the airways are obstructed or restricted (Reynolds, 2011). There are different types of spirometer, which use different methods for measurement, for example a pneumotachometer measures the flow rate of gases by detecting pressure differences across the fine mesh, and electronic spirometers can measure airflow rates using ultrasonic transducers, or by measuring pressure differences in the channel (Reynolds, 2011).

1.4. COPD classification and severity

The severity and classification of COPD is determined by measuring FEV1 and its ratio to the FVC (Hogg, 2008). COPD can be classified into five stages according to the Global Initiative on Obstructive Lung Disease (GOLD) (.

Table 1.1).

Table 1.1: Summary of COPD different stages.

FEV1 = Forced Expired Vital capacity in 1 second, FEC = Forced Vital Capacity (Hogg, 2008).

Stage	Definition
Stage 0	To be at risk, where a subject experiences chronic cough and sputum.
Stage I Mild COPD	FEV1/FVC < 70% • FEV1 ≥ 80%
Stage II Moderate COPD	FEV1/FVC < 70% • 50% ≤ FEV1 < 80%
Stage III Severe COPD	FEV1/FVC < 70% • 30% ≤ FEV1 < 50%
Stage IV Very severe COPD	FEV1/FVC < 70% • FEV1 < 30% predicted or FEV1 < 50%

1.5. Diseases classified as COPD

There are three types of diseases that are covered by the COPD definition: chronic bronchitis, emphysema and asthmatic bronchitis, all of which can lead to serious health issues and, if untreated, eventually death (Murarescu, Mitrofan and Mihailovici, 2007). COPD patients suffer from chronic infections, exaggerated inflammatory responses, have a progressive decline in respiratory function, and show symptoms of protease imbalance (Rab *et al.*, 2013). Many patients with COPD have all the pathologic conditions (chronic obstructive bronchiolitis, emphysema, and mucus plugging), but the relative extent of each can vary (Vlahos and Bozinovski, 2014).

1.5.1. Chronic bronchitis

Approximately two-thirds of patients with COPD exhibit chronic bronchitis, which is diagnosed after the patient has a continuous cough for over 3 months that occurs over 2 successive years (Murarescu, Mitrofan and Mihailovici, 2007) and leads to the formation of a chronic, persistent cough (Sutherland and Martin, 2003). Alongside the persistent cough, chronic bronchitis symptoms include mucus accumulation (Figure 1.2), chronic bacterial infections, and persistent neutrophilic airway inflammation (Rab *et al.*, 2013). Mucus hypersecretion and stasis can increase bacterial infections and the subsequent inflammatory response and are therefore considered a major contributor to lung function decline and dyspnoea (Rab *et al.*, 2013). Studies have shown that the inflammation in chronic bronchitis can affect the mucosal layer and the associated glands and ducts in the airway that are less than 2 mm in diameter, which leads to a further narrowing of the airways and subsequent airflow limitation (Hogg, 2008).

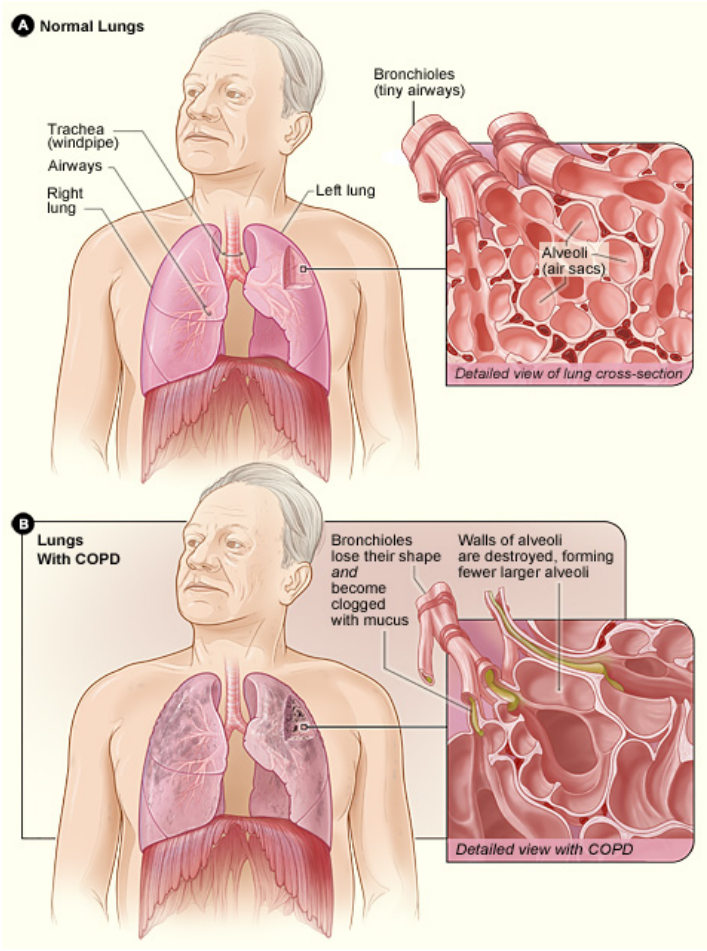


Figure 1.2: Pathogenesis of COPD.

A) The structure and location of the lungs in the body, with a detailed cross-section showing normal bronchioles and alveoli. The alveoli are the microscopic blood vessel-lined sacks in which oxygen and carbon dioxide gas are exchanged. B) The effect of COPD on the lungs, specifically the damaged bronchioles and alveolar walls which are no longer elastic and can be filled with mucus. Reproduced from: http://www.nhlbi.nih.gov/health/health-topics/topics/copd/

1.5.2. Emphysema

Emphysema describes the abnormal enlargement of the lung alveoli (Figure 1.2), thereby causing destruction to their walls without detectable fibrosis (Snider, 1989). Most COPD patients present a combination of emphysema and inflammation with a relatively high incidence of bronchiectasis (Rab *et al.*, 2013). COPD patients with emphysema but without chronic bronchitis can display mucus obstruction in the small airways, which can lead to an increase in bacterial infections and an altered lung tissue

microbiome (Rab *et al.*, 2013). Emphysema has two types that have been studied extensively: centrilobular emphysema (CLE) and panlobular emphysema (PLE). In CLE lung alveolar destruction occurs in the upper lobes of the lungs, which has been shown to be as a result of cigarette smoking, whereas PLE leads to the destruction of the lower lobes of the lungs (Anderson and Foraker, 1973).

The pathogenesis of emphysema results from the inflammation which recruits immune cells, such as neutrophils and macrophages, to the damaged area where they respond by releasing proteases, for example neutrophil elastase (Hogg and Senior, 2002). The proteases digest components of the extra-cellular matrix in the wall of the alveoli, which leads to alveolar destruction and enlargement (Hogg and Senior, 2002).

1.6. Risk factors involved in the development of COPD

COPD is regarded as a complex disease with contributions from both genetic and environmental factors, but the leading risk factor is cigarette smoke (Rab *et al.*, 2013). Occupational exposure to dust or chemical fumes causes 15–20 % of the cases of COPD, and genetic factors such as mutations in the alpha 1-antitrypsin gene cause about 3 % of all cases of COPD (Rab *et al.*, 2013). The different factors involved can affect the development and severity of COPD.

1.6.1. Working environment

Working in clean areas and using eco-friendly products can help reduce the incidence of COPD. The repeated exposure of factory workers to air polluted with chemicals, irritants, fumes, metal or dust increases the risk of developing the disease when compared to people who work in eco-friendly environments (Fishwick, Barber and Darby, 2010). Air pollutant exposure (such as dust and chemicals, which lead to airway hyper-responsiveness) can occur in two different environments: a) outdoors and b) indoors, where the pollutants accumulate due to lack of ventilation (Fishwick, Barber and Darby, 2010). The exposure to these irritants can increase the severity of COPD, especially if combined with other factors such as cigarette smoke, which in turn can lead to increases in mortality (Fishwick, Barber and Darby, 2010).

1.6.2. Age and gender

The ability and the efficiency of the immune system to eliminate infection decreases gradually with age, which means that, as people become older, they are more susceptible to developing COPD as their immune systems cannot clear lung infections, which can lead to bronchitis and emphysema. Research by Fukuchi (2009) showed patients in their 60s develop COPD at a higher rate compared to those in their 40s due to the lack of the effectiveness of the immune system, where the activity of lymphocytes and some immune signalling pathways become impaired (Fukuchi, 2009). Research has also shown that some older patients lose some important factors which are involved in immune defence mechanisms, for example there is a reduction in expression of the anti-inflammatory protein SIRT-1, which leads to increased levels of inflammation after exposure to irritants or infection, which are common triggers for COPD (Taylor, 2010). The increase in COPD can also occur due to a reduction in muscle strength in elderly people. This can mean they have a reduced ability to clear build-up of mucus in the lungs by coughing, which in turn increases infections by bacteria and viruses which can cause inflammation and the formation of bronchitis (Taylor, 2010).

Research indicates gender can be a risk factor for the development of COPD in the general population, with males having a slightly higher incidence than females (De Torres *et al.*, 2005; Davis *et al.*, 2013). Other research shows that gender only correlates to COPD development when it is related to smoking status (Massaro and Massaro, 2004; De Torres *et al.*, 2006). For example, in the NHANES III database, airflow obstruction of current smokers was present in 14.2 % of white males and 13.6 % white females (Tamimi, Serdarevic and Hanania, 2012). The larger risk factor is smoking rather than gender, as male smokers are 12 times more likely to die from COPD when compared to non-smoking men (Rab *et al.*, 2013).

1.6.3. Alpha-1 antitrypsin

One of the key genetic factors to have a direct effect on the pathogenesis of emphysema is alpha-1 antitrypsin deficiency (Abboud and Vimalanathan, 2008). Alpha-1 antitrypsin works by inhibiting neutrophil elastase. Neutrophil elastase is a protease which is activated by inflammation, it causes destruction of alveoli walls and can also

increase mucus retention, which are both important factors in the development of COPD (Rab *et al.*, 2013). If the patient is deficient in alpha-1 antitrypsin, then the activity of neutrophil elastase is not controlled, which can lead to the increased destruction of lung alveoli (Rab *et al.*, 2013). Alpha 1-antitrypsin gene mutations can also exacerbate the progression of COPD in smokers, as there is an increase in inflammation due to the cigarette smoke, which causes increased levels of proteases to be produced and this is not counteracted by alpha-1 antitrypsin activity (Rab *et al.*, 2013). There is some evidence that when people smoke they are unable to produce alpha-1 antitrypsin (MacNee, 2005), and that cigarette smoke contributes to an anti-protease imbalance, allowing for the formation of COPD due to excessive protease activity (Wanner, 2009).

1.6.4. Other genetic risk factors associated with COPD

Currently, the only proven genetic risk factor for COPD is alpha-1 antitrypsin, however genome-wide association studies and genome-wide linkage analysis suggests there are other candidate genes that could be involved in the genetic pathologic background of COPD (Teramoto, 2016; Hersh *et al.*, 2008). Teramoto (2016) identified hedgehog-interacting protein, family with sequence similarity 13-member A, cholinergic nicotine receptor alpha 3/5, and iron regulatory binding protein 2 (IREB) as potential novel gene candidates in multiple populations, but the pathological roles in COPD are not yet understood (Teramoto 2016). The study by Hersh *et al.* (2008) identified two potential genes – serpin peptidase inhibitor E2 and Transforming growth factor (TGFβ1), but these genes have not been universally replicated across all studies. There is also some evidence that individuals with a genetic deficiency in the protease inhibitor Z allele are at increased risk of emphysema, and possibly also asthma, especially if the person is a smoker; these individuals show a rapid decline in lung function after massive environmental exposure when compared to the general population (Al Ashry & Strange, 2017).

There is also a suggestion that there are other genetic factors that can increase the susceptibility of alpha-1 antitrypsin deficient patients to lung disorders including emphysema and COPD (Brebner & Stockley, 2013). These include IREB2, IL-10,

TNF-α, cholinergic receptor nicotinic alpha 3 subunit (CHRNA3), glutathione S-transferase, and nitric oxide synthase 3 (NOS3) (Brebner & Stockley, 2013).

COPD is not the only lung disease that has an associated genetic risk; another example is idiopathic pulmonary fibrosis (IPF). This progressive and often fatal lung disease has also been linked to four genes that could cause an inherited form known as familial interstitial pneumonia (FIP) (Kropski *et al*., 2013). The genes are surfactant protein A2, surfactant protein C, the RNA component of telomerase and telomerase reverse transcriptase, which all play important roles in the progression and severity of the disease (Kropski *et al*., 2013).

1.6.5. Smoking cigarettes

Although just 15-20 % of smokers develop COPD, several studies[1] have shown that smoking cigarettes is an important factor in the development of COPD (Global Initiative for Chronic Obstructive Lung Disease (GOLD), 2004). Current estimates suggest that the prevalence of cigarette smoking in Europe is 29 % and 21 % in the US (Tamimi, Serdarevic and Hanania, 2012), with a total of more than 1 billion cigarette smokers worldwide, which accounts for the high, and increasing, levels of COPD seen (Rab *et al.*, 2013). There is also a large population that is exposed passively to smoke, which is associated with considerable morbidity both in adults and children (Rab *et al.*, 2013). Although not all smokers develop the disease, the smokers' current status depends on the time when they started smoking (most patients that develop COPD have smoked for more than 20 years) and the number of cigarettes per day, which, in turn, can affect the severity of the disease (Rab *et al.*, 2013).

It has been shown that the release of toxic compounds from tobacco (in both smoke and tar) can lead to the development of more severe stages of COPD by causing changes within the immune system (Parkes *et al.*, 2008; Taylor, 2010). Smoke contains very high concentrations of short-lived reactive oxygen intermediates (superoxide, hydrogen peroxide, and nitric oxide), and a large number of volatile compounds; and the tar

contains very high concentrations of stable free radicals (semiquinones, hydroquinones, and carbon-centred free radicals) and can produce large amounts of H_2O_2 in aqueous extracts, all of which need to be detoxified (Rab *et al.*, 2013). Antioxidants in the respiratory epithelial lining fluid (ascorbate and reduced glutathione) can detoxify some of the reactive oxygen species, but when antioxidants are depleted, the reactive species interact with biological targets on respiratory epithelial cells and cause damage (Rab *et al.*, 2013). Cigarette smoke and its reactive oxygen intermediates also have a high inflammatory potential and can promote recruitment of activated inflammatory cells in the lung, which further contribute to tissue injury (Rab *et al.*, 2013).

Cigarette smoke affects gene expression in human airway epithelium, and some of these changes can be attributed to differential expression of microRNAs (miRs) (Rab *et al.*, 2013). miRs are noncoding RNAs that post-translationally down-regulate gene expression by degrading mRNA or inhibiting translation. Studies using rats and mice indicated the expression of a number of miRs is decreased in response to smoke exposure, which would lead to an increase in mRNA and protein of the miR targets (Rab *et al.*, 2013). Schembri *et al.* (2009) compared whole-genome miR and mRNA expression in the bronchial airway epithelium of smokers and non-smokers; they found that 28 miRs were differentially expressed and confirmed that the levels of the target mRNAs were increased (Schembri *et al.*, 2009). The changes in the genes and the encoded proteins can be associated with changes observed in the progression of COPD (Rab *et al.*, 2013).

1.7. The effect of COPD in the lungs

In the lungs of COPD patients, the presence of irritants that cause oxidative stress, such as cigarette smoke, trigger an inflammatory and immune cell response co-ordinated by the epithelial cells and macrophages (Figure 1.3) (Barnes, 2008a).

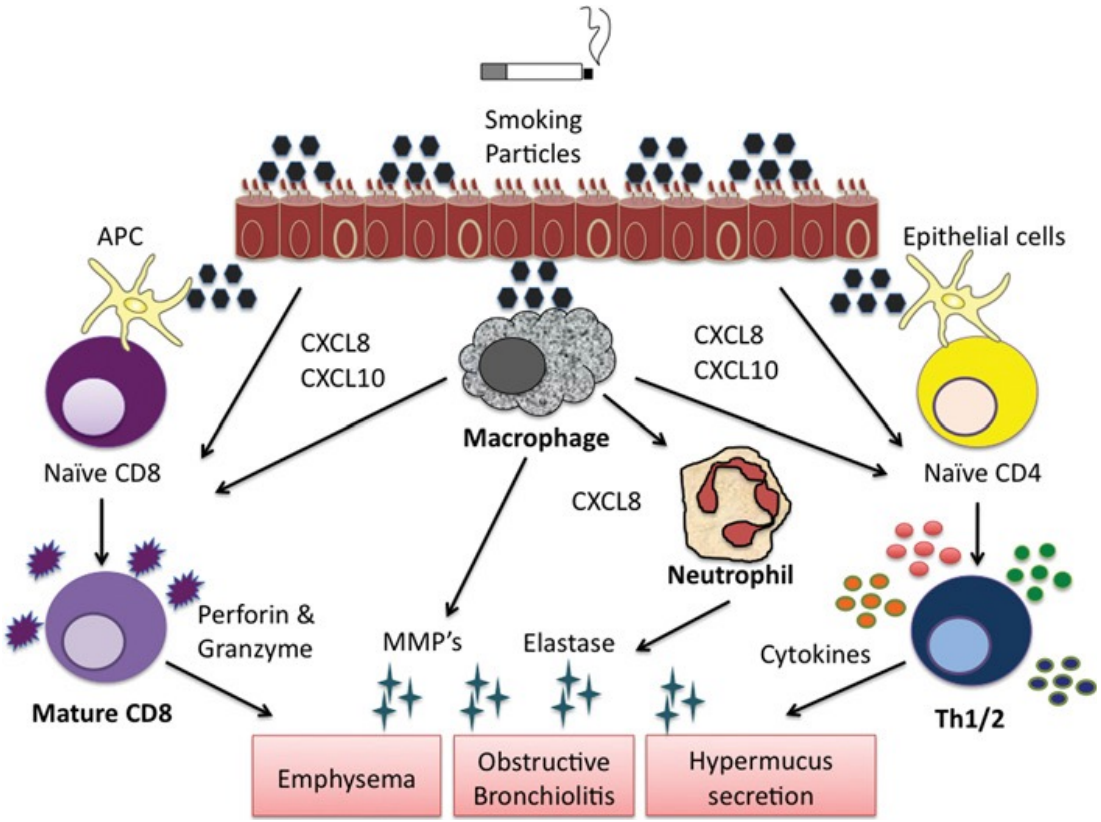


Figure 1.3: Inflammatory and immune cells involved in COPD.

Cigarette smoke and other airway irritants activate epithelial cells and macrophages to release chemotactic factors that can attract inflammatory cells to the lungs and are taken up by antigen presenting cells (APCs). Alveolar macrophages (AM) can release CXC-chemokine ligand 8, which act on CC-chemokine receptor 2 (CCR2) to attract neutrophils and monocytes (which can differentiate into macrophages) and CXCL9, CXCL10 and CXCL11, which can all act on CXCR3 attracting T helper 1 (Th1) cells and type 1 cells. CD8 T-lymphocytes potentially play a role in lung destruction via perforin and granzyme. All the inflammatory cells, macrophages and epithelial cells release proteases, such as matrix metalloproteinase 9 (MMP9), which can cause elastin degradation which is seen in emphysema. The protease, neutrophil elastase, also causes mucus hypersecretion, which is involved in bronchitis. Epithelial cells and macrophages also release transforming growth factor-β (TGFβ), which stimulates fibroblast proliferation, resulting in fibrosis in the small airways restricting air flow (Lane *et al.*, 2010).

A key feature of COPD is compromised mucociliary clearance, which results in the inefficient removal of inhaled chemicals, particles, and pathogens from the airways and contributes to chronic inflammation (Rab *et al.*, 2013). The reduction in movement of the mucous film that coats the airway surfaces allows pathogens and other irritants to remain in the lung where they activate immune responses including the recruitment of neutrophils and macrophages, and they can also activate the unfolded protein response (UPR) (Rab *et al.*, 2013).

The UPR is induced by reactive oxygen species, such as those present in cigarette smoke, which causes the elevation of UPR markers such as binding immunoglobulin

protein (BiP) and protein disulphide isomerase (PDI) which can initiate downstream effects (Rab *et al.*, 2013). Irritants, such as cigarette smoke, can also cause the down-regulation of histone deacetylase 2, an inflammatory response inhibitor, which can exacerbate the pathology of COPD (Rab *et al.*, 2013). The inhibition is due to post-translational modification caused by oxidative stress from exposure to the irritant (Rab *et al.*, 2013).

1.7.1. Inflammation

Inflammation in the lungs is caused by exposure to different irritants, for example cigarette smoke or pathogens, and over time continuous inflammation can lead to severe damage to structures of the lung and results in deterioration of lung function (GOLD, 2004). Inflammation is a major cause of lung fibrosis, gland hypertrophy and decreased smooth muscle tone (Gosens *et al.*, 2006), and lung inflammation will persist in smokers even after cessation associated with severe damage caused to the extracellular matrix and the endodermis (Barnes and Stockley, 2005).

During the progression of COPD, sustained inflammation occurs due to epithelial cells and macrophages releasing chemotactic factors (cytokines and chemokines) that attract inflammatory cells to the lungs (Figure 1.3) (Barnes, 2008a). This causes infiltration of a large numbers of immunological cells into the lungs including $CD8^+$ T cells, monocytes and neutrophils, which cause a number of changes within airways, including the activation of proteases and fibroblasts (Roth, 2008).

1.8. The role of cytokines in COPD

Cytokines and chemokines act by binding to their receptors, which lead to the secretion of other cytokines and chemokines, or they can induce their own production, known as the autocrine effect (Chung, 2001; Barnes, 2008b, 2009). Cytokines work together, not individually, to modulate the inflammatory response, and because COPD is an inflammatory disease, a large variety of these proteins have been detected in the plasma and sputum of COPD patients (Chung, 2001). They can by classified according to their role in the disease (Table 1.2) as pro-inflammatory cytokines, T cell cytokines, growth factors, chemokines and anti-inflammatory cytokines, and each class can have a different effect on the pathogenesis of (Chung, 2001; Barnes, 2008b, 2009).

Table 1.2: Cytokines in COPD.

Cytokines organised into the different groups according to their role (pro-inflammatory cytokines, T cell cytokines, growth factors, chemokines and anti-inflammatory cytokines) and their effect on the pathogenesis of COPD (Banes, 2008; Zlotnik & Yoshie, 2000; Mantovani *et al.,* 2004; Chung, 2001).

Cytokines	Effect on COPD	Producing cell types
Pro-inflammatory cytokines		
TNF-α	Enhances disease	Produced by macrophages, T cells, mast cells and airway smooth muscles; activates epithelium, endothelium, APCs, monocytes and macrophages, increases inflammation, IL-8 expression increased, bronchial hyper-responsiveness.
IL-1β	Enhances disease	Produced by epithelial cells, macrophages and numerous other cell types; activates macrophages, MMP9 secretion, and increases inflammation.
IL-6	Enhances disease	Produced by macrophages, T cells, B cells, and airway epithelial cells; increases IgE, acts as T and B cell growth factor, increases inflammation with other cytokines.
IL-32	Enhances disease	Produced by epithelial cells, macrophages, and CD8 T cells; induced by IFN-γ to increase the inflammation, activates the secretion of TNF-α and IL-1β.
Thymic stromal lymphopoietin (TSLP)	Enhances disease	Produced by epithelial cells; controls dendritic cell secretion of CCL17 and CCL22 that attract Th2, and IP10 that attracts Th1 and Tc.
Lymphocyte and T cells regulatory cytokines		
IFN-γ	Enhances disease	Produced by Th1 cells and NK cells; Control Th1 and Tc cell recruitment to the site of infection, activates alveolar macrophages and monocytes, and epithelial cells, endothelial cells, decreases Th2 cell and eosinophil influx after allergen, decreases bronchial hyper-responsiveness and IgE.
IL-12	Enhances disease	Produced by B cells, macrophages, dendritic cells, eosinophils, and epithelial cells; decreases Th2, regulates Th1 differentiation and secretion of IFN-γ by NK and T cells.
IL-13	Reduces disease	Produced by Th2 cells, basophils and eosinophils; activates eosinophils, decreases apoptosis, increases the production of IgE, increases mucin expression, induces airway re-modelling.
IL-17	Unknown	Produced by Th17 cells; induces release of epithelial CXCL1 and CXCL8 that attract neutrophils.

IL-18	Unknown	Produced by macrophages, dendritic cells, NK cells, CD8 T cells and airway epithelium; induces IFN-γ secretion, induces Th1 cell development with IL-12.
IL-21	Enhances disease	Produced by Th17 cells; important for the differentiation of Th17 cells and the inhibition of FOXP3 expressed by Treg.
IL-22	Enhances disease	Produced by Th17; regulates the release of IL-10.
IL-23	Enhances disease	Produced by dendritic cells; important in the regulation of Th17.
IL-25	Unknown	Produced by Th2 cells, granulocytes, epithelial cells; increases number of Th2 cells.
IL-4	May enhance disease	Produced by Th2 cells, mast cells, basophils and eosinophils; increases the production of IgE, increases number of Th2 cells, decreases Th1 cells, increases mucin expression.
IL-5	Unknown	Produced by Th2, mast cells and eosinophils; increases the numbers of eosinophils and Th2 cells, decreases apoptosis, increases bronchial hyper-responsiveness.
IL-9	Unknown	Produced by Th2, and eosinophils; increases activated T cells, increases the number of mast cells, increases mucin expression, causes eosinophilic inflammation and bronchial hyper-responsiveness.
Growth Factors		
Granulocyte-macrophage colony stimulating factor (GM-CSF)	Enhances disease	Produced by macrophages, eosinophils, fibroblasts, epithelial, airway smooth muscle cells and T cells; important for the differentiation and survival of neutrophils and macrophages, eosinophil apoptosis, increases number of neutrophils, increases number of eosinophils.
Transforming growth factor β (TGF-β)	Enhances disease	Produced by epithelial cells, eosinophils and macrophages; induces proliferation of fibroblasts, airway smooth muscle cells, and epithelial repair, increases fibrosis, suppresses inflammation (Tregs) and decreases T-cell proliferation.
Vascular endothelial growth factor (VEGF)	Reduces disease	Produced by smooth muscle cells; induces angiogenesis, increased vascular leakage, involved in pulmonary vascular remodelling.
Anti-inflammatory/Inhibitory cytokines		
IL-10	Decreases diseases	Produced by Th2 cells, Treg cells, $CD8^+$ T cells, monocytes and macrophages; decreases eosinophil survival, decreases

		Th1 and Th2, inhibits the release of other inflammatory cytokines (TNF-α, IL-1β, GM-CSF), chemokines and MMPs, decreases inflammation and bronchial hyper-responsiveness.	
Transforming growth factor β (TGF-β)	Enhances disease	Produced by epithelial cells and macrophages and induces proliferation of fibroblasts, airway smooth muscles cells, and epithelial repair.	
Chemokines			**Receptors or agonists and cell expression pattern**
CCL2	Enhances disease	Produced by macrophages, epithelial, and T cells; activates monocytes and T cells.	Receptor CCR2 expressed on monocytes, NK, basophils, immature DCs, B, activated T cells.
CCL5	Enhances disease	Produced by macrophages; activates Th1 and Tc and recruits eosinophils, T cells, and macrophages.	CCR-1 on NKs, monocytes, macrophages, immature DCs. CCR-3 on Th2, eosinophils, immature DCs, basophils. CCR-5 on Th1, NK, activated T cells, monocytes, macrophages.
CXCL1 and CXCL8	Enhances disease	Produced by macrophages and epithelial cells; recruits neutrophils.	CXCL1 receptor CXCR2, and CXCL8 receptors CXCR1 and CXCR2 expressed on neutrophils.
CCR2 agonists	Enhances disease	Increases numbers of monocytes.	CCL-2, -7, -12, -13.
CCR3 agonists	Enhances exacerbations	Increases numbers of eosinophils.	CCL-5, -7, -8, -11, -13, -15, -24, -26.
CCR4 agonists	Unknown	Increases numbers of Th2 cells.	CCL-17, -22.
CCR5 agonists	Enhances disease	Increases numbers of T cells, and eosinophils.	CCL-3, -4, -5.
CXCR2 agonists	Enhances disease	Increases numbers of neutrophils and monocytes.	CXCL-1, -2, -3, -5, -6, -7, -8.
CXCR3 agonists	Enhances disease	Increases numbers of Th1 cells, and Tc1 cells.	CXCL-9, -10, -11, expressed on Th1, NK.

1.9. Epithelial cell co-ordinated response

1.9.1. Epithelial cells

The epithelium represents the first line of defence for the upper respiratory tract against pathogens, allergens, pollutants, and microorganisms (Calderon-Garciduenas *et al.*, 2001). Epithelial cells play an important role in the passive immunity against pathogens infecting the airways; for example, mucus produced from goblet cells traps bacteria and inhaled particulates (Adler and Li, 2001).

The epithelial cells also secrete antioxidants, cationic peptides with antimicrobial effects, and anti-proteases, for example secretory leuko-protease inhibitor (SLPI), as part of the innate defence system (Aarbiou, Rabe and Hiemstra, 2002). In addition, epithelial cells transport immunoglobulin A (IgA) and are therefore also involved in adaptive immunity (Pilette *et al.*, 2001). It is possible that irritants, such as cigarette smoke, may impair these immune responses of the airway epithelium increasing the susceptibility to infection (Sutherland and Martin, 2003).

Airway and alveolar epithelial cells are an important source of inflammatory mediators and proteases in COPD. Epithelial cells are activated by irritants (such as cigarette smoke) to produce chemotactic factors, including TGF-β, TNF-α, IL-1β, GM-CSF and IL-8, which attract inflammatory cells and fibroblasts to the lungs (Takizawa *et al.*, 2001; Floreani *et al.*, 2003; Barnes, 2008b). TGF-β is an important chemotactic factor as it recruits fibroblasts that cause fibrosis which can block the small airways (Barnes, 2008b; Takizawa *et al.,* 2001).

Airway epithelium in COPD often shows signs of squamous metaplasia, which could be an effect of increased proliferation of epithelial cells. Proliferation, measured by Proliferating Cell Nuclear Antigen (PCNA), was shown to be slightly increased in some normal smokers, but it was markedly increased in patients with chronic bronchitis, and was consistent with the degree of squamous metaplasia (Takizawa *et al.*, 2001). The metaplastic alterations can lead to an increase in the number and size of the goblet cells, which can lead to an increase in the secretions of the upper airway (Wright *et al.*, 1984; Rab *et al.*, 2013). Studies also show that epithelial growth factor receptors have increased expression in airway epithelial cells of smokers, which may contribute to

basal cell proliferation and result in squamous metaplasia (Franklin *et al.*, 2002). Vascular Endothelial Growth Factor (VEGF) also appears to be necessary to maintain alveolar cell survival, as inhibition of VEGF receptors in rats induces apoptosis of alveolar cells and an emphysema-like pathology (Kasahara *et al.*, 2000).

Studies have shown that irritants, including tobacco smoke, can affect epithelial cells in different ways. They can cause a reduction in cell viability and induction of apoptosis in respiratory hair cells (Lan *et al.*, 2007), produce opposite mitogenic or pro-apoptotic effects depending on the irritant concentration (Luppi *et al.*, 2005), inhibition of chloride transport (Cohen *et al.*, 2009), or even cause impairment of epithelial regeneration upon injury (Van Winkle *et al.*, 2001). Studies have shown that chronic and intermittent exposure to cigarette smoke can lead to changes in the epithelium including: hyperplasia (loss of cilia and metaplasia with keratinisation), sub-mucosal thickening, neutrophilic inflammation and the stimulation of the mononuclear inflammatory cells (Wanner, Salathe and O'Riordan, 1996; Hamm *et al.*, 2007; Gerhart, 2008).

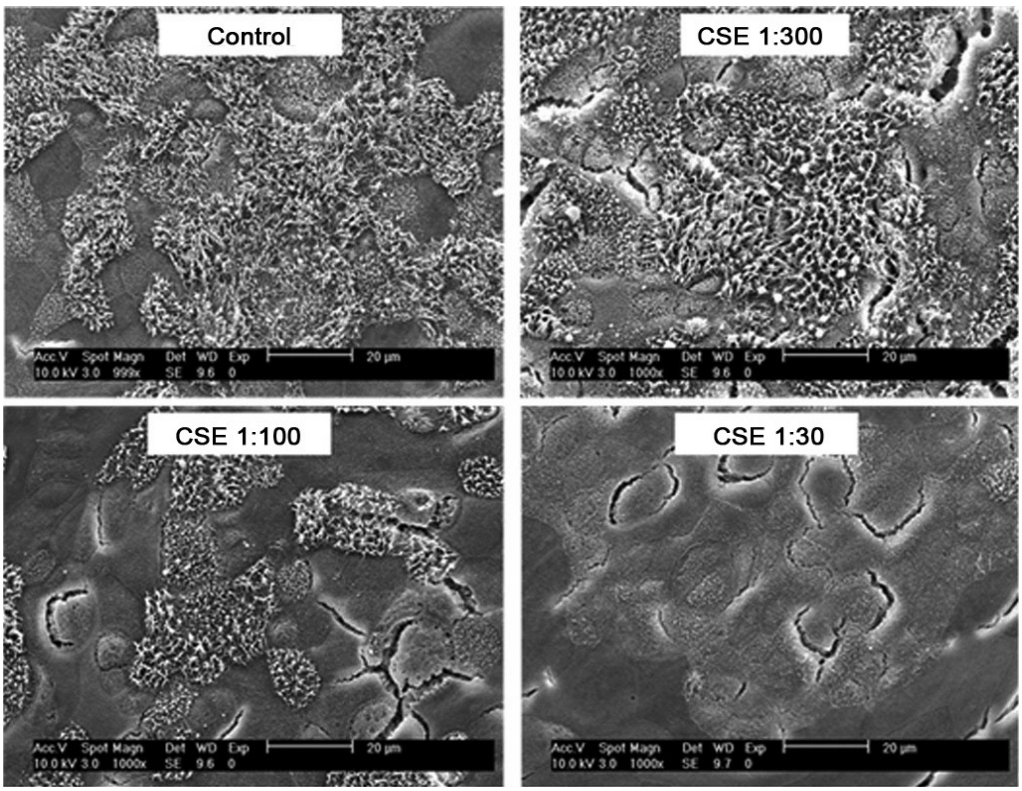


Figure 1.4: Scanning electron micrographs of cilia in alveoli showing reduced development.
As the concentration of Cigarette Smoke Extract (CSE) increases, there is a decrease in number and density of cilia (Tamashiro, Cohen, *et al.*, 2009).

Irritants and their toxic metabolites have been shown to be able to significantly reduce the beating of the cilia of epithelial cells, have a negative impact on the ciliogenesis in a dose-dependent fashion (in particular maturation and differentiation) and reduce ciliary beating (Tamashiro, Xiong, *et al.*, 2009; Ambarus *et al.*, 2012). This has been demonstrated by Tamashiro *et al*. (2009a) who used mouse respiratory epithelium cell cultures to show that the exposure to irritants such as cigarette smoke can cause serious damage to the epithelial cells, including a significant loss of cilial development (Figure 1.4), as well as a reduction in their size (Figure 1.5). Alongside the *in vitro* studies, results *in vivo* also show that acute and chronic exposure to irritants such as cigarette smoke can damage mucociliary transport as well as increase mucus production in humans (Navarrette *et al.*, 2012).

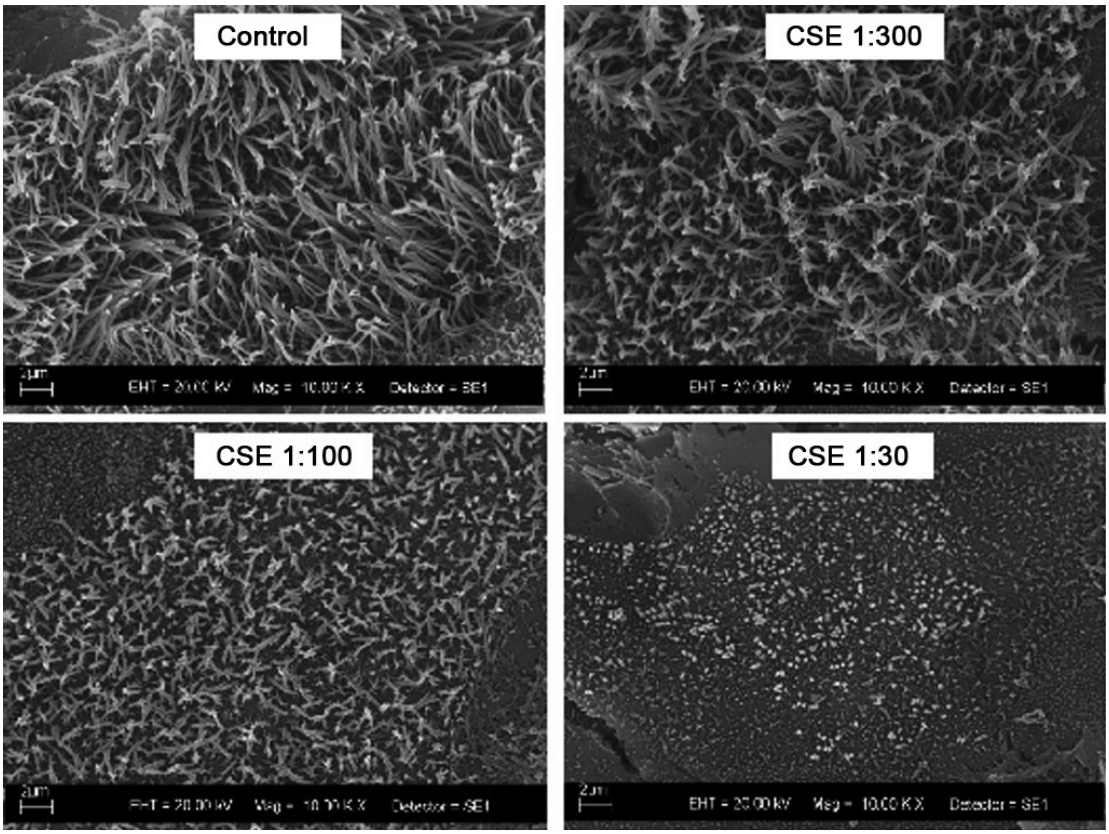


Figure 1.5: Scanning electron micrographs of cilia in alveoli showing reduction in size.
As the concentration of Cigarette Smoke Extract (CSE) increases, there is a decrease in in the size and density of cilia (Tamashiro, Cohen, *et al.*, 2009).

1.9.2. Smooth muscle

In the pathogenesis of COPD, inflammatory cells can interact with the airway smooth muscle to increase the progression of the disease. There is evidence to suggest that there is an increase in the number of neutrophils and $CD8^+$ cells in the bundles of smooth muscle in the peripheral airways of smokers with COPD compared to healthy controls, and that this neutrophilic infiltration was inversely related to FEV1 (Baraldo *et al.*, 2004). These results suggest the micro-localisation of the inflammatory cells can contribute to the observed airflow limitation in COPD.

Research has also shown that there is a significant reduction in glycosaminoglycans in the airway smooth muscle cells of COPD patients (Klagas *et al.*, 2009). These are essential extracellular matrix molecules involved in the regulation of tissue flexibility, and include chondroitin sulphate, dermatan sulphate, heparin, heparan sulphate, hyaluronic acid (HA), and keratin sulphate (Klagas *et al.*, 2009). Within the smooth muscle cells of COPD patients, secretion of HA was significantly decreased (when compared to healthy controls), which correlated to a decrease in the HA receptor and HA metabolising enzymes, all of which may regulate the pathological airway remodelling seen in COPD (Klagas *et al.*, 2009).

1.9.2.1. Smooth muscle in health and disease

Smooth muscle contractile activity has been shown to play a major role in the regulation of the function of the gastrointestinal system, respiratory system, genitourinary system and vascular system, and alterations to smooth muscle cell phenotype and function can be linked to tissue remodelling and many different disorders including asthma, cardiovascular disease, pre-term labour and functional bowel disease (Kim *et al*., 2008). Studies have shown that regulation of smooth muscle activity is exerted through phosphorylation and dephosphorylation of myosin which alters its activity, through calponin and caldesmon controlling the accessibility of actin to myosin, and through remodelling of the actin cytoskeleton (Kim *et al*., 2008).

The extracellular matrix (ECM) is an important support structure for smooth muscle cells and can influence its biology by activating biochemical and mechano-transduction signalling pathways, which control smooth muscle cell migration, growth, survival,

contraction, cytokine production and stiffness (Schuliga, 2015). The interaction between the smooth muscle and the ECM is a dynamic multidirectional process; smooth muscle cells can produce ECM proteins, cross-linking enzymes and proteases, all of which regulate the ECM structure and act through activating receptors such as integrins and protease-activated receptor-1 (Schuliga, 2015).

During COPD progression, there is chronic inflammation and structural remodelling, which is related to aberrant function of the airway smooth muscle (ASM) cells controlled by TGF-β and can result in hyperplasia (O'Leary *et al*., 2016; Michaeloudes *et al*., 2017). Studies in primary human ASM cells isolated from COPD patients, healthy smokers and healthy non-smokers showed that TGF-β stimulation caused an increase in IL-6 and CXCL8 release, with the highest levels in the COPD patients, and an increased SMAD3 expression in COPD patients only (O'Leary *et al*., 2016). Studies have also shown that ASM stimulated with TGF-β have an impaired energy balance, with accumulation of glutamine, lactate, amino acids and fatty acids, which may lead to changes in biosynthesis and redox balance (Michaeloudes *et al*., 2017).

1.10. Macrophage co-ordinated response

1.10.1. The importance of macrophages

Macrophages are a type of leucocyte that fights foreign substances by phagocytosis (engulfing and then digesting them), and were discovered in 1884 by a Russian bacteriologist called Élie Metchnikoff (Gordon, 2008). They can be found in all tissues and they can move in an amoeboid manor in order to fight potential pathogens such as bacteria, viruses and cancer cells (Ovchinnikov, 2008).

In the immune system, macrophages play a crucial role not only in innate defence (phagocytosis), but they also to help initiate specific adaptive defence mechanisms by engaging other immune cells such as lymphocytes (antigen presentation), and have a key role as anti-inflammatory regulatory agents by decreasing immune reactions through the release of cytokines (Lohmann-Matthes, Steinmuller and Franke-Ullmann, 1994; Barnes, 2004). In humans, several diseases can be exacerbated by macrophage disorders, such as COPD, and they can lead to death (Ovchinnikov, 2008). Human

macrophages are a key component of the innate immune response; they can be produced by differentiation of monocytes in different tissues

Table 1.3) and can be classified into several groups depending on the site of their function (Figure 1.6). They are estimated to be about 21 micrometres (0.00083 in) in diameter (Krombach *et al.*, 1997). By using flow cytometry or immune-histochemical staining, macrophages can be identified by their specific expression of proteins such as CD14, CD40, CD11b, CD64, F4/80(mice)/EMR1 (human), lysozyme M, MAC-1/MAC-3 and CD68 (Khazen *et al.*, 2005).

Table 1.3: Locations and functions of different tissue macrophages.

Data are simplified and do not account for the heterogeneity in many tissues; phenotypic markers listed are those expressed by the majority of cells, those expressed on sub-types are not included (adapted from Davies *et al*., 2013; Varol *et al*., 2015; Gordon *et al*., 2014; Gordon & Plüddemann, 2017).

Tissue	Cell type	Functions	Phenotypic markers
Adipose tissue	Adipose tissue-associated macrophages	Control of insulin sensitivity and adaptive thermogenesis.	F4/80^{+}, CD45^{+}
Blood	Ly-6C^{lo} monocytes	Act as "intravascular housekeepers" clearing endothelial cell debris.	CX3CR1^{+}, Ly-6C^{lo}, F4/80^{+}, CSF1R^{+}
Bone	Osteoclasts	Multinucleated cells that resorb bone by disruption of the mineralized matrix.	Calcitonin receptor^{+}
	Bone marrow macrophages	Erythropoiesis. Maintenance of hematopoietic stem cells.	CD169^{+}, F4/80^{+}, ER-HR3^{+}
Breast	Mammary gland macrophages	Immune surveillance, support of branching morphogenesis.	CSF-1^{+}, TGF-β^{+}
Cardiovascular system	Heart macrophages	Clearance of apoptotic cardiomyocytes, vasculogenesis, angiogenesis, and regulate cardiomyocyte electrical activity.	CD68^{+}
Central nervous system	Microglia	Promotes neuronal survival, immune-surveillance, removal of dead neurons, synaptic remodelling.	F4/80^{+}, CD11b^{+}, CD45^{lo}
	Perivascular macrophages	Immune surveillance.	F4/80^{+}, CD11b^{+}, CD163^{+}, CD45^{hi}
	Meningeal macrophages	Immune surveillance.	F4/80^{+}, CD11b^{+}, CD45^{hi}
Gastrointestinal tract	Intestinal macrophages	Maintenance of intestinal homeostasis and regulation of immune responses to commensals.	CX3CR1^{hi}, F4/80^{+}, CD11b^{+}, CD11c^{+}, CD64^{+}
	Muscularis gut macrophages	Regulation of smooth muscle contractions.	IL-10^{+}
	Intestinal lamina propria macrophages	Immune surveillance, gut homeostasis, luminal antigen uptake.	IL-10^{+}
Liver	Kupffer cells (sessile)	Clearance of microorganisms and cell debris from the blood. Clearance of aged erythrocytes.	F4/80^{hi}, CD11b^{lo}, CD169^{+}, CD68^{+}, Galectin-3^{+}, CD80$^{lo/-}$
	Motile liver macrophages	Immune surveillance, detoxification, iron and cholesterol recycling.	F4/80^{+}, CD11b^{+}, CD80^{hi}

Lung	Alveolar macrophages	Immune surveillance of the lung for inhaled pathogens, homeostatic regulation of tissue function, phagocytosis of excessive surfactants and surfactant-opsonized particles	F4/80^{lo}, CD11b^{lo}, CD11c^{hi}, CD68^{+}, Siglec F^{+}, MARCO^{+}, CD206^{+}, Dectin-1^{+}, Galectin-3^{+}
	Interstitial macrophages	Regulates DC maturation/activation.	F4/80^{+}, CD11c^{-}, CD68^{+}, MHCII^{+}
Serosal tissues	Peritoneal macrophages: F4/80^{hi} majority	Immune surveillance and regulation of homeostatic environment. Apoptotic cell clearance.	F4/80^{hi}, CD11b^{hi}, Tim4^{+}, MHCII^{lo}
	Peritoneal macrophages: F4/80^{lo}	Immune surveillance.	F4/80^{lo}, CD11b^{+}, Tim4^{-}, MHCII^{hi}, CD11c$^{+/-}$
	Pleural macrophages: F4/80^{hi} majority	Immune surveillance.	F4/80^{hi}, CD11b^{hi}, Tim4^{+}
	Pleural macrophages: F4/80^{lo}	Immune surveillance.	F4/80^{lo}, CD11b^{+}, Tim4^{-}
Skin	Dermal macrophages	Immune surveillance.	F4/80^{+}, CD11b^{+}, CD11c^{lo}, CD206^{+}, MHCII^{lo}, CD169^{+}, Dectin-1^{+}, CD301^{+}, Dectin-2^{+}
	Langerhans cells	Interaction with T lymphocytes.	F4/80^{+}, CD11b^{+}, CD11c^{+}, Langerin^{+}
Spleen	Marginal zone macrophages	Immune surveillance of the circulation, detoxification.	CD68^{+}, CD209b^{+}, MARCO^{+}, Dectin-2^{+}, Tim4^{+}
	Metallophilic macrophages	Immune surveillance.	CD68^{+}, CD169^{+}
	Red-pulp macrophages	Erythrocyte clearance and iron metabolism.	F4/80^{+}, CD206^{+}, Dectin-2^{+} (Spi-C)
	White pulp (tingible body) macrophages	Clearance of apoptotic cells resulting during the germinal centre reaction.	CD68^{+}

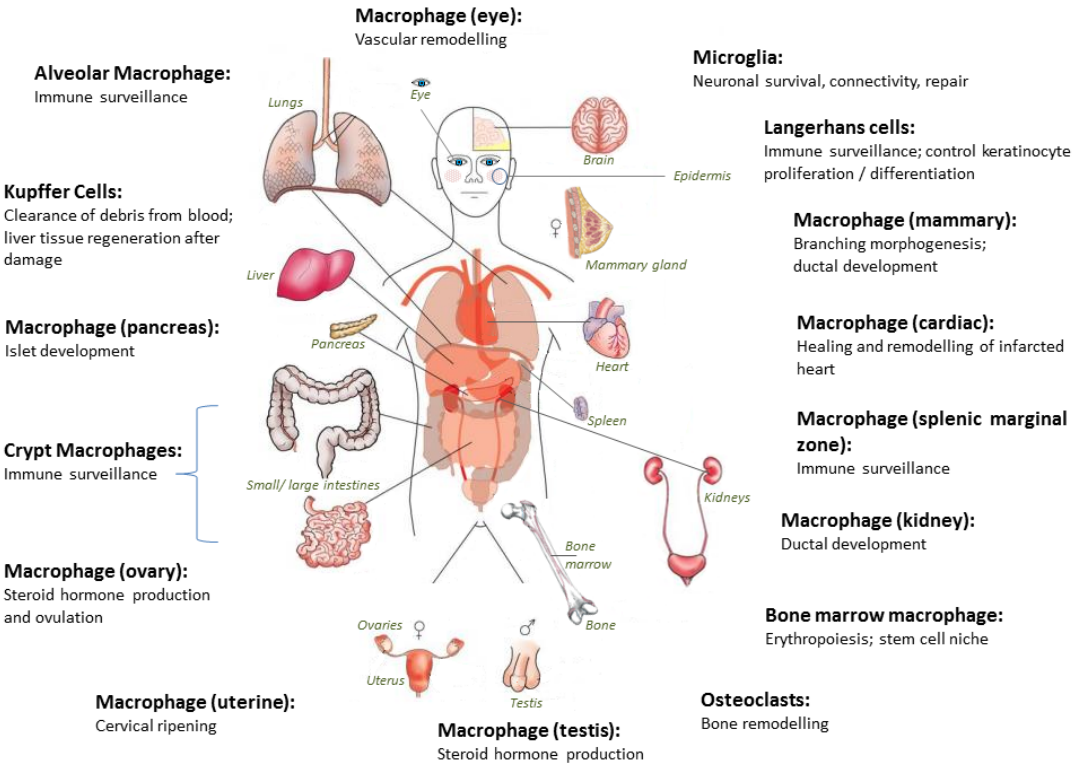


Figure 1.6: Nomenclature, spatial organisation and role of the different human macrophage populations.

Note that an additional sub-population of macrophages known as Lymph Node Macrophages have been reported by Gray and Cyster (2012), and it includes: Subcapsular Sinus Macrophages (SSMs), Medullary Sinus Macrophages (MSMs), and Medullary Cord Macrophages (MCMs). (Adapted from (Hume, 2012)).

Within the pulmonary system alveolar macrophages (AM) make up 90 % of the macrophage population. There are also other populations, for example airway and resident lung tissue macrophages, present in the lung (Liu *et al.*, 2008). The functions of AMs include the production of signalling mediators, phagocytosis (organic and inorganic particulates, including bacteria/viruses/ fungi), processing of phagocytosed material, removing dying and dead cells, and removal of cell debris (Yamasaki & van Eeden, 2018; Vlahos and Bozinovski, 2014). AMs play a principle role in the innate response against inhaled irritants through phagocytosis (Franke-Ullmann *et al.*, 1996).

AMs are unique leukocytes that constitute over 90 % of the pulmonary population and recently it has been shown that AMs originate from foetal blood monocytes within the first week of life and their maintenance is dependent on their self-renewing capacity (Vlahos and Bozinovski, 2014). They respond to airborne irritants and microbes and

this lung macrophage pool, once developed, is long lived in the absence of an inflammatory insult in humans. Peripheral blood monocytes can also replenish lung macrophages in a stimulus-specific manner (Vlahos and Bozinovski, 2014). In addition to mounting an appropriate immune response during infection and injury, AMs actively coordinate the resolution of inflammation through apoptotic cells and any alteration of this process can lead to deleterious effects (Vlahos and Bozinovski, 2014).

Mucosal immunity is crucial for the correct functioning of the body's immune response (Hardy *et al.*, 2013). This response is determined by the interaction between the epithelial cells of the mucosal surface and the immune cells underneath (for example, macrophages). In this interaction, and in keeping with their role as part of the innate immune response, the macrophages play a crucial role in priming the immune response by activating, deviating or suppressing different components, depending on the incoming stimuli (internal or external pathogens, immune regulation, tumour surveillance, etc.) (Foey, 2012).

Several studies have been carried out to evaluate the function of macrophages in COPD, but the results are not consistent (White *et al.*, 2003). This is mainly due to the fact that the importance of macrophages in COPD is not limited to their role in fighting the disease, but they also have a role in increasing the severity of the disease, as chronic accumulation of macrophages at sites of inflammation is consistent with the chronicity and the slow development of COPD (Tetley, 2002).

As a consequence of the continuous activation by chemokines during the progression of the disease, the macrophages are ultimately changed (Russell *et al.*, 2002). Hodge *et al*. (2003) showed that macrophages from COPD patients had a decreased ability to phagocytose airway pathogens, therefore, they proposed that this response would be partly related to the pathogenesis of COPD due to the accumulation of apoptotic epithelial cells. Studies proved that the number of apoptotic epithelial cells is higher in COPD patients than in healthy smokers and non-smokers, which is consistent with their role in COPD development (Hodge *et al.*, 2003). It has been suggested that it is through secreting a number of proteins that activate neutrophilic inflammation (like tumour necrosis factor alpha (TNF-α), leukotriene B4 and IL-8) that macrophages mediate their function in COPD (Barnes, 2004).

1.10.2. Maintaining homeostasis of AMs and their activation in lung tissues

Alongside activating the AMs, which is crucial in an immune response to pathogens and irritants, maintaining their homeostasis under normal conditions is also important. Under homeostatic conditions, AMs exhibit tumoricidal, microbicidal, and parasiticidal activities at a lower level than tissue-resident macrophages. Compared to tissue macrophages, AMs display a diminished capacity to present antigen to T cells, reduced phagocytic capacity and respiratory burst (Morris *et al.*, 2003; Vlahos and Bozinovski, 2014). They are closely associated with alveolar epithelial cells (AECs), this induces the expression of epithelial restricted αvβ6 integrin which binds and activates latent TGFβ and inhibits macrophage activation implicated in alveolar wall destruction (Vlahos and Bozinovski, 2014). Recognition of antigens by toll like receptors (TLRs) promotes AMs to detach from AECs, and the subsequent production of proteases by activated AMs activates latent TGFβ, reinstating AMs to their resting state. This intricate mechanism of micro-environmental macrophage specialization keeps the macrophage response in check, and research suggests that alterations to these mechanisms may cause AMs to fail to effectively resolve inflammation and restore normal lung homeostasis in COPD, as shown by elevated numbers of macrophages in affected tissues (Tetley, 2002; Morris *et al.*, 2003).

1.10.3. Role of AMs in innate host defence

As macrophages have an important role in responding to various challenges, they must be primed specifically, both spatially and temporarily, via controlled signals. This leads to the expression of specific molecules on their surface, which in turn is responsible for the macrophages secreting unique chemokines and cytokines which allows them to carry out their specific roles (Vlahos and Bozinovski, 2014).

1.10.4. The role macrophages as antigen presenting cells

Studies have shown that macrophages can act as APCs either under classical activation or alternative activation (Goerdt & Orfanos, 1999). Macrophages are part of the mononuclear phagocyte system; to act as an APC, macrophages need to express MHC II glycoproteins on their surface, synthesize and release IL-1 and process antigens (Unanue, 1984). They can act as APCs after stimulation with growth factors including IFN- γ, CSF-1, as this causes them to express cell surface markers including CD11c, IL-12 and CD14 which activates Th1 cells (Fiorentino *et al*., 1991; Desmedt *et al*., 1998; Hume, 2008). Macrophage antigen presentation occurs with tight regulation and can be controlled by the synthesis and expression of the MHC II proteins (Unanue, 1984).

Alveolar macrophages are a good example of alternatively activated macrophages as they represent the first-line of defence; if they function successfully, they do not produce a strong Th1 response, but instead they produce Th2-associated effector functions, including endocytotic clearance, reduced proinflammatory cytokine secretion and antigen presentation, with anti-inflammatory (Th1 immunosuppressive) effects being produced by PGE2, and IL-10 secretion (Goerdt & Orfanos, 1999).

With regard to COPD, evidence indicates that alveolar macrophages show reduced expression of MHC class I and MHC class II, CD80 and CD86 (Pons *et* al., 2005; Lofdahl *et al*., 2006; Hodge *et al*., 2011). This indicates that alveolar macrophages have reduced antigen presenting capacity in COPD.

1.10.5. Macrophage activation

Macrophages can be activated by irritants, including cigarette smoke, to release inflammatory mediators

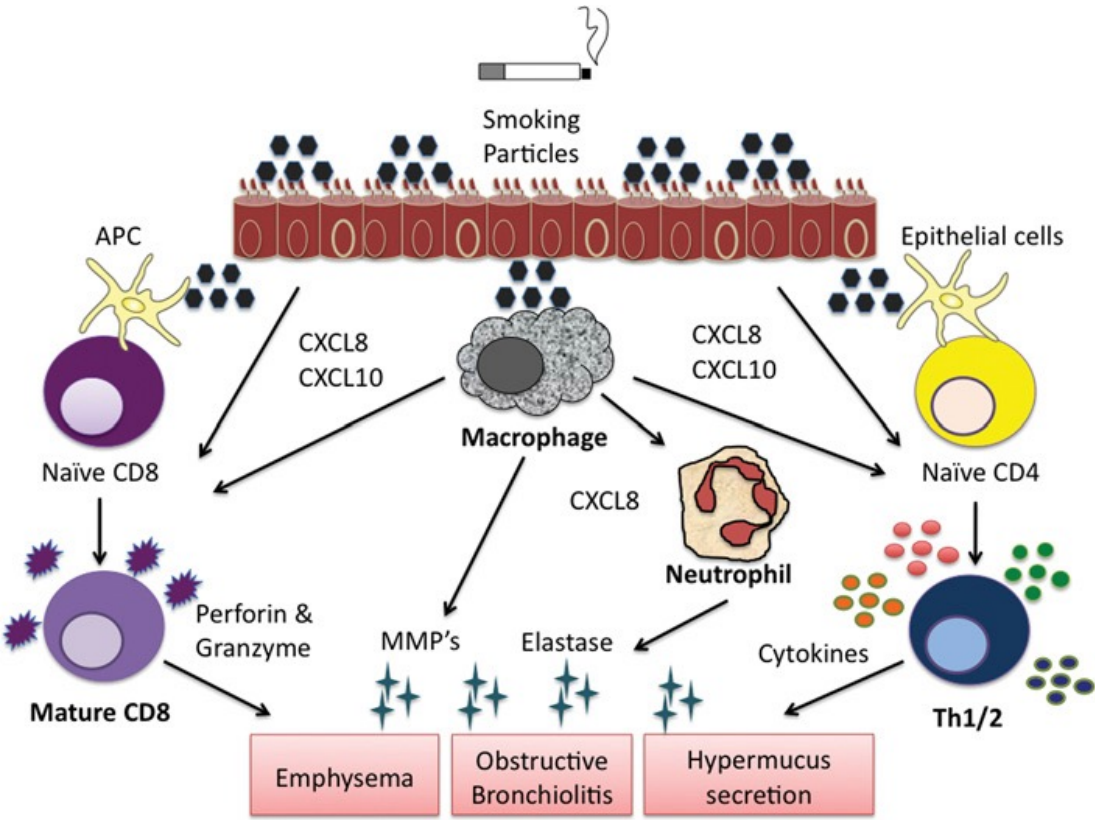


Figure 1.3) such as: CC-chemokine ligand 2 (CCL2) to attract monocytes, CXC-chemokine ligand 1 (CXCL1) and CXCL8 to attract neutrophils and monocytes, and CXCL9, CXCL10, CXCL11 and Interleukin-1 (IL-1) to attract T cells (Th1 and TC1) (Tetley, 2002; Barnes, 2008a). By secreting a number of proteins that activate neutrophilic inflammation, like tumour necrosis factor alpha (TNF-α), leukotriene B4 and IL-8, these cells mediate their function in COPD (Barnes, 2004).

In the same way that T helper cells can be polarised to express certain markers on their cell surface, macrophages can be activated into several subclasses producing different kinds of cytokines and chemokines (Martinez *et al*., 2013). Generally, and upon certain signals, monocytes can give two kinds of macrophages (Figure 1.7); the first are pre-inflammatory cells called M1 macrophages, which have a "classical" phenotype, are activated in the pro-inflammatory Th1 response, and are triggered by IFN-γ and LPS. The others decrease inflammation and act to repair tissues, called M2 macrophages,

which have an “alternate” phenotype, and are activated in anti-inflammatory response (Vlahos and Bozinovski, 2014; Mills, 2012).

The differences between the populations of M1 and M2 macrophages can be observed in their metabolism, for example, they digest the amino acid arginine to either an "M1" molecule (nitric oxide) or an "M2" molecule (ornithine) (Gordon, 2008).

The expression of M1 macrophages can be induced by the activity of IFNγ, cytokines, or microbial stimuli (such as lipopolysaccharide (LPS)) and granulocyte macrophage colony stimulating factor (GM-CSF). M2 macrophages can be activated by exposure to the Th2 cytokines IL-4 and IL-13, which induce production of high levels of IL-10 and IL-1RA and a low expression level for IL-12, in addition they can be activated by the immune complex and the macrophage colony stimulating factor (M-CSF) (Mills *et al.*, 2000). After activation M1 macrophages can increase NO production, and the expression of MHC-II, IL-12, IL-23 and STAT-1; whereas after activation M2 macrophages produce the opposite effect, they show reduced expression of all the factors that M1 increases, and M2 macrophages can also increase the killing and encapsulation of parasites upon activation (Stein *et al.*, 1992; Doyle *et al.*, 1994).

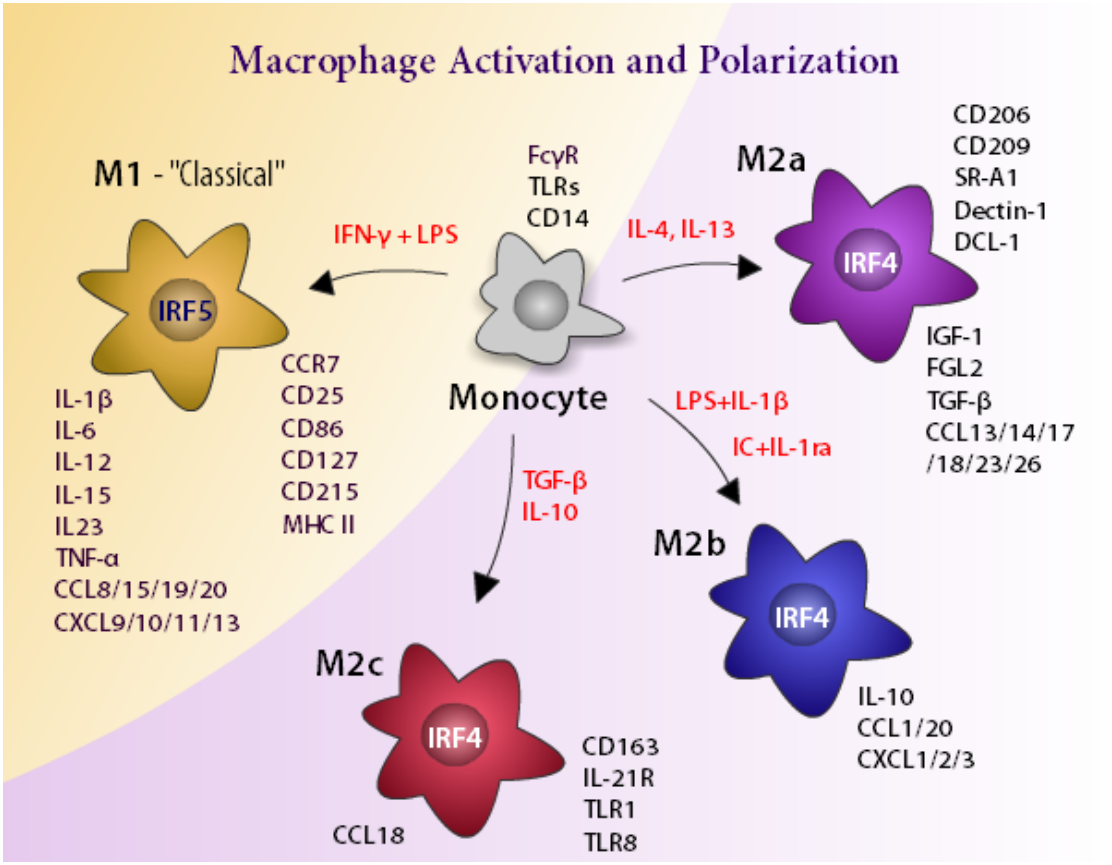


Figure 1.7: Macrophage activation and polarisation.

The figure shows what each type of cell interacts with and releases, and what signals trigger the monocyte to form the different populations of macrophage. Within the macrophages is the name of the transcription factor involved in maintaining the phenotype, IRF5 is responsible for maintaining M1 phenotype, and IRF4 is responsible for the M2 phenotype. Adapted from (BioLegend, 2011).

The different populations of M1 and M2 macrophages can be detected by the exclusive phenotype markers they produce on the cell surface (Table 1.4): M1 macrophages are characterised by the production of IL-12^{high}, IL-23^{high} and IL-10^{low}, and M2 macrophages express IL-2^{low}, IL-23^{low} and IL-10^{high} on their surface. M2 macrophages also express high levels of scavenger, mannose and galactose receptors (Davis *et al.*, 2013). These macrophage types also have distinctive chemokine and chemokine receptor profiles; M1 macrophages can secrete chemokines CXCL9 and CXCL10 which attract TH-1 cells, and M2 macrophages express chemokines CCL17, CCL22 and CCL24 (Jaguin *et al.*, 2013).

The M2 type of macrophage can be further divided into four subsets: M2a, M2b, M2c and M2d according to their gene expression profiles (Mantovani *et al.*, 2004). The M2a subtype is triggered by IL-4 or IL-13 (common IL-4R-alpha, CD124), M2b is induced by IL-1R ligands or exposure to immune complexes with LPS or IL-1β, and the M2c subtype can be elicited by IL-10, TGF-beta and glucocorticoid hormones (BioLegend, 2011; Hao *et al.*, 2012). M2d differ from M2a-c: they can be described by a $IL\text{-}10^{high}$ $IL\text{-}12^{low}$ M2 profile with some features of tumour-associated macrophages (TAMs), and have been shown to have some phenotype and functional similarities to ovarian TAMs (Duluc *et al.*, 2007).

Table 1.4: Macrophage polarisation: classically activated (M1) and alternatively activated (M2) subset phenotypes.

Arg-1, arginase-1; FIZZI, resistin-like molecule-alpha (Relm-alpha); GCs, glucocorticoids; ICs, immune complexes; IL1-ra, IL-1 receptor antagonist; LIF, leukocyte inhibitory factor; TGM2: transglutaminase 2; TNF-alpha: transforming growth factor-beta; MR (CD206), mannose receptor; NO, nitric oxide; iNOS, nitric oxide synthase; SR, scavenger receptor; VEGF, vascular endothelial growth factor; Ym1 (also known as chitinase-3-like protein-3 (Chi3l3). Adapted from (Duluc *et al.*, 2007; Hao *et al.*, 2012).

	Macrophages Subtypes				
	M1	**M2a**	**M2b**	**M2c**	**M2d TAM**
Stimulation and Activation	IFN-gamma TNF-alpha LPS	IL-4 IL-13	ICs LPS LTR/IL-1R	IL-10 TGF-β GCs	IL-6 LIF MCF
Expression	CD86 CD80 MHC II↑ IL-1R I TLR2 TLR4 iNOS (converts arginine to L-citrulline and NO)	CD163 MHC II SR CD206↑ (MR↑) TGM2↑ DecoyR -IL-1R II Mouse only: Ym1-Fizz1 - Arg-1	CD86 MHC II	CD163 TLR1 TLR8	VEGF
Cytokines: Produced by macrophage sub-sets	TNF-alpha IL-1beta IL-6 IL-12 IL-23	IL-10 TGF-beta IL-1ra	IL-1 IL-6 IL-10 TNF-alpha	IL-10 TGF-beta	IL-10 IL-12 TNF-alpha TGF-beta
Chemokines: induced to express	CCL11 CCL10 CCL9 CCL8 CCL5 CCL4 CCL3 CCL2	CCL24 CCL22 CCL17	CCL1	CCR2	CCL5 CXCL10 CXCL16

After the macrophages are triggered to become either the M1 or M2 type their status is not fixed. Recent studies have shown that macrophages can undergo re-polarisation from M2 to M1 and back again in response to changes in the cytokine environment *in vitro* (Davis *et al.*, 2013). Over time in the body the level of gene, protein, and metabolite expression can change along with microbicidal activity, which means the macrophages need to be dynamic to respond to changes in the environment by causing changes in polarisation. During pulmonary diseases, macrophages do not always follow the usual division criteria, and it has been suggested that the inflammatory environment in the airways can drive the activation of M1 and M2 macrophages (Vlahos and Bozinovski, 2014).

In COPD patients, there is evidence that there is an increased number of macrophages and different macrophage sub-populations exist in the inflamed lung due to alterations in phenotype of highly plastic airway macrophages (Vlahos and Bozinovski, 2014). Currently, the importance of these sub-populations in COPD is unknown, but their ongoing characterisation demonstrates that they do not conform to the classic M1/M2 dichotomy (Vlahos and Bozinovski, 2014). It has been suggested that endogenous mediators can promote an imbalance in inflammatory M1 vs. healing M2 macrophages at a transcriptional level and this could be a plausible mechanism underlying why AMs fail to effectively resolve inflammation and restore normal lung homeostasis in COPD (Vlahos and Bozinovski, 2014). Recent studies suggest that deficiencies in the processes that switch off inflammation and restore lung homeostasis in patients with COPD who smoke, leads to an increase in M2 transcription (detected by CD163 induction) and a down regulation of M1 genes (Vlahos and Bozinovski, 2014). There is a need to better characterise the distinct AM populations present in COPD and their contribution to pathology, as their highly plastic nature offers an opportunity to reprogram macrophages to facilitate restoration of lung homeostasis (Vlahos and Bozinovski, 2014).

1.10.6. Downstream events of AMs activation

The role of the AMs can be further elucidated by investigating what occurs after activation. Activated macrophages can produce an array of cytotoxic compounds

including reactive oxygen species (ROS) such as superoxide anions, hydroxyl radicals, hydrogen peroxide, and nitric oxide (NO), which can promote macrophage accumulation, retention, and activation, independently of other pro-inflammatory stimuli, which facilitate the antimicrobial defence mechanism (Nathan and Hibbs Jr., 1991; Pires *et al.*, 2012). AMs expression of phagocytosis facilitating receptors (scavenger, β-glucan, immunoglobulin (F_cR), mannose, complement) can also lead to antimicrobial defence (Taylor, Gordon and Martinez-Pomares, 2005).

In order to maintain an optimal oxidant/antioxidant balance, endogenous antioxidants including superoxide dismutase, glutathione peroxidase and catalase are upregulated to protect against molecular and cellular damage caused by oxidants (Pires *et al.,* 2012).

Recent studies have suggested oxidants found in CS reduce the ability of AM to release NO and express nitric oxide synthetase causing NO dependent-immunosuppressive effects, which impair mechanisms by directly causing cytoskeletal instability and carbonyl modification which is important in developing a severe COPD pathology (Pires *et al.*, 2012; Vlahos and Bozinovski, 2014). iNOS (expressed by M1 macrophages) and arginase-1 (expressed by M2 macrophages) are elevated in the airways of COPD patients, leading to exacerbations and worsened lung functions (Bhowmik *et al.*, 2005).

AMs can act as a reservoir for neutrophil elastase, which, when released, localises to lung elastic fibres and activates MMPs. This triggers an inappropriate cascade of proteinase activity, degrading extracellular matrix components and promoting the release of mucins through epidermal growth factor receptor (EGFR)-dependent mechanisms, causing injury and abnormal repair seen in emphysematic patients (Stockley, 2002; Abboud and Vimalanathan, 2008). The increase in proteinase activity overwhelms the native inhibitors and contributes to the proteolytic processes in COPD (Abboud and Vimalanathan, 2008; Roth, 2008).

In bacterial product-induced acute lung injury, AMs release pro-inflammatory cytokines, leading to the early phase of neutrophil influx (Maus *et al.*, 2002). It has also been suggested that by regulating MCP-1 and by engulfing neutrophils through phagocytosis, AMs would control, or limit, the neutrophil influx (Beck-Schimmer *et al.*, 2005).

In response to irritation, AMs can also secrete enzymes including the gelatinases MMP-2, MMP-9, and MMP-12, the cysteine proteinases cathepsin K, L, and S, which together are responsible for destruction of lung parenchyma (Tetley, 2002; Vlahos and Bozinovski, 2014). COPD patients have increased expression of MMP-1, MMP-9, and cathepsins K, L, and S, which correlates with the increased destruction of the lung parenchyma and tissue remodelling seen in patients with emphysema (Barnes, Shapiro and Pauwels, 2003; Abboud and Vimalanathan, 2008). Studies have shown that MMP-9 is expressed at a higher level in AMs from smokers with normal lung function than non-smoking healthy subjects, and MMPs persist for a long time during the disease process, thereby reflecting the importance of the longevity of macrophages in the lungs, which is also supported by the chronic nature and slow progression of this disease (Russell *et al.*, 2002; Tetley, 2002; Abboud and Vimalanathan, 2008).

The expression of some scavenger receptors, such as CD163, are down regulated in COPD patients as a result of the low secretion of its inducers (IL-10 and serum amyloid A (SSA)). Consequently, this leads to further deterioration in the lung function as a result of decreased phagocytosis and activity of proteolytic enzymes (Van Gorp *et al.*, 2010).

Macrophages have also been shown to control $CD8^+$ T cells, iBALT formation, and Th17, however the exact mechanism is still to be elucidated (Vlahos and Bozinovski, 2014). The interaction between macrophages and T cell subsets can have an impact on COPD progression, as T cells can control the polarisation state (M1/M2) of macrophages, and there is some evidence that macrophages can regulate T cell biology (Vlahos and Bozinovski, 2014). As certain T cell subsets, including $CD8^+$ T cells and Th17 cells, are implicated in COPD pathology, further investigation into the role of macrophages in regulating T cell biology would be beneficial (Vlahos and Bozinovski, 2014).

Impaired AM function is key to the high colonisation rates and increased susceptibility observed in COPD (Barnes, 2004). Excessive oxidative stress is particularly deleterious to AM function, leading to a deficiency in phagocytosis of bacteria, and impaired clearance of apoptotic cells through oxidant-dependent activation of RhoA and

inhibition of Rac1, this impairment is problematic as neutrophils are persistently recruited into the airways (Hodge *et al.*, 2003; Vlahos and Bozinovski, 2014).

To conclude, within the lungs, AMs coordinate the efficient clearance of inhaled irritants and microbes to resolve inflammation, and can produce large amounts of NO, and other ROS when activated by pathogens or pro-inflammatory cytokines (Hodge *et al.*, 2003; Vlahos and Bozinovski, 2014).

Interaction of macrophages with their environment plays a key role in their regulation and involvement in ongoing processes. One of the mechanisms used by macrophages to detect pathogens or cell signalling molecules are intracellular and cell surface pattern recognition receptors (PRR); these detect molecular patterns associated with microbes and cell damage (Gordon and Martinez, 2010).

1.11. Macrophages and pattern recognition receptors

PRRs have been shown to recognise unique, highly conserved motifs and molecules present on microorganisms, known as pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs) (Janeway, 1992). These patterns are often essential microbial components that can be shared by different microorganism groups, which allows a small number of receptors to detect a large variety of pathogens (Medzhitov, 2007). There are several commonly found examples of PAMPs including peptidoglycan, flagellin, LPS, β-glucan, and unmethylated 2′-deoxyribo(cytidine-phosphate guanosine) (CpG) motifs, which are all found in bacteria or fungi and can trigger an immune response (Medzhitov, 2007).

PRRs can also detect and be activated by the presence of host cell damage, in the presence and absence of pathogens, through danger-associated molecular patterns (DAMPs) (Chen and Nuñez, 2010). DAMPs are endogenous host molecules which are normally intracellular and protected from the immune system, but they can be released due to damage, cell stress, or cell death (Bianchi, 2007). DAMPs can include heat shock proteins (HSP), the high-mobility group box 1 protein (HMGB1), uric acid crystals, adenosine triphosphate (ATP), and poly- and oligo-nucleotides (Bianchi, 2007).

The PRRs themselves are composed of different types of molecules and receptors depending on the pattern they recognise and the signal they activate within the

macrophage; they can induce the expression of chemokines and cytokines, antimicrobial peptides and reactive oxygen species (ROS), cause endocytosis, phagocytosis and co-stimulation of APCs (Medzhitov, 2007). The most common PRRs are intracellular receptors such as the nucleotide-binding oligomerisation domain-containing (NOD) proteins; cell surface bound receptors such as mannose receptor (MR) and TLR-4; and secreted molecules such as LPS-binding protein (LBP) or mannan-binding lectin (MBL) (Lee and Kim, 2007). All these PRRs are important in immune regulation and the level of cross-talk between the signalling pathways initiated upon receptor binding plays a role in immune regulation (Lee and Kim, 2007).

A wide variety of DAMPs and PAMPs can be recognised by the leucine-rich repeat (LRR)-containing cytosolic NOD-like receptors (NLRs), and these receptors can form multi-protein complexes (inflammasomes) that activate inflammatory caspases (Martinon, Mayor and Tschopp, 2009). One of the classes that are key for anti-fungal immunity are the C-type lectin receptors (CLRs), for example MR, Dectin-1, and dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin 1 (DC-SIGN), which are a diverse group of membrane bound and soluble proteins that recognise specific carbohydrate containing molecules (Hardison and Brown, 2012). Viral RNA is detected by the retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), which are cytosolic RNA helicases made up of RIG-I, melanoma differentiation-associated protein 5 (Mda5), and laboratory of genetics and physiology 2 (LGP2) (Kawai and Akira, 2010).

One of the first groups of PRRs discovered were the TLRs; type I transmembrane proteins with a LRR extracellular domain and an intracellular Toll/IL-1 receptor (TIR) domain that can be intracellular or plasma membrane-bound; they recognise a diverse variety of PAMPs and DAMPs including lipids, nucleic acids and proteins from fungi, bacteria, parasites and viruses (Chen and Nuñez, 2010; Kawai and Akira, 2010). TLRs act through recruitment of different TIR domain-containing adaptor molecules to initiate signalling cascades (Table 1.5) (Kawai and Akira, 2010). These include myeloid differentiation primary response protein 88 (MyD88), TIR-domain-containing adaptor protein inducing IFN-β (TRIF, also known as TIR-domain-containing molecule 1 (TICAM1)); and the 'sorting adaptors' TRIF-related adaptor molecule (TRAM, or

TICAM2)), and TIR-domain-containing adaptor protein (TIRAP, or MyD88-adaptor-like protein (MAL)) (Kawai and Akira, 2010). These adaptor molecules can lead to the activation of different immune responses through separate signalling molecules; MyD88-dependent signalling results in activation of mitogen-activated protein kinase (MAPK), some interferon regulatory factor (IRF) pathways, nuclear factor κB (NF-κB), and production of pro-inflammatory cytokines. Whereas TRIF-dependent signalling causes late-phase activation of NF-κB and IRF3 to produce type I interferons and pro-inflammatory cytokines (Kawai and Akira, 2010).

Table 1.5: Characteristics of Human TLR and molecules involved in signalling.

The location, associated ligands and adaptor molecules recruited of the different human TLRs are listed; some of the endogenous ligands are still putative as microbial contamination of preparations cannot be ruled out. Abbreviations: ECM = extracellular matrix; ER = endoplasmic reticulum; GIPL = glycoinositolphospholipid; LDL = low-density lipoprotein; poly(I:C) = polyinosinic-polycytidylic acid; tGPI-mucin = Glycosylphosphatidylinositol-anchored mucin-like glycoproteins from *Trypanosoma cruzi* trypomastigotes. Information compiled from: (Kawai and Akira, 2005, 2010; Yarovinsky *et al.*, 2005; Lee and Kim, 2007; Chen and Nuñez, 2010; Guan *et al.*, 2010; Pifer *et al.*, 2011; Shi *et al.*, 2011; Liu, Gao and Ji, 2012; Oldenburg *et al.*, 2012).

TLR/ Location	Ligands – Exogenous	Ligands - Endogenous	Adaptor molecules recruited
1 Plasma membrane	Forms heterodimers with TLR2 which recognise tri-acyl lipoproteins from Gram-negative bacteria and mycoplasma (e.g. Pam3CSK4)	TLR1-TLR2 heterodimers recognise HMGB1, heat shock proteins (putative), and uric acid crystals	TIRAP, MyD88
2 Plasma membrane	Bacterial lipopeptides, peptidoglycan and lipoteichoic acid from Gram-positive bacteria, mycobacterial lipoarabinomannan, fungal zymosan, hemagglutinin from measles virus, tGPI-mucin from *Trypanosoma cruzi*	HMGB1, numerous ECM components, HSP (putative)	TIRAP, MyD88
3 Endosome	Viral dsRNA and the synthetic RNA analogue poly(I:C)	mRNA, stathmin	TRIF

4 Plasma membrane	LPS from Gram-negative bacteria, viral envelope proteins, GIPLs from *Trypanosoma*	HMGB1, numerous ECM components, β-defensin 2, heat shock proteins (putative). Oxidised LDL and amyloid-β recognised by putative TLR4-TLR6 heterodimer	TIRAP, MyD88, TRAM, TRIF
5 Plasma membrane	Flagellin (major structural component of bacterial flagella)	Unknown	MyD88
6 Plasma membrane	Forms heterodimers with TLR2 which recognise di-acyl lipoproteins from Gram-positive bacteria and mycoplasma	TLR2-TLR6 heterodimer recognises versican (ECM proteoglycan) and ω-(2-carboxyethyl)pyrrole Oxidised LDL and amyloid-β recognised by putative TLR4-TLR6 heterodimer	TIRAP, MyD88
7 Endosome	Viral ssRNA, group B *Streptococcus* RNA, synthetic poly(U) RNA, imidazoquinoline derivatives (imiquimod, resiquimod), guanine analogues (e.g. loxoribine)	Self-derived RNA	MyD88
8 Endosome	Viral ssRNA, resiquimod (human TLR8 only)	Self-derived RNA	MyD88
9 Endosome	Unmethylated CpG DNA motifs, hemozoin from *Plasmodium falciparum*	HMGB1, self-derived DNA	MyD88
10 Plasma membrane	Can recognise tri-acyl lipoproteins in a heterodimer with TLR2, but may lack signalling ability	Unknown	MyD88

PRRs can also be used in the innate immune system to assess how severe the detected threat is, and respond in an appropriate manner, for example not responding to commensal organisms despite them expressing some of the same MAMPs (e.g. LPS) as pathogens, and a strong reaction to the presence of organisms in otherwise sterile tissues (Blander and Sander, 2012). Studies have suggested that the innate immune system can fine tune the response to differentiate between varying levels of risk based on five checkpoints, which allows maintenance of the optimal balance between low inflammation caused collateral damage and pathogen eradication (Blander and Sander, 2012). Within the checkpoints the immune system can discriminate between particulate and soluble PAMPs, invasive and non-invasive microorganisms, viable and dead

microorganisms, pathogenic and non-pathogenic organisms, and tissue specific signals (Blander and Sander, 2012).

1.11.1.1. The role of PRRs in COPD

There is increasing evidence of the importance of PRRs in the development and progression of COPD; studies have shown CS can induce inflammatory processes by activating some innate immune cells, such as AM and epithelial cells, by directly or indirectly triggering PRRs on the AM surface through DAMPs released from dying cells or stressed cells, and also PAMPs (Brusselle, Joos and Bracke, 2011). DAMPs release can be triggered by oxidative stress, inhaled irritants and toxic agents, and tissue hypoxia after CS exposure (Brusselle, Joos and Bracke, 2011). CS exposure can also include LPS in the particulate phase, which can play a role in the pathogenesis of COPD and allow the growth of respiratory pathogens, which are a significant cause of exacerbations in COPD (Berenson *et al.*, 2015). These pathogens not only cause exacerbations, they can also perpetuate and amplify chronic inflammation via PAMPs even in stable COPD, which leads to the persistence of inflammation even after the cessation of smoking (Brusselle, Joos and Bracke, 2011). There is evidence to suggest that an altered regulation of PRR on AM may affect their ability to recognise and react to pathogens, and therefore any change in expression may be involved in bacterial colonisation and chronic inflammation (Droemann *et al.*, 2005).

Studies have shown there are changes in the expression of some TLR including TLR2 and TLR4 in AM between non-smokers, healthy smokers and COPD patients (Droemann *et al.*, 2005). By comparing the *ex vivo* expression of CD14, TLR2 and TLR4 in AMs from healthy smokers, COPD patients and healthy non-smokers Droemann *et al.* (2005) found that there was a reduced expression of TLR2 in smokers (13.98 ± 2.54 relative mean fluorescence intensity (rMFI)) and COPD patients (12.07 ± 3.5 rMFI) when compared to non-smokers (6.59 ± 1.425 rMFI, $p < 0.01$), and after exposure to LPS there was only an increase in TLR2 protein expression in non-smokers. Overall these results suggest that smoking and COPD caused a change in the AM phenotype, which resulted in an impaired response to microbial stimulation and could explain the persistent infections observed in some COPD patients (Droemann *et al.*,

2005). The further study of these types of changes and their functional relevance could provide targets for creating more effective treatments for COPD.

Further evidence for the importance of TLR in COPD severity comes from the study by Berenson *et al*. (2015) which investigated the effect of TLR polymorphisms on the innate immune response to respiratory pathogens. The effect of single nucleotide polymorphisms (SNP) of TLR is increasingly being recognised as playing a role in inflammatory diseases, and as an impaired or dysfunctional innate response to some pathogens, including *Streptococcus pneumoniae*, *Moraxella catarrhalis*, and non-typeable *Haemophilus influenza* can contribute to COPD morbidity, the effect of TLR SNP was investigated (Berenson *et al.*, 2015). They found there were no significant inter-group differences for the SNPs investigated, but there was a high frequency of expression of both SNP for TLR9, and there was a significant reduction ($p = 0.02$; $p = 0.008$; $p = 0.02$) in IL-8 responsiveness to the pathogens investigated (non-typeable *H. influenzae, M. catarrhalis* and *S. pneumoniae*) in COPD ex-smokers who had the TLR9(T1237C) SNP, which also correlated with a reduced FEV-1 % predicted ($p = 0.037$). (Berenson *et al.*, 2015). Overall, the results showed an association of TLR9(T1237C) expression with COPD severity and dysfunctional AM responses to respiratory pathogens, suggesting this could be used as a predictor of COPD severity for patients.

TLRs are not the only members of the PRR signalling pathways that are affected in COPD; there is evidence of increased bronchoalveolar lavage fluid (BAL) concentrations of uric acid, HMGB1, extracellular ATP16, receptor for advanced glycation end products (RAGE), when compared to smokers without COPD (Brusselle, Joos and Bracke, 2011). The activation of PRRs such as RAGE and TLRs increases pro-interleukin 1β expression, which is cleaved by NLR family pyrin domain-containing (NLRP) inflammasomes to form into mature interleukin 1β, which is involved in inflammation and emphysema (Brusselle, Joos and Bracke, 2011).

1.12. Involvement of other immune cells in COPD

1.12.1. Neutrophils

In COPD, neutrophils are attracted to the site of lung infection by the release of chemo-attractants from macrophages such as CXC-chemokine ligand 1 (CXCL1), CXCL8 and leukotriene B (MacNee, 2005; Barnes, 2008b; Taylor, 2010). Irritants such as cigarette smoke can elevate IL-8 by increasing the stability of IL-8 mRNA, which increases protein expression (Rab *et al.*, 2013). Neutrophils enter the lungs from the general blood circulation and they localise to the airways (Stockley, 2002). Within bronchial secretions high levels of neutrophils can be detected in smokers with COPD, but there is also a slight increase in their number in healthy smokers (Tamimi, Serdarevic and Hanania, 2012; GOLD, 2004). There is a strong positive correlation of neutrophil counts and IL-8 levels with smoking history, and also a negative correlation with predicted FEV1, which can help predict disease and inflammation severity (Tetley, 2002; Tamimi, Serdarevic and Hanania, 2012).

Neutrophils adhere to the endothelial cells by the action of molecules such as E-selectin that is up-regulated in COPD patients (Stockley, 2002), and they can produce a variety of proteases, such as cathepsin G, MMP-8, MMP-9, and proteinase 3, all of which can cause deterioration of the lung's parenchyma and mucus hypersecretion (Russell *et al*., 2002; Barnes, 2008a).

1.12.2. T cells

There is evidence to suggest that the tissue remodelling and destructive changes observed in COPD are caused by complex interactions between the adaptive and innate immune systems (Fairclough *et al.*, 2008). T cells play an important role in the pathogenesis of the disease, especially in emphysema (O'Donnell *et al.*, 2006). After activation by irritants (for example cigarette smoke, or pathogens), both epithelial cells and macrophages release chemotactic factors such as CXCL9, CXCL10 and CXCL11, which can all act on CXCR3 attracting T helper 1 (Th1) cells and type 1 cytotoxic T (TC1) cells to the lungs (Fairclough *et al.*, 2008; Gosman *et al.*, 2006; Barnes, 2008a). These cells then release proteases, for example MMP9, which cause elastin degradation

and the alveolar destruction seen in emphysema (Barnes, 2008b). The importance of T cell responses in COPD have also been shown by T cell depletion in a mouse model of COPD, as this inhibited alveolar destruction (Rab *et al.*, 2013).

T cells originate from thymocytes, which are multipotent DN (double-negative) cells that express αβ TCRs (T-cell receptors) and are the precursors for both αβ $CD8^+$ (cytotoxic) and $CD4^+$ (helper) T-cells. During development, they go through three main stages which are defined by expression of the CD8 and CD4 co-receptor molecules, forming the DN $CD4^-CD8^-$, then the double positive (DP) $CD4^+CD8^+$ and finally the single positive (SP) $CD4^+CD8^-$ or $CD4^-CD8^+$. Once the cells are committed to forming $CD4^+$ or $CD8^+$ cells, they can further differentiate into a type 1 or type 2 cell (Fairclough *et al.*, 2008). Of particular interest are $CD8^+$ T cells as these are strongly associated with emphysema and the Th1 response via IFN-γ secretion, which is crucial in response to bacterial and viral infections, and also macrophage activation (Cosio and Majo, 2002; Chrysofakis *et al.*, 2004; Urbanowicz *et al.*, 2010). Their importance is supported by studies that have shown that there is an increased number of $CD8^+$ T-cells in both the lower respiratory tract and the peripheral airways of patients with COPD (Wang *et al.,* 2013). After activation, the $CD8^+$ T cells can express cytotoxic products like perforins and granzymes, which can lead to apoptosis and cell destruction of the parenchyma of the lung (Costa *et al.*, 2008).

In addition, $CD4^+$ T cells play an important role in COPD during the differentiation of Th1 cells, $CD4^+$ T cells prime $CD8^+$ T cells to activate them, help in their survival and maintain their memory (Costa *et al.*, 2008). Another subtype of $CD4^+$ T cells (Th17) are important in COPD progression as they produce IL-17, which recruits neutrophil infiltration through the stimulation of lung epithelial cells to release CXCL1 and CXCL8 (Gadgil and Duncan, 2008; Gosman *et al.*, 2006). The development of Th17 cells required the transcription factor ROR*γ* t (retinoic orphan receptor *γ* t, also known as RORC2). After development Th17 cells can express IL-6R, IL-23R and TGF-*β*R1 on their cell surface, along with chemokine receptors such as CCR4 and CCR6, which have been shown to be important in translocating to inflamed tissues (Lane *et al.,* 2010).

Both $CD4^+$ and $CD8^+$ T cell subtypes can release IFN-γ which activates alveolar macrophages to secrete a number of cytokines including IL-12, which is necessary to maintain T cell replication and activation (Gadgil and Duncan, 2008).

1.12.3. Natural Killer Cells (NKC)

NK cells are important in the innate immune response, as they are cytotoxic lymphocytes capable of killing viral infected or transformed cells due to the secretion of their granules, which contain mediators like perforins and granzymes (Caligiuri, 2008). These mediators, along with cytokines, fight pathogens and help to activate T cells, which stimulate the adaptive immune response (Caligiuri, 2008). By connecting the innate and the adaptive immune responses, NK cells act as the first line of defence against microbial infections that occur frequently in COPD patients (Stefanska and Walsh, 2009). NK cells have the ability to recognise stressed cells that do not present MHC and they can be activated by a range of activating receptors such as Ly49 homodimers, NCR (natural cytotoxicity receptors), and CD94: NKG2 heterodimers (Hong and Lee, 2010).

Despite its predicted importance in COPD, the function of NK cells in this disease is not fully understood. Studies have shown the number of NK cells is either the same or decreased and that they have reduced phagocytic activity in both smokers who have COPD and healthy smokers, indicating that there is no specific change in COPD, which suggests other mechanisms that cause cell death, such as $CD8^+$ and NKT cells, are more important in COPD pathogenesis (Fairclough *et al.*, 2008). Other studies have shown that the role of cytotoxic effector cells were affected in COPD patients, due to the decreased numbers of NK ($CD56^+$ $CD3^-$) and NKT-like cells ($CD56^+$ $CD3^+$) in their blood, and conversely, an increase in these cells in the sputum of COPD subjects (compared to healthy non-smokers and smokers), which again suggests T cells might have a more significant cytotoxicity role in COPD (Urbanowicz *et al.*, 2009, 2010). This suggestion is backed up by the fact that there is an increase in T and Th17 cells (a subset of $CD4^+$ T-lymphocytes) that secrete cytokines such as IL-10 and IL-17 which have a significant cytotoxicity role in the COPD (Urbanowicz *et al*., 2010).

Studies show that upon cessation of smoking in COPD patients, the cytotoxic ability of NK cells is not upregulated, meaning that there are other regulators involved in their suppression (Culley, 2009). Studies suggest that modulating the activation of killer cells (including NK, NKT and $CD8^+$) could be a goal for COPD therapy (Wang *et al.,* 2013).

1.12.4. Dendritic cells (DCs)

Dendritic cells (DCs) are leukocytes that are found abundantly at sites of higher antigenic exposure like skin and mucosal surfaces and they spread through the peripheral blood and lymphatic systems (Holt and Stumbles, 2000). DCs are APCs and their main role is the uptake of invading antigens, via phagocytosis, and presenting them to T cells. As they are located at mucosal surfaces and present antigens to T cells, it has been suggested they may play a role in COPD progression (Tamimi, Serdarevic and Hanania, 2012). They are activated by a wide range of PAMPS which affect the markers expressed on their surface, for example: CD80, CD83, CD86, CD40 and high levels of Major Histocompatibility Class 2, (MHC II) (Mohamadzadeh and Luftig, 2004). These cells do not have a long life span and are replaced by a new set of cells (Mohamadzadeh and Luftig, 2004), and young DCs can express molecules that relate to antigen absorption such as mannose and Fc receptors (Stefanska and Walsh, 2009).

DCs have two main subtypes in both humans and mice: Myeloid DCs (mDCs), which are also known as conventional or classical DCs, and plasmacytoid DCs (pDCs). These classes are differentiated by the type of markers that are expressed on the surface of the cell as well as their function (Ueno, Palucka and Banchereau, 2010). mDCs express CD11b (important for T cell activation) as a surface marker, and they are important in the maintenance of self-tolerance and autoimmunity induction; whereas pDCs express CD45 isoform (B22), which is usually found on B cells. mDCs can upregulate co-stimulatory proteins and MHC expression after pathogen or DAMP activation, which can activate naïve $CD4^+$ and $CD8^+$ T cells (Benvenuti, 2016). Thus, mDCs play a crucial role in the activation of T cells and antigen presentation, which is important in COPD due to the high level of infections seen and the increased levels of T cell activation (Zuniga *et al.*, 2004). Another important factor contributing to COPD is the effect of exposure to CS; studies have shown that mDCs are significantly affected by

CS, which leads to T-cell stimulation suppression, and a reduction in the immunogenic properties of DCs (Stampfli and Anderson, 2009). Studies both *in vitro* and *in vivo* in murine and humans have provided evidence of the changes that occur in DCs; CS impaired the maturation of mDCs in the lungs in humans, and reduced the expression of co-stimulatory molecules in a mouse model, which suggests they would have a reduced ability to stimulate T-cells (Robbins *et al*., 2008; 2004; Liao *et al.*, 2015; Arellano-Orden *et al.,* 2016). CSE has been shown to differentially affect T-cell stimulation depending on the cell type ($CD4^+$ or $CD8^+$) and length of treatment in mouse bone marrow-derived DCs (bmDCs) (Mortaz *et al.*, 2009; Givi *et al.*, 2015). Studies in human monocyte-derived DCs (moDCs) with CSE have shown there is a reduced maturation rate and lower ability to stimulate Th17 and Th1 cells, which means there are lower levels of T-cell stimulation (Vassallo *et al.*, 2005; Kroening *et al.*, 2008; Le Rouzic *et al.*, 2016). Another study, conducted by Alkhattabi (2015), supports this as it showed that CS and nicotine can inhibit the expression of proteins and signalling molecules essential for the activation, migration and survival of DCs, and their ability to activate T-cells, which overall suggests CS can suppress the immunogenic potential of DCs. All these studies point toward DCs being important in the development and progression of COPD, but more research is required to ascertain what the changes are in terms of abundance of DCs and their profile in COPD patients.

1.12.5. B cells

There is evidence that the total number of B cells increases in the central airways of moderate to severe COPD patients (Zeimpekoglou, 2008; Brandsma *et al.*, 2009), but the actual role of B cells, and what stimulates them, in COPD is still not fully understood.

Studies have shown that $CD4^+$ T cells (activated by cytokines) are involved in the activation of $CD8^+$ T cells (Urbanowicz *et al.,* 2010), but they can also cause differentiation of the Th2 subset of T cells which can recruit B cells and stimulate them to produce antibodies (Brandsma *et al.*, 2009). This suggests the increase in $CD4^+$ T cells may be having a dual effect in response to bacterial and viral colonisation of COPD patients (Zeimpekoglou, 2008).

The overreaction of the immune system seen in COPD may be in part due to the increased number of B cells observed. Recent research suggests that B cells can be involved in the development auto-immunity in COPD due to oxidative stress from irritants, such as cigarette smoke, triggering B cells to secrete auto-reactive antibodies against self-antigens (Jones and Ostrem, 2011; Price *et al.*, 2011). Studies have shown that in the lungs of COPD patients, there can be high concentrations of B cells in the inflammatory infiltrate, especially in patients with emphysema (Gosman *et al.,* 2006; Faner *et al.,* 2016). An increase in B cells has also been linked to an increase in autoantibodies in COPD patients, with the production of specific autoantibodies to multiple autoantigens varying between patients (Lee *et al.*, 2007; Feghali-Bostwick *et al.*, 2008; Leidinger *et al.*, 2009; Stefanska and Walsh, 2009; Karayama *et al.*, 2010; Kuo *et al.*, 2010; Kirkham *et al.*, 2011; Núñez *et al.*, 2011; Packard *et al.*, 2013). There is also evidence to suggest there is an association of both natural and disease-specific autoantibodies in COPD (Daffa *et al.*, 2015).

1.13. Management and treatment of COPD

COPD is a chronic disease that can be treated and prevented by taking some precautions and following the instructions of the physicians. In order to control COPD, there are two main approaches that are followed: non-therapeutic and therapeutic.

The non-therapeutic treatments mainly depend on increasing the patients' awareness of the causes of COPD by: educating the patients to avoid risk factors of COPD (Sutherland and Cherniack, 2004), and stopping smoking as this is the leading cause of COPD and other serious diseases. The cessation of smoking can not only improve lung function, but also enhance the quality of the patient's life. However, in more advanced forms of COPD, stopping smoking may not stop or reverse the effects of the disease, some patients still suffer from chronic bronchitis due to irreversible functional and anatomic changes, in which case other treatments are required (Price *et al.*, 2011; Rab *et al.*, 2013).

Mucus hypersecretion and stasis directly contribute to bacterial infections and subsequent inflammatory responses and are therefore considered causative for the lung function decline seen in COPD patient populations (Tamimi, Serdarevic and Hanania,

2012). There are a large number of medications that are used to treat COPD, but they must be used systematically in a step-by-step approach depending on the severity of the disease. One of the most common treatments is bronchodilators, such as long acting muscarinic antagonist (LAMA), used in combination with inhaled corticosteroids. LAMA works by inhibiting muscarinic receptors on the bronchial airways, which leads to muscle relaxation, bronchodilation and a small improvement in lung function. Although these treatments can reduce the number of acute exacerbations, increase quality of life and improve dyspnoea, they do not significantly improve the mortality associated with COPD (Price *et al.*, 2011; Rab *et al.*, 2013). Other treatments such as long acting B2-agonists (LABA), which work by improving the mucociliary component of COPD, and mucolytics, such as *N*-acetyl-cysteine and carbocysteine, which reduce mucus secretions can be beneficial to some patients but again do not reduce mortality (Tamimi, Serdarevic and Hanania, 2012). Supplemental oxygen can be life-saving in those who are hypoxic, but does not alter COPD pathogenesis (Rab *et al.*, 2013).

The use of corticosteroids to treat COPD is a controversial issue and a number of studies showed that inhaled steroids enhance the risk of pneumonia and have no effect on mucus obstruction or its clearance (Tamimi, Serdarevic and Hanania, 2012). As more information is discovered about the underlying causes of COPD and how the different immune and epithelial systems interact, other potential treatments can be investigated which can both alleviate symptoms and reduce the mortality associated with COPD.

1.14. Modelling macrophages and COPD

1.14.1. Animal models of COPD

As there are ethical issues in using patients with COPD for studies, one alternative is to use animal models, as they can provide information about pathophysiology, physiology, and the basic mechanisms underlying COPD, even though they can only model some aspects of the disease (Vahideh *et al*., 2017). Animal models have been induced mainly in rats, guinea pigs and mice, using various doses and time courses of intra-tracheal LPS, intranasal elastase and more commonly CS, and have measured lung inflammation (mediators and cells) and lung pathological data (Vahideh *et al*., 2017).

Emphysema has been modelled in dogs, monkeys, sheep, rodents and guinea pigs and can be induced by administration of particulates, chemicals, proteinases, as well as exposure to CS, and the results produce some of the characteristics associated with human COPD (Mahadeva & Shapiro, 2002). Genetic manipulation, particularly in mice, can also produce relevant models for emphysema and COPD, and these are sometimes the accidental product of studies in other areas (Mahadeva & Shapiro, 2002). Some examples include mice lacking expression of pulmonary surfactant protein-D (SP-D-/- mice) generated to measure the importance of SP-D in the defence of the lung, and a mouse model that over-expresses RAGE in the pulmonary epithelium has been used to model features of COPD in the adult lung (Yoshida & Whitsett, 2006; Wright & Churg, 2008; Mahadeva & Shapiro, 2002; Robinson *et al*., 2012). There are some naturally occurring mice strains that develop emphysema such as the tight skin, beige, blotchy, osteopetrotic and pallid strains (which has decreased levels of α-1-antitrypsin) (Yoshida & Whitsett, 2006; Wright & Churg, 2008; Mahadeva & Shapiro, 2002). There are other knockout mouse models that can be used to model some of the changes in COPD including platelet derived growth factor A (–/–), fibroblast growth factor receptors 3 and 4 (–/–), elastin (–/–), retinoic acid receptor-g (–/–), tumour necrosis factor-α converting enzyme (–/–), tissue inhibitor of metalloproteinases-3 (–/–),and MMP-12 (–/–), all of which produce different aspects associated with emphysema and COPD development (Yoshida & Whitsett, 2006; Wright & Churg, 2008; Mahadeva & Shapiro, 2002). The final group of mouse models that need to be considered are the models where an over-expression of a protein leads to emphysema symptoms, examples of these include IL-13, IFN-γ, and MMP-1 (human interstitial collagenase) (Yoshida & Whitsett, 2006; Wright & Churg, 2008; Mahadeva & Shapiro, 2002).

1.14.2. An *in vitro* cell line model

Macrophages have important functions in many diseases, which make understanding their biology critical if these diseases are to be managed effectively. One way to understand their functions is to use *in vitro* cell lines (for example THP-1 human pro-myeloid cell line), which allow researchers to manipulate the interactions that macrophages have with their environment and with other immune cells (Tsuchiya *et al.*, 1980).

Early studies showed significant similarities in the morphology and differentiation properties of THP-1 cells when compared to primary monocytes and macrophages (Tsuchiya *et al.*, 1980). THP-1 grows as single round cells in suspension that contains Fc receptors, C3b receptors and surface or cytoplasmic immunoglobulins; they produce IL-1, stain positive for α-naphthyl butyrate esterase, produce lysosomes and are phagocytic (Tsuchiya *et al.*, 1980).

THP-1 cells can restore the response of purified T-lymphocytes to Con A, they show increased CO_2 production upon phagocytosis and can be differentiated into macrophage-like cells using certain reagents like phorbol 12-myristate 13-acetate (commonly known as PMA or TPA) or vitamin D3 [1, 25 (OH) 2D3], which causes them to adhere to the bottom of the culture plates (Tsuchiya *et al.*, 1980; Schwende *et al.*, 1996). After differentiation THP-1 undergo morphological changes to form cells that have a macrophage-like phenotype; for example, the cells have flat and amoeboid shapes with larger numbers of free ribosomes in the cytoplasm, in addition to well-developed Golgi apparatus and rough endoplasmic reticulum (Tsuchiya *et al.*, 1982). PMA treated THP-1 cells are frequently used to investigate the functions of macrophages, and a comparison between primary human monocyte–macrophages and PMA or 1,25 (OH) 2D3 treated THP-1 cells by Daigneault *et al.* (2010) highlighted the phenotypical changes of THP-1 and similarities between the cell types (Daigneault *et al.*, 2010).

In the lab, the use of THP-1 cells has some advantages over the use of human primary monocyte–macrophage cells, including low genetic variability, ease of transformation and storage; but for studying some interactions, for example where genetic variability is an advantage, it is more practical to use human primary monocytes or macrophages (Daigneault *et al.,* 2010).

1.14.3. Investigating the role of macrophages in COPD using Peripheral Blood Mononuclear Cells

Another technique used to understand macrophage functions is the use of *in vitro* Peripheral Blood Mononuclear Cells (PBMCs), which allow researchers to manipulate

the interactions that macrophages undergo with their environment in general, and also with other immune cells in particular.

Human PBMCs have been widely used as controls, both positive and negative, for example in ELISA and in flow cytometry assays (Slota *et al.*, 2011). This type of cell is characterised by a round nucleus, such as a lymphocyte, or a monocyte. Such cells have a crucial role in the immune system in fighting infection. The population of lymphocyte contains different kinds of cells, including $CD4^+$ and $CD8^+$ T cells, B cells, NK cells. By using histopaque 1077 (ficoll) all these cells can be isolated from whole blood, and by using a Buffy coat underneath, a layer of plasma is formed by a hydrophilic polysaccharide that separates layers of blood, with monocytes and lymphocytes detectable (Vissers *et al.*, 2013).

To detect macrophages in the culture the presence of different cell surface markers can be investigated using flow cytometry, such as CD14, CD16, CD206 and CD11b:

CD14 is known as LPS receptor that can interact with Endotoxin, Lipopolysaccharide (LPS), TLR4, LBP, LY96, and TLR2. It is a co-receptor of innate immunity that is strongly expressed on monocytes, macrophages, and weakly on neutrophils (Khazen *et al.*, 2005). CD16 is known as FCRIIIA, Fc γ receptor, which can associate directly with activated neutrophils. Generally, CD14 and CD16 are found together on secretory vesicles of neutrophils, but CD16 is also expressed on macrophages and NK cells (van de Velde, Mottram and Hogarth, 2006).

CD206, also known as the macrophage mannose receptor (MMR), is a scavenger receptor that is found principally on the surface of tissue macrophages, lymphatic and hepatic endothelial cells in both humans and mice (Stahl, 1992; Taylor, 2001). This marker is identified by the carbohydrate pattern and it plays an important role in phagocytosis of microorganisms in both host defence and homeostasis (McKenzie *et al.*, 2002).

CD11b, the alpha-chain of the leucocyte integrin Mac-1, can be found on the surface of macrophages, neutrophils, monocytes, NK cells, and subclasses of lymphocytes (Khazen *et al.*, 2005). This marker not only plays a crucial role in the migration of

leukocytes from peripheral blood to the sites of infection (Springer, 1990), but is also implicated in adhesion (Butcher, 1990).

In vitro, both granulocyte macrophage colony stimulating factor (GM-CSF) and macrophage colony stimulating factor (M-CSF) are involved in the differentiation of monocytes to macrophages (Jaguin *et al.*, 2013). Stimulating monocytes with GM-CSF produces the M1 macrophage subtype with a "pro-inflammatory" cytokine profile such as IL-23 and TNF production. On the other hand, treating monocytes with M-CSF produces M0 macrophages, which has some similarities to the "anti-inflammatory" cytokine profile (Lathrop *et al.*, 2015).

1.14.4. Activation of AMs in model systems

There are also other methods of investigating the effect of macrophages, and in particular AMs, in model systems. In the literature, the activation of AMs has been studied in different model organisms and using different activation methods, such as:

- Treating chimeric mice, parabiosis, and adoptive cellular transfer models with lethal irradiation, leads to generation of AMs from bone marrow-derived blood monocytes (Guilliams *et al.*, 2013). These macrophages can adapt to produce various functional and morphological properties (Vlahos and Bozinovski, 2014);
- Inoculating mice with influenza virus leads to the generation of AMs through self-renewal proliferative mechanisms (Hashimoto *et al.*, 2013);
- TLRs recognise antigens, which leads to the induction of actin polymerisation, and thus AMs will detach from alveolar epithelial cells (Takabayshi *et al.*, 2006).
- AMs, through a negative feedback mechanism, can produce TGF-β directly, or produce proteases, which would lead to TGF-β production, thereby leading to suppression of T cell activation and returning AMs to homeostasis respectively (Coleman *et al.*, 2013).
- Surfactant protein A induces the release of reactive oxygen and nitrogen intermediates (Hickman-Davis *et al.*, 2002).

- Engulfing dying cells has been suggested to induce anti-inflammatory or suppressive properties in AMs due to the detection of low levels of GM-CSF, TNFα, IL-8, and IL-1β (Fadok *et al.*, 1998).
- Expression of the scavenger receptor MARCO leads to priming AMs against *Streptococcus pneumonia* in the lungs (Arredouani *et al.*, 2004).
- Cigarette smoke and other irritants induce AMs to release inflammatory mediators (Vlahos and Bozinovski, 2014).

All these methods have different advantages and disadvantages, allowing different aspects of the involvement of the macrophages in disease progression, but for this study we are particularly interested in the effect of cigarette smoke on the role of macrophages and how this might affect COPD progression.

1.15. The effects of cigarette smoke extract (CSE) on macrophages

As cigarette smoke extract (CSE) had been shown to play a role in COPD progression, the effect on macrophages needs to be elucidated. In the literature, there have been many studies on the effects of CSE on different cells involved in the inflammatory response including lung macrophages from humans and animals, and on cell lines, and also bronchial and lung epithelial cells directly (Smith *et al.*, 2010). Studies have generally measured the phenotypic and transcriptomic effects of CSE on pro-inflammatory mediators, pro-inflammatory cytokines, reactive oxygen and reactive nitrogen species (ROS, RNS) and also enzymes such as proteases (Smith *et al.*, 2010; Yin *et al*. 2014; Kojima *et al*. 2013; Boorsma 2013; Wright *et al*. 2012; Robinson *et al*. 2012).

A selection of pro-inflammatory mediators have been measured in macrophages from different sources exposed to CSE (Table 1.6), and these included the chemokines CCL-2, CCL-3 monocyte chemotactic protein-1 (MCP-1), LPS, NF-κB, Ras, leukotriene B4 (LTB4) involved in chemotactic mediator synthesis, SAA which can induce expression of CD163, and also RAGE, which are multi-ligand cell surface receptors of the immunoglobulin family expressed by epithelium and macrophages (Vlahos and Bozinovski, 2014; Smith *et al.,* 2010; Yin *et al.,* 2014; Boorsma, 2013; Robinson *et al.*, 2012).

Many different pro-inflammatory cytokines were investigated (Table 1.6), but the ones that were most studied include IL-6, IL-8, IL-1β, IFNγ, TNFα, macrophage inflammatory protein-1α (MIP-1α), and GROa (Robinson *et al.,* 2012; Smith *et al.*, 2010; Yin *et al*., 2014; Boorsma, 2013).

Table 1.6: The mediators and cytokines that are released by macrophages when exposed to CSE. CCL = CC-chemokine ligand, CXCL = CXC-chemokine ligand, MCP = monocyte chemotactic protein, LPS = Lipopolysaccharide, NF-κB = nuclear factor kappa-B, LTB4 =Leukotriene B4, SAA = serum amyloid A, RAGE = receptors for advanced glycation end-products, IL = interleukin, IFNγ = Interferon gamma, TNFα = tumour necrosis factor-alpha, MIP-1α = macrophage inflammatory protein-1α, and GROa = growth regulated oncogene-alpha.

Mediators	Cytokines
CCL-2, CCL-3	IFNγ
CXCL1, CXCL8, CXCL9, CXCL10, CXCL11	IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-17
MCP-1	IL-1α, IL-1β
LPS	TNFα
NF-κB	MIP-1α
Ras	GROa
LTB4	
SAA	
RAGE	

The other factors involved that were measured included ROS, RNS, matrix metalloproteases (MMP-9, MMP-2, MMP-12), NF-κB, AP-1, MAPK activation/inhibition including p38 MAPK, ERK, peroxisome proliferator-activated

receptor gamma (PPARγ), toll-like receptors (TLR2, TLR4), apoptosis inhibitor of macrophage (AIM), and B-cell lymphoma extra-large (Bcl-xL) (Vlahos and Bozinovski, 2014; Smith *et al*., 2010; Yin *et al*., 2014; Kojima *et al.,* 2013; Boorsma, 2013).

Changes at the transcriptome level (measured by RT-PCR and micro-array) were generally mirrored by changes at the phenotypic level (measured by Western blot, immuno-histochemical evaluations and ELISA). These studies found that there was no change observed for cytokines IL-5, IL-3, or IL-17 in the CSE-exposed animals (Smith *et al.*, 2010).

CSE initially delayed the production of MIP-1b, MCP-1, and the cytokines IL-1β, and IL-6, and the LPS-induced response (Smith *et al.*, 2010; Yin *et al.,* 2014). CSE exposure also increased the production of IL-8, TNFα (regulated primarily by the Erk 1/2 pathway), IL-1α, IL-6, MIP-1α, LTB4, Ras, p38 MAPK, MMP-1 and MMP-9, and this was associated with increased ROS release; whereas IL-2, IL-4, IL-10 and PPARγ were down-regulated, and this corresponded to a reduced particle endocytosis (Vlahos and Bozinovski, 2014; Smith *et al.,* 2010; Yin *et al*., 2014; Beck-Schimmer *et al*., 2005). The results for IFNγ varied between the studies; expression either did not change (Smith *et al.*, 2010), or decreased after CSE exposure (Boorsma, 2013), suggesting more research is needed to determine its involvement in CSE induced inflammation.

CSE is a potent stimulator and activator of IL-18 and the IL-18 pathway, and IL-18R signalling was shown to play a critical role in CSE inflammation (Smith *et al.*, 2010) The studies also showed an increased expression of TLR4 and TLR2, suggesting that TLR4-mediated inflammatory signalling is important in the CSE-related inflammatory pathogenesis (Yin *et al*., 2014).

Another study focused on the effect of CSE on AIM, and found that AIM-positive macrophages were significantly increased after CSE exposure, which was also linked to a significant increase in Bcl-xL expression (Kojima *et al*., 2013). SAA has been shown to increase on CSE exposure, which can target the ALX/FPR2 receptor and reduce the activity of pro-resolving ligands such as LipoxinA4, which normally stimulates the non-phlogistic clearance pathways (Vlahos and Bozinovski, 2014). CSE

significantly increased RAGE expression and the data suggests pro-inflammatory cytokine secretion occurs via RAGE-mediated mechanisms (Robinson *et al.,* 2012).

A large study of the effect of CSE on gene expression in THP-1 and human PBMCs, showed that the CSE concentration and sample time can have an effect on the number of genes altered, with a large number of genes being affected after 24 h (311 up-regulated, 306 down-regulated) than after 8 h (199 up-regulated, 206 down-regulated), which shows that the timing of the gene assay can affect the genes highlighted (Wright *et al*., 2012). The results showed that the major pathways altered were associated with cell survival, such as inducible antioxidants, protein chaperone and folding proteins, and the ubiquitin/ proteasome pathway (Wright *et al*., 2012).

Studies suggest that CSE exerts its immunosuppressive effect through an oxidant-dependent mechanism (Smith *et al.*, 2010). CSE can result in oxidative stress in lungs which leads to disruption of GSH metabolism, and causes a decreased ability of AMs to phagocytose bacteria (Marti-Lliteras *et al.*, 2009). This can also cause a decreased ability to perform efferocytosis of dying cells (Hodge *et al.*, 2007) as a result of a defect in actin polymerisation due to an oxidant-dependent inhibition of Rac1 (Minematsu, Blumental-Perry and Shapiro, 2011) and activation of RhoA (Richens *et al.*, 2009).

Some studies have shown that cigarette smoke led to an increased change of AMs into the M2 gene profile (Figure 1.8), with a higher level in smokers with a normal lung function than in smokers with COPD (Shaykhiev *et al.*, 2009). In addition, research has shown that the number of macrophages in the lungs, airways, Broncho alveolar lavage fluid (BALF), parenchyma, and sputum of patients with COPD is increased 5 to 10-fold in comparison with healthy subjects (Pesci *et al.*, 1998). However, this increase is not necessarily beneficial, as it has been shown that depletion of lung macrophages can results in signs of recovery from emphysema, for example (Beckett *et al.*, 2013).

Overall the studies suggest CSE caused inflammation and had detrimental effects on the metabolism and function of innate immune cells, and these studies suggest that the signalling pathways that should be investigated by RPPA in this project to determine the effect of CSE on signalling in macrophages are the LPS-induced response, the IFNγ pathway, the cytokine pathway and TNFα.

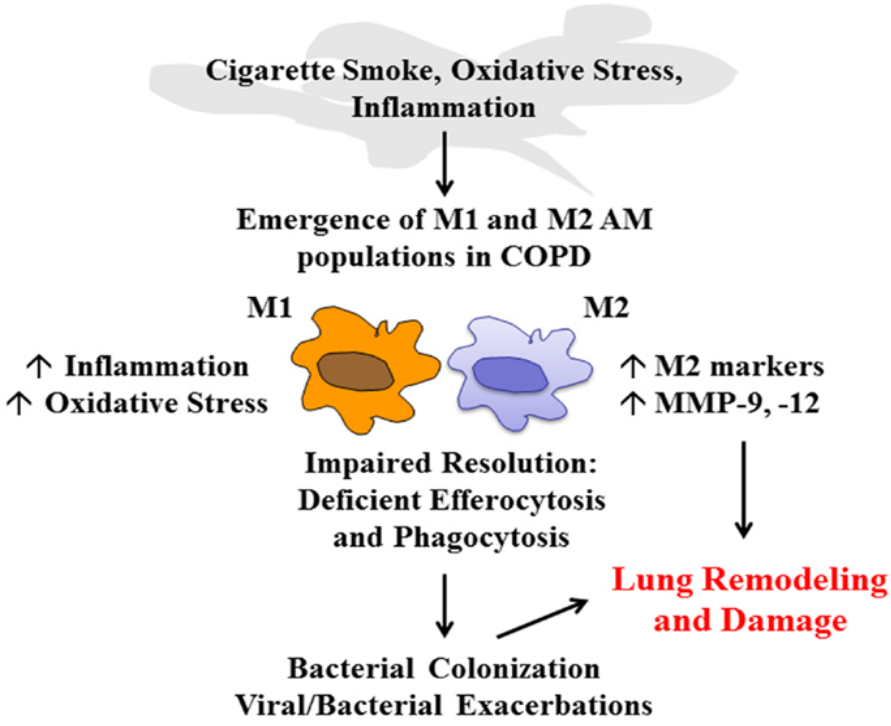


Figure 1.8: AMs in COPD.

The generation of M1 or M2 AMs will depend on the external signal (cigarette smoke, oxidative stress, or airway inflammatory microenvironment). A higher M1/M2 ratio will result in more oxidative stress and inflammation, leading to disruption of phagocytosis and efferocytosis, and hence exacerbation in COPD. A higher M2/M1 ratio will lead to, among other things, increased protease production, which can lead to deleterious lung damage and remodelling. (Adapted from (Vlahos & Bozinovski, 2014)).

1.15.1. Contents of cigarette smoke extract

Studies into the components of cigarette smoke have shown that it contains more than 5,000 chemicals, some of which are carcinogenic or toxic. One paper produced an electronic database for different smoke components which had over 2,200 entries, containing emission level details for 542 of the entries and assessed the human inhalation risks for 98 smoke components that were at detectable levels (Table 1.7) (Talhout *et al*., 2011). This high level of toxic and carcinogenic components in smoke can be linked to respiratory, carcinogenic, and cardiovascular effects observed in smokers, which could lead to death (Talhout *et al*., 2011).

Table 1.7: List of hazardous tobacco smoke components with their cancer and non-cancer inhalation risk values.

[1]Cancer inhalation risk values provide an excess lifetime exposure risk, in this case the human lung cancer risk at a 1 in 100,000 (E-5) level. [2]Non-cancer inhalation risk values indicate levels and exposure times at which no adverse effect is expected; here values for continuous lifetime exposure are listed (Adapted from Talhout *et al*., 2011).

Smoke component	**Cancer risk value[1] (mg m^{-3})**	**Non-cancer risk value[2] (mg m^{-3})**	**Effects and targets**
1,1,1-Trichloro-2,2-bis(4-chlorophenyl)ethane (DDT)	1.00E-04		
1,1-Dimethylhydrazine	2.00E-06		
1,3-Butadiene	3.00E-04	2.00E-03	reproduction
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TEQ)	2.60E-04		
2-Amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeAaC)	2.90E-05		
2-Amino-3-methylimidazo[4,5-b]quinoline (IQ)	2.50E-05		
2-Amino-6-methyl[1,2-a:3',2"-d]imidazole (GLu-P-1)	7.10E-06		
2-Aminodipyrido[1,2-a:3',2"-d]imidazole (GLu-P-2)	2.50E-05		
2-Aminonaphthalene	2.00E-05		
2-Nitropropane		0.02	liver, focal vacuolization and nodules
2-Toluidine	2.00E-04		
3-Amino-1,4-dimethyl-5H-pyrido [4,3-b]indole (Trp-P-1)	1.40E-06		
3-Amino-1-methyl-5H-pyrido[4,3-b]-indole (Trp-P-2)	1.10E-05		
4-Aminobiphenyl	1.70E-06		
5-Methylchrysene	9.10E-06		
7H-Dibenzo(c,g)carbazole	9.10E-06		
2-Amino-9*H*-pyrido[2,3-b]indole (AaC)	8.80E-05		
Acetaldehyde	4.50E-03	9.00E-03	nasal olfactory epithelial lesions
Acetamide	5.00E-04		
Acetone		30	neurological effects
Acetonitrile		0.06	mortality
Di(2-ethylhexyl) phthalate	4.20E-03		

Dibenzo[a,i]pyrene	9.10E-07		
Dibenzo[a,h]acridine	9.10E-05		
Dibenzo[a,h]anthracene	8.30E-06		
Dibenzo[a,j]acridine	9.10E-05		
Dibenzo[a,h]pyrene	9.10E-07		
Dibenzo[a,l)pyrene	9.10E-07		
Dibenzo[a,e]pyrene	9.10E-06		
Dibenzo[c,g]carbazole	9.10E-06		
Dimethylformamide		3.00E-02	digestive disturbances; minimal hepatic changes
Ethyl carbamate	3.50E-05		
Ethylbenzene		0.77	liver and kidney effects
Ethylene oxide	1.10E-04		
Ethylenethiourea	7.70E-04		
Formaldehyde	7.70E-04	1.00E-02	nasal irritation
Hexane		0.7	neurotoxicity
Hydrazine	2.00E-06	5.00E-03	fatty liver changes
Hydrogen cyanide		3.00E-03	CNS and thyroid effects
Hydrogen sulfide		2.00E-03	nasal lesions
Indeno(1,2,3-c,d)pyrene	9.10E-05		
Isopropylbenzene		0.4	increased kidney, adrenal gland weights
Lead	8.30E-04	1.50E-03	not applicable
Manganese		5.00E-05	neurobehavioral
m-Cresol		0.17	CNS
Mercury		2.00E-04	nervous system
Methyl chloride		0.09	cerebellar lesions
Methyl ethyl ketone		5	developmental toxicity
Naphtalene		3.00E-03	nasal effects
N-nitrosodi-n-butylamine (NBUA)	6.30E-06		
N-nitrosodimethylamine (NDMA)	7.10E-07		
Nickel		9.00E-05	chronic active inflammation and lung fibrosis
Nitrogen dioxide		1.00E-01	not applicable
N-nitrosodiethanolamine	1.30E-05		
N-nitrosodiethylamine	2.30E-07		
N-nitrosoethylmethylamine	1.60E-06		

N-Nitrosonornicotine (NNN)	2.50E-05		
N-Nitroso-N-propylamine	5.00E-06		
N-nitrosopiperidine	3.70E-06		
N-nitrosopyrrolidine	1.60E-05		
n-Propylbenzene		0.4	increased organ weight
o-Cresol	C- possible human carcinogen	0.17	decreased body weight, neurotoxicity
p-, m-Xylene		0.1	respiratory, neurological, developmental
p-Benzoquinone	C- possible human carcinogen	0.17	CNS
p-Cresol	C- possible human carcinogen	0.17	CNS
Phenol		0.02	liver enzymes, lungs, kidneys, and cardiovascular system
Polonium-210	925.9		
Propionaldehyde		8.00E-03	atrophy of olfactory epithelium
Propylene oxide	2.70E-03		
Pyridine		0.12	odour threshold
Selenium		8.00E-04	respiratory effects
Styrene		0.092	body weight changes and neurotoxic effects
Toluene		0.3	colour vision impairment
Trichloroethylene	82	0.2	liver, kidney, CNS effects
Triethylamine		7.00E-03	n.a.
Vinyl acetate		0.2	nasal lesions
Vinyl chloride	1.10E-03		

1.15.2. Effect of cigarette smoke on immune function and other lung diseases

Studies have shown that cigarette exposure can cause many different pathological changes which can result in disease and tissue damage (Cao *et al*., 2017). Cigarette smoking over the long-term (either passive or active) can cause pathological changes

in the airways, including inflammation, impaired mucociliary clearance, mucus hypersecretion, and altered ECM composition, which can lead to malignant transformation, remodelling, chronic bronchitis, asthma, lung cancer, idiopathic pulmonary fibrosis and COPD (Van der Vaart *et al*., 2004). The effect of cigarette smoke on mucus cells is of interest as mucus secretion and clearance is a highly regulated and balanced process that involves beating ciliated cells in the epithelium, mucus-producing goblet cells, and inflammatory cells, therefore any perturbations to this balance can result in cilia paralysis and goblet cell hyperplasia and lead to higher risks of respiratory problems (Haswell *et al*., 2010). These changes have been shown by different studies, including the study by Cao *et al.* (2017) in a well-differentiated human air-liquid-interface (ALI) *in vitro* airway tissue model using two different commercial cigarette smoke solutions (4 h exposure per day for 1–5 days); they found no cytotoxicity, but did find an increased mucin induction and hypersecretion (specifically of MUC5AC and MUC5B), MMP secretion and oxidative stress that reacted in a time- and dose-dependent manner (Cao *et al*., 2017). The secretion of MUC5B by non-mucous cells has been linked to the onset of airway diseases, and therefore may be an important indicator of airway health, along with ECM integrity disruption which has been linked to the initiation of cigarette smoke-associated respiratory diseases (Cao *et al*., 2017).

Other studies into the effects of CS on epithelial cell phenotype and pro-fibrotic responses in primary epithelial cells from COPD patients and healthy controls show that CS induced proliferation, apoptosis, caspase activity, and IL-8 release in a dose-dependent manner, with a higher response in normal epithelial cells than cells from COPD patients, suggesting CS promotes epithelial cell hyperplasia and activation, and this proliferative response reached a maximum at 10 % CSE then dropped sharply, probably due to toxicity (Murray *et al*. 2017). There were also CSE-induced expression increases for TGFβ1, GSK3β and col1α1, which fits with other studies (Heijink *et al*., 2011; Kode *et al*., 2006; Murray *et al*. 2017). This was further supported by work from the same group in a mouse model using CSE and Poly-IC to model viral exacerbations, which showed Poly-IC induced lung inflammation, enhanced CSE related inflammation, with increased caspase-3 expression in areas of alveolar inflammation,

but without any effect on lung tissue caspase-3/-7 activity with or without CSE (Murray *et al.* 2017). These results agree with the work of Lee *et al.* (2001) who showed smoking generates a pro-proliferative dose-dependent response in the epithelial cells of smokers, and the work of Kang *et al*. (2008) who found that Poly-IC in combination with CSE induced IL-18, type I IFN and IFN-γ, and that CSE enhanced the effects of influenza, which overall suggest CSE selectively enhances the alveolar and airway remodelling and inflammatory responses induced in the murine lung by viruses and Poly-IC (Kang *et al*., 2008; Lee *et al*., 2001).

Within the lung, cyclic adenosine monophosphate (cAMP), a secondary messenger, has been shown to play a key role in signalling cascades that are involved in a wide variety of pathophysiological and physiological processes, and its localisation and availability is controlled by adenylyl cyclases and phosphodiesterases (PDEs) (Zuo *et al*., 2018). Studies using a novel cAMP Förster resonance energy transfer (FRET) biosensor *in vivo* in mice, *ex vivo* precision cut lung slices and *in vitro* in human cell models have shown that CS exposure up-regulated activity and expression of PDE3 and PDE4, which reduce the availability of cAMP and can influence CS-induced pulmonary pathophysiology (Zuo *et al*., 2018).

The effect of CSE on asthma is of importance as in most developed countries 25 % of asthmatic adults are smokers, which can lead to more severe symptoms, increased inflammation, impairment of the short-term therapeutic response to corticosteroids and an accelerated decline in lung function (Thomson *et al*., 2004; Chalmers *et al*., 2002). Studies have shown there is a strong correlation between parents who smoke and the severity of the children's asthma symptoms; they had a 13 % lower mean FEV %, a 23% lower mean FEF and 47 % more symptoms, showing that indirect smoking can exacerbate the symptoms of asthma (Murray *et al*., 1986).

1.15.3. Effects of nicotine

There is some evidence that nicotine can suppress the immune system, which can be detrimental in many cases, but it could have therapeutic potential to be used as a neuroprotective and anti-inflammatory agent (Sopori 2002).

The main mode of action of nicotine is as an agonist of nicotinic acetylcholine receptors (nAChRs), which are a heterogeneous family of ligand-gated ion channels consisting of five membrane-spanning subunits that can mediate fast synaptic transmission and modulate the release of neurotransmitters, including glutamate and dopamine at synapses (Egleton *et al*., 2008; Wen *et al*., 2016). nAChRs are also expressed in keratinocytes and bronchial epithelium as they have other functions besides neurotransmission, including regulation of migration, angiogenesis, cell proliferation, secretion, apoptosis, and invasion, which are important in cardiovascular disease, lung disorders and cancer (Egleton *et al*., 2008; Improgo *et al*., 2011; Ambrosi & Becchetti 2013). There is evidence to suggest that that subunits α5, α4 and β2 are involved in nicotine addiction, and the α7 subunit of nAChR mediates proliferative effects of nicotine in lung cancer and mesothelioma cells (Egleton *et al*., 2008; Wen *et al*., 2016). Studies have suggested the underlying molecular mechanisms for the effect of nicotine in lung cancer include increased intracellular calcium levels, activation of cancer signal transduction pathways, and acetylcholine acting as a growth factor (Improgo *et al*., 2011; Ambrosi & Becchetti 2013).

1.16. Conclusions

COPD is a complex and chronic disease that affects the lives of many of humans all around the world. As the disease progression and interactions are not yet fully understood, more research is needed in order to uncover the detailed pathogenesis, which will hopefully allow researchers to design treatments not only to cure symptoms but also reduce the associated mortality. The severity of COPD ranges from mild to severe with different levels of bronchitis and emphysema observed in patients and as the disease progresses there are increasing death rates. There are a number of risk factors that are involved in the development of COPD, however smoking cigarettes carries the highest associated risk for all forms of the disease. The overreaction of immune and epithelial responses can play a negative role, which increases the complexity of the disease. So, ongoing research is trying to understand the role of the immune system in COPD.

Macrophages have a significant role in the pathogenesis of COPD and other lung related diseases as they recruit inflammatory cells to the lungs and release proteases that cause some of the damage seen in COPD (for example alveolar destruction and mucus hypersecretion); thus, more research is needed to identify what activates the macrophages, their possible role in the development of this disease, and the mechanism of their interaction with other immune cells in this disease.

As PBMCs are a convenient model of human primary monocyte–macrophages, they were used in this study. CD14 positive cells were isolated from buffy coats and stimulated with M-CSF to form macrophages, which were then stained with CD markers specific for macrophages to confirm the identity of the cells before exposing them to cigarette smoking extract (CSE) in order to investigate the changes to macrophages.

1.17. Aims and objectives

Current research into COPD suggests that macrophages, and particularly AMs, play an important role in disease progression, but the underlying mechanisms have not been fully elucidated. Therefore, to investigate this, the hypothesis of this thesis was that AMs are affected by CSE or nicotine in combination with LPS or Poly-IC, which causes an altered expression of key cell surface markers and results in changes in AM regulation and activity. The particularly novel aspect of this work is determining the effects of CSE on a wide range of signalling molecules in macrophages.

The first step in investigating this hypothesis was to find a suitable macrophage cell line to be used to allow changes in the cell surface markers to be analysed. Initially, THP-1 cells were investigated for this project, by culturing and differentiating cells with PMA, followed by staining for CD markers specific for monocytes (CD45RA, CD49a, HLA-DR and CD69), macrophages (CD44 and CD11b) or both (and CD14 and CD36) to confirm the identity of the cells.

As the results for the THP-1 cell surface markers were inconsistent, PBMCs were then isolated from the blood of donors, monocytes differentiated into macrophages and investigated for CD markers (CD14, CD16, CD206 and CD11b).

To investigate if the number of recovered macrophages could be increased, PBMCs were plated using a novel coating material called HDMS. Increasing concentrations of HDMS in different solvents were investigated to find the optimum conditions.

To analyse how CSE and nicotine can alter macrophage cell surface markers, macrophages generated from PBMCs were exposed to CSE and nicotine in combination with Poly-IC or LPS and changes in the CD cell surface markers (CD14, CD16, CD206 and CD11b) were detected.

To further analyse how CSE and nicotine affect macrophages, several cell signalling and macrophage-related proteins (pSTAT6, pSTAT3, IRF4, KLF-4, BMP-7, FABP-4, pHSP27, NFκB-P50, PPAR-γ, HIF2α, pP38 MAPK) were selected which have a variety of roles relating to macrophage function and regulation. Changes in these proteins were detected using a previously optimised RPPA in macrophages isolated from PBMCs after they had been treated with the different combinations of CSE or nicotine with LPS or Poly-IC.

Based on the results of the RPPA, an ELISA of the supernatants used for the RPPA was carried out to measure TNF-α and IL-10 levels to differentiate between the activity of M1 and M2 macrophages, respectively.

2. Materials and methods

2.1. Materials

RPMI 1640 medium (Sigma Aldrich Company Ltd. Gillingham, UK RNBB7148) was supplemented with (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100 IU/ml Penicillin, 100 μg/ml Streptomycin, 2 mM L-Glutamine and 10 % Foetal bovine serum (FBS)). Other material included: Histopaque-1077 (Sigma H8889), $CD14^+$ microbeads human (Miltenyi Biotec Ltd. Surrey, UK, 130-050-201), LS column (Miltenyi Biotec 120-000-472), magnet for CD14 cell separation (Miltenyi Biotec), macrophage colony stimulating factor (M-CSF) (R & D systems), Lipopolysaccharide (LPS) (Sigma L4391), and Poly-IC (Invivo Gen). Buffers (Table 2.1), antibodies (Table 2.2) and macrophage stimuli (Table 2.4) were all from Sigma unless otherwise stated.

	Catalogue Number	**Company**
Human IL-10	DY217B-05	R&D systems, Abingdon, UK
Human TNF-α	DY210-05	R&D systems
Streptavidin	893975	R&D systems

Table 2.1: Buffers used during the generation and phenotyping of macrophages (MΦs) and ELISA.

Buffer	**Preparation**	**Company**
Bovine Serum Albumin (BSA)	----------	Sigma (101M7021V)
Phosphate buffered saline (PBS)	1 tablet per 100 ml distilled water	Sigma (RNBC9334)
Phosphate buffered saline (PBA)	500 ml PBS containing 0.1 % sodium azide, and 1 % BSA	----------

Formaldehyde	0.5 % formaldehyde (625 ml methanol (40 % formaldehyde solution) in 50 ml PBS	----------
Benzonase diluent 50 % glycerol, 20 nM Tris HCl pH 8, 2 nM $MgCl_2$, 20nM NaCl	In 50 ml tube, add 25 ml 100 % glycerol + 25 ml distilled water, discard 2 ml. Add 1 ml Tris HCl pH 8 + 1 ml NaCl + 100 µl $MgCl_2$	
Benzonase enzyme	1 µl of benzonase in 250 µl of benzonase dilution buffer	
Radioimmunoprecipitation assay buffer (RIPA)	----------	ThermoFisher Scientific (89901) Life Technologies Ltd, Paisley, UK
98+ % Hexamethyldisilazane (HMDS) $(CH_3)_3SiNHSi(CH_3)_3$	1 %, 3 % and 5 % HMDS were diluted in ethanol (70 % and 100 %) and Propanol 100 %	Alfa *A*esar (10158871) Alfa Aesar, Thermo Fisher Scientific, Heysham, UK
CD14 MicroBeads human	----------	MACS Miltenyi Biotec (130-050-201)
MACS buffer	20 ml PBS, 80 µl EDTA and 100 µl FBS	----------
SuperG Blocking Buffer	In 50 ml tube, add 500 µl of NAF and 500 µl NaVO3 stock solutions to 5 ml of 10x PBS stock, and make up to 50 ml with distilled water. Take an empty 50 ml tube, put 5 ml of 30 % BSA + 45 ml PBS which supplemented with NAF & $NAVO_3$ and keep the tube in the fridge.	Ready to use from GRACE BIO-LABS SKU: 105100 – 100 ml Grace Bio Labs, Oregon, USA.
MSDS (β-mercaptoethanol sodium dodecyl sulphate)	In 50 ml tube, add 3.3 ml 1.5 M Tris HCL pH 6.8 to 6 ml Glycerol + 6.4 ml 20 % SDS + 2.3 ml of dH_2O. Vortex the mixture and aliquot 450µl in Eppendorf tubes	----------

	and store in -20 °C. In the day of using, add 50 µl of β-mercaptoethanol to the Eppendorf tube in the Flammable hood.	
Reagent Diluent Concentration 2 (10x) for ELISA	----------	R&D systems (291195)
1-Step™ Ultra TMB-ELISA Substrate Solution	----------	Thermo Fisher (34029)
Carbonate- Biocarbonate Buffer- capsule	----------	Sigma Aldrich (C3041-100CAP SLBK5141V)
Antibody Diluent, Background Reducing	----------	Dako (s3022) Agilent Technologies LDA UK Stockport, UK
Pierce™ BCA Protein Assay Kit	----------	ThermoFisher Scientific (23225)
TWEEN 20	----------	Sigma Aldrich (038K00915)
Sulfuric acid	----------	Sigma Aldrich (339741-500ML)

Table 2.2: Antibodies used for flow cytometry staining of Macrophages.

Antibody	**Label**	**Isotype**	**Company (catalogue number)**
CD14	FITC	IgG2, mouse	Beckman Coulter, IM0645U Indianapolis, USA
CD16	PE	IgG1, mouse	Beckman Coulter, A07766
CD206	CD49aPE	IgG1, mouse	Beckman Coulter, IM2741
CD11b	FITC	IgG1, mouse	Beckman Coulter, IM0530
CD36	PE	IgG2ak, mouse	BioLegend, 336206
CD49a	FITC	Ms IgG 1, K	AbD Serotec (MCA1133FT) Kidlington, UK
CD45RA	PE	IgG1, mouse	Beckman coulter (IM1834U)
CD69	PC5	Ms IgG2b	Beckman coulter (IM2656)

HLA-DR	ECD	Ms IgG1	Beckman coulter (IM3636)

Table 2.3: Antibodies used for ELISA of Macrophages.

	Catalogue Number	**Company**
Human IL-10	DY217B-05	R&D systems, Abingdon, UK
Human TNF-α	DY210-05	R&D systems
Streptavidin	893975	R&D systems

Table 2.4: Stimuli used for Macrophages.

	Catalogue Number	**Company**
M-CSF	216-MC-005	R&D systems
Poly-IC	Tlr-pic-5	Invivo Gen
LPS	L4391	Sigma Aldrich
Nicotine	N3876	Sigma Aldrich
Cigarette		Marlboro

2.2. Methods

2.2.1. THP-1 cell culture

The THP-1 monocyte cell line was cultured in tissue culture flasks with complete RPMI 1640 medium for three days at 37 °C. This RPMI 1640 medium was supplemented with 10 % FBS, 2 mM L-Glutamine and 1 mM sodium pyruvate, plus penicillin (100 units/ml) and streptomycin (100 μg/ml) to inhibit bacterial contamination.

2.2.2. Stimulating THP-1 cells with PMA

Phorbol 12-myristate 13-acetate (PMA), (also known as 12-O-tetradecanoylphorbol 13-acetate (TPA) is a specific activator of Protein Kinase C (PKC) and hence of NFκB.

PMA is the most commonly used phorbol ester. It is active at nano-molar concentrations. PMA causes an extremely wide range of effects in cells and tissues. After dissolving PMA in DMSO, it was aliquoted and stored at -20 ° C in the dark.

Cells were seeded in 6-well plates, 2 ml in each well, and PMA (5 ng/ml) was added to cells in different concentrations (50 pg/ml, 100 pg/ml, 150 pg/ml, 250 pg/ml and 500 pg/ml). We started with 50 pg/ml PMA and stained with all the CD markers of both monocytes and macrophages for three days. Based on the results from the first concentration, some markers, such as CD45RA, CD69, CD44 and HLA-DR were excluded as treating with PMA decreased their expression. THP-1 cells were stimulated with PMA and stained with CD49a, CD14, CD36 and CD11b.

2.2.3. Isolation of PBMC

Buffy coats and leucocyte cones were used. Blood from four different donors (with different ages, 1 in their 20s, 1 in their 30s, and 2 between 50-60 years old) was decanted into 50 ml sterile tubes and diluted 1:3 using PBS. Half the blood was added gently on the wall of a 10 ml Histopaque tube and a clear layer appeared at the bottom of the tube. The samples were centrifuged at 800g for 22 min. PBMCs were transferred from the surface of the Histopaque into a new 50 ml sterile tube. 30 ml PBS buffer was added to wash the cells, which were centrifuged at 300 g for 10 min. The supernatant was discarded, and the wash step was repeated twice more. After the final wash 10 ml RPMI medium was added and a cell count was performed. Finally, the cells were centrifuged at 300 g for 5 min and resuspended as $5x10^5$ cells/ml.

2.2.4. Cell counting

After the second wash, cells were centrifuged in a 50 ml tube at 300 g, for 5 min. The supernatant was discarded, and cells were re-suspended in 30 ml complete medium. Equal volumes of cells (pellet) and Trypan blue were mixed in an Eppendorf tube (10 µl each) and pipetted into one side of a haemocytometer. Cells were counted in three of the four squares found in the plate using the microscope and their mean was calculated. Cell concentration was calculated as follows:

Number of cells/ ml = means of cells counted x dilution factor x volume of chamber (1×10^4) cells/ml.

2.2.5. $CD14^+$ monocyte magnetic separation

By using human CD14 Micro-Beads (human) and LS MACS columns (Miltenyi Biotec, UK), $CD14^+$ cells (monocytes) were purified from the PBMC fraction. According to the number of cells after the second wash, MACS buffer and CD14 micro beads were added, MACS buffer at a concentration of 80 µl MACS buffer and 20 µl CD14 micro beads were added per 1×10^7 cells. Purified monocytes were re-suspended at a density of $2x10^6$ monocytes/ml in RPMI-1640 (Sigma-Aldrich, UK) containing 15 % human AB serum (Sigma-Aldrich, UK or PAA Laboratories Ltd, UK), 2 mM L-glutamine (Sigma-Aldrich, UK), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich, UK), and 10 mM HEPES (Invitrogen, UK) – known as "RPMI complete medium". 50 ng/ml recombinant human (rh) M-CSF (R&D Systems, UK) was added to the monocyte cultures.

2.2.6. Checking the purity of $CD14^+$ monocytes

Flow cytometric measurements were performed using a two-colour FC500 flow cytometer (Becton Dickinson). Forward and side scatter of light was used to identify cell populations and exclude debris. Analysing unstained cells recorded auto fluorescence which could be subtracted from the sample measurements. On day 0 of cell culture, both CD14 negative and positive cells were stained with antibodies for markers that are reportedly expressed on macrophages (such as CD14, CD16, CD206 and CD11b) to check the purity of cells. On day 8, the same markers were used to stain macrophages to assess the effects of the different concentrations of CSE and nicotine in combination with and without Poly-IC and LPS (Mittar, Paramban and McIntyre, 2011).

2.2.7. Staining for flow cytometry analysis

After cells were harvested, 300 µl of the samples were placed in the FACS tubes, 500 µl of PBA was added and the tubes were centrifuged at 300 g for 5 min. The pellets were re-suspended, then Fc receptors were blocked by adding 150 µl 5 % mouse serum to the cells and incubating for 45 min at 4 °C prior to antibody staining.

Cells were stained with antibodies to markers that are reportedly expressed on THP-1 monocytes (CD49a, CD69, CD45RA and HLA-DR), antibodies to markers that are reportedly expressed on THP-1 macrophages (CD44 and CD11b), and those markers that are expressed on both (CD14, and CD36) (Mittar, Paramban and McIntyre, 2011). To confirm the kind of cells before treating with cigarette smoke extract, cells were stained with monocyte and macrophage markers as follows. Anti-CD49a-FITC, anti-CD69-PC5, anti-CD45RA-PE and anti-HLA-DR-ECD were used to stain reputed monocyte cell surface markers, whereas anti-CD44-PC-7, and anti-CD11b-FITC were used to stain reputed macrophage cell surface markers, and anti-CD14-ECD and anti-CD36-PE were used to stain both.

For detection of the cell surface markers, 5 µl of monoclonal mouse anti-human antibodies were added as follows: IgG1κ CD49a FITC (AbD Serotec), IgG1κ anti-CD45RA-PE (Beckman Coulter) and 2.5 µl anti-CD69-PC5 (Beckman Coulter) and IgG1κ anti-HLA-DR-ECD (Beckman Coulter). 5 µl of monoclonal mouse anti-human antibodies IgG2aκ anti-CD36-PE (Bio-Legend), IgG1κ anti-CD11b-FITC (eBioscience) and 2.5 µl IgG2aκ anti-CD14- ECD (Immuno-teck) and IgG2bκ anti-CD44-PC7 (eBioscience) were used as macrophage markers, together with relevant isotypes IgG1κ-FITC (BD Biosciences) and IgG2b-PE (BD Pharmingen). Incubation took place in the dark at 4 °C for 60 min. 1 ml PBA was added to wash off excess unbound antibodies then the cells were centrifuged at 300 g for 5 min. Finally, the cells were fixed using 200 µl of 0.5 % formaldehyde and stored at 4 °C until analysed.

2.2.8. Flow cytometry

Flow cytometric measurements were performed using a four colour FC500 flow cytometer (Becton Dickinson). Forward and side scatter of light was used to identify cell populations and exclude debris. Analysing unstained cells recorded auto fluorescence.

2.2.9. Coating culture plates with HMDS

In order to minimise the adhesion of macrophages and increase the number of cells harvested, a novel method was investigated, the wells of a 24-well plate were coated

with 1 ml of 1 % Hexamethyldisilazane (HMDS) in 100 % propanol for 30 minutes, then washed with 1 ml ethanol 70 % and dried before use.

2.2.10. Generation of macrophages from $CD14^+$ monocytes

750,000 monocytes were seeded per well in HMDS coated 24 well culture plates and incubated at 37 °C for 7 days. On day 0 all CD14 negative and positive cells were stained with macrophage markers such as CD14, CD16, CD206 and CD11b to check the purity of cells. On Day 3 of culture, cells were fed with 500 µl per well of fresh RPMI complete medium containing the appropriate M-CSF.

2.2.11. Cigarette smoke extract (CSE) preparation

It should be noted that there are more than four thousand components in cigarettes that contribute to the diversity of cigarette brands. Marlboro red was chosen to produce the CSE in this project because it is the brand used most often by 42.4 % smokers (Sharma *et al.*, 2016). Some of the most common chemicals in CSE include nicotine, ammonia, carbon monoxide, benzopyrenes, acrolein, nitrogen oxides, acetone, and hydroquinone (Sopori, 2002) [177]. The CSE concentrations used in the cultures in the present study (1 % and 3 %) were chosen to be consistent with those used in numerous previous published studies on the effects of CSE on cells in vitro (Aoshiba *et al.*, 2001; Mian *et al.*, 2008; Mian *et al.,* 2009; Su *et al.,* 2004; Wongtrakool *et al*., 2014). The chosen nicotine concentrations used (50 µg and 150 µg) were based on 50 µg representing approximately 4 % of the nicotine inhaled per cigarette (i.e. about 1.2 mg per cigarette [Jarvis *et al*., 2001]) and 150 µg representing approximately 1 % of the nicotine inhaled by smoking 10-15 cigarettes per day (Jarvis *et al*., 2001) these concentrations were also consistent with those used in previous studies (Wongtrakool *et al*., 2014; Cheng *et al*., 2018; Daffner *et al*., 2012; Tinti & Soory, 2012).

On day 7 of the macrophage generation, a CSE collection apparatus was set up 30 minutes prior to cell culture for each experiment in a fume hood (Figure 2.1). The procedure for generating CSE was based on that used in previous studies (Aoshiba *et al.*, 2001; Bernhard *et al.*, 2004; Liu *et al*., 2005; Mian *et al.*, 2008; Mian *et al.,* 2009; Stringer *et al*., 2007; Su *et al.,* 2004; Wongtrakool *et al*., 2014). To collect CSE, firstly, the three-way stopcock was switched to the syringe-cigarette position while CSE from

a single lit commercial cigarette (with filter removed) were aspirated into the 50 ml syringe at a rate of 125 ml in 60 s. The tap was then switched to the syringe-flask position to allow the smoke into the medium and it was slowly bubbled through the 10 ml sterile phenol red free RPMI medium 1640 (pre-warmed to 37 °C for 30 min) until the entire 50 ml syringe of smoke was finished. This process was repeated until the cigarette was finished, then the tube was closed and shaken well. The crude CSE was filtered through a 0.45 µm pore filter to remove bacteria and/or large particles. A dilution series of the CSE was made and the absorbance at 320 nm was measured. By using a standard curve of known concentrations of CSE, the dilution needed to obtain an absorbance of 0.15 for each batch was calculated and this diluted extract was taken as 100 % CSE. The 100 % CSE solution was then used to create 10 %, 20 % and 50 % CSE solutions, and phenol red free RPMI medium 1640 was used as a blank. Since the CSE was produced in RPMI medium, the negative control cultures for CSE addition were simply the cells cultured in RPMI medium without CSE.

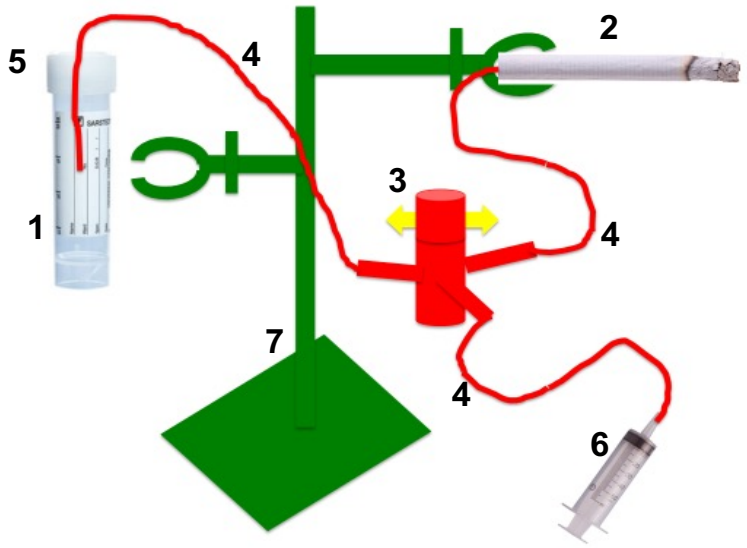


Figure 2.1: Set up of the apparatus to produce the CSE.

1) 10 ml of phenol-red free RPMI-1640 (GIBCO, Invitrogen, Paisley, UK) in a 30 ml sterile container 2) cigarette Marlboro, EU; Tar 10 mg, Nicotine 0.8 mg, carbon monoxide 10 mg) with filter cut off; 3) 3-way tap; 4) Three sections of flexible tubing (one from the cigarette to the tap, second from the tap to the syringe and third from the tap into the hole of the sterile container) ; 5) sterile lid with hole cut in, for tubing to fit through; 6) 50 ml syringe to aspirate the smoke; 7) two clamps and one clamp stand.

2.2.12. Cigarette smoke stimulation

On day 7 of macrophage culture, different concentrations of CSE (1 % and 3 %) and nicotine (50 and 150 μg/ml) were used to treat cells in combination with or without LPS Sigma-Aldrich (at 100 ng/ml) or Poly-IC InvivoGen (30 μg/ml). The cells were incubated for 24 h at 37 °C with 5 % CO_2. On day 8 the tissue culture plate was placed on ice for 15 min, then cells were collected in FACS tubes and stained with the same antibodies used previously (Table 2.2) for the detection of cell surface molecule expression by flow cytometry. Preparation of Cell Lysates after stimulating macrophages with CSE and Nicotine to be prepared for time-course Reverse-Phase Protein Microarray (RPPA) experiments

Cell lysis (splitting of cells) is used to break open cells when it is desirable to avoid shear forces that would denature or degrade sensitive macromolecules, such as proteins and DNA; for example, lysates are used in RPPA and Western blotting to analyse the composition of specific proteins and nucleic acids individually or as complexes. In this experiment, cells lysates were prepared after stimulating MΦ with CSE and nicotine in the presence or absence of Poly-IC and LPS to be analysed by RPPA.

The procedure (Figure 2.2) occurred over three days (days 7, 8 and 9 of cell growth), and the cells from the different donors were initially grown for one week in a 24-well tissue culture plate coated with 1 % HMDS in 100 % propanol. On day 7 of cell growth, the macrophages were harvested by incubating plates on ice for 15 min and cells were collected by scraping the wells into a 50ml tube, followed by addition of 1 ml of cold EDTA in PBS to all wells, with 15 min incubation followed by scraping of the wells into the same 50 ml tubes to remove any remaining cells. The tubes were then centrifuged at 350 g, 4 ^{o}C, acceleration 3, deceleration 3 for 5 min, the medium discarded, and fresh complete RPMI-1640 medium was added before cell counting was undertaken (Section 2.2.2).

2 ml of cells were added to each well; the 3 plates were treated with different concentrations of CSE and nicotine in the presence and absence of Poly-IC and LPS, and then the plates were incubated at 37 °C. After 2 hours, plate 1 was removed from the incubator, the supernatants kept in labelled Eppendorf tubes and the cells washed

by addition of 1 ml cold PBS per well. The PBS was removed, then 200 µl of the lysis buffer (10 µl of phosphatase and protease inhibitor cocktail and 5 µl of Benzonase per 1 ml of RIPA buffer 1 X) kept on ice was added, and the plate was incubated on ice for 15 minutes with frequent shaking. The cell lysates were collected by scraping the wells, which were put into labelled PCR tubes and stored at -80 °C.

On day 8, plate 2 was removed from the incubator, the supernatant from the wells were removed and kept, and the cell lysis process was followed as before. On day 9 plate 3 was removed from the incubator, the supernatant from the wells were removed and kept, and the cell lysis process was followed as before.

The concentration of protein in the lysates was determined using the BCA assay kit, and the results then analysed to find the minimum and the maximum concentrations, and set the concentration of dilution according to the minimum concentration. 50 µl of each lysate was diluted with RIPA buffer as required to make all the samples the same concentration, and store at -80 °C.

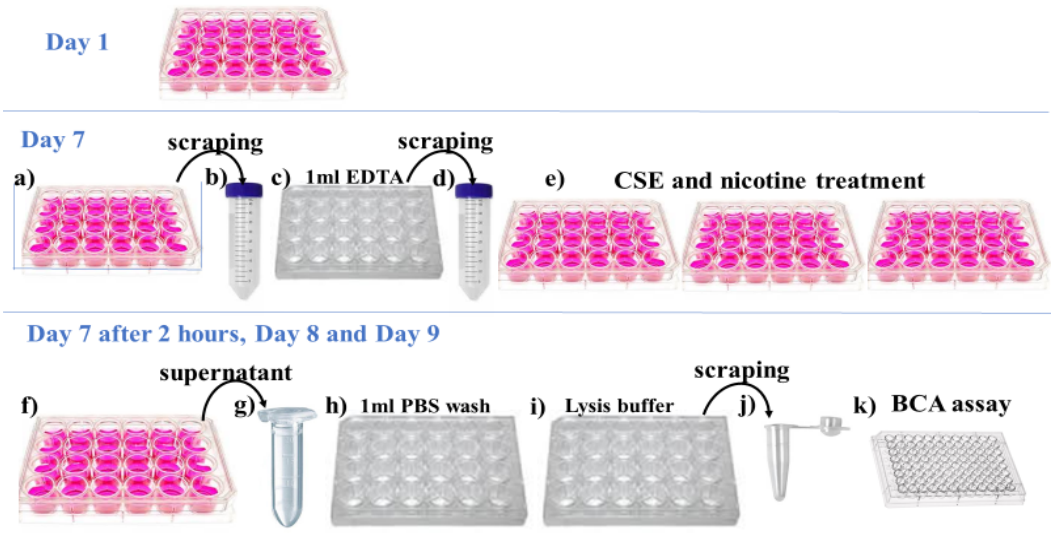


Figure 2.2: Timeline for the preparation of cell lysates.

Day 1 cells from the different donors were initially grown in a 24-well tissue culture plate coated with 1 % HMDS in 100 % propanol. **Day 7 a)** macrophages were harvested by incubating plates on ice for 15 min, **b)** cells were collected by scraping the wells into a 50 ml tube, **c)** addition of 1 ml of cold EDTA in PBS to all wells for 15 min **d)** scraping of the wells into the same 50 ml tubes to remove any remaining cells. The tubes were then centrifuged at 350 g, 4 ^{o}C, for 5 min, the medium discarded, and fresh complete RPMI-1640 medium was added before cell counting. **e)** 2 ml of cells were added to each well and the 3

plates were treated with different concentrations of CSE and nicotine in the presence and absence of Poly-IC and LPS, and then the plates were incubated at 37 °C. **Day 7 + 2 hours f)** plate 1 was removed from the incubator, **g)** the supernatants kept in labelled Eppendorf tubes, **h)** cells washed by addition of 1 ml cold PBS per well. **i)** PBS was removed, then 200 µl of the ice-cold lysis buffer (10 µl of phosphatase and protease inhibitor cocktail and 5 µl of Benzonase per 1 ml of RIPA buffer 1 X) was added and incubated on ice for 15 min with frequent shaking. **j)** cell lysates were collected by scraping into labelled PCR tubes and stored at -80 °C. **Day 8** plate 2 was removed from the incubator and steps **f** to **j** were followed. **Day 9** plate 3 was removed from the incubator and steps **f** to **j** were followed. **k)** protein concentration was determined by BCA assay, then 50 µl of each lysate was diluted with RIPA buffer as required to make all the samples the same concentration, and store at -80 °C.

2.2.13. Bicinchoninic acid (BCA) assay for protein concentration determination

The BCA working reagent (WR) was prepared by mixing 50 parts of BCA reagent (A) with 1 part of reagent (B) to produce the desired volume calculated using the following formula:

Total volume of WR = (number of standards + number of unknowns) X (number of replicates) X (volume of WR per sample)

The standards were prepared (Table 2.5) and replicates of 10 µl were pipetted into a sterile microtitre plate, replicates of 10 µl of the samples were added to the plate, then all the wells had 200 µl of WR added and the plate was mixed for 30 seconds. The plate was then covered and incubated at 37 ^{o}C for 30 min. After incubation, the plate was cooled at room tempersature for 10 min, then the absorbance at or near 562 nm was measured on a plate reader.

Table 2.5: Preparation of the BCA standards.

Dilute the contents of one BSA standard ampule into several clean vials using the same diluent as the sample. Each 1 ml ampule of BSA is sufficient to prepare 3 replicates of each diluted sample. The volume of diluent = lysis buffer.

Dilution Scheme for Standard Test Tube Protocol and Microplate Procedure (Working Range = 20–2,000 µg/ml)

Vial	Volume of Diluent	Volume and Source of BSA	Final BSA Concentration
A	0	300 µl of Stock	2,000 µg/ml
B	125 µl	375 µl of Stock	1,500 µg/ml
C	325 µl	325 µl of Stock	1,000 µg/ml
D	175 µl	175 µl of vial B dilution	750 µg/ml
E	325 µl	325 µl of vial C dilution	500 µg/ml
F	325 µl	325 µl of vial E dilution	250 µg/ml
G	325 µl	325 µl of vial F dilution	125 µg/ml
H	400 µl	100 µl of vial G dilution	25 µg/ml
I	400 µl	0	0 µg/ml = Blank

A BCA standard curve was prepared by plotting the average blank-corrected 562 nm measurement for each BCA standard against its concentration in µg/ml, which allowed the concentrations of the samples to be determined.

2.2.14. Microarray set up

For all the microarray work, the PBS used was supplemented with 5 mM NaF and 2 mM $NaVO_3$ even when used for making blocking and washing buffers, and diluting antibodies. The microarray process (Figure 2.3) can be divided into different stages of preparation, printing, blocking and detection.

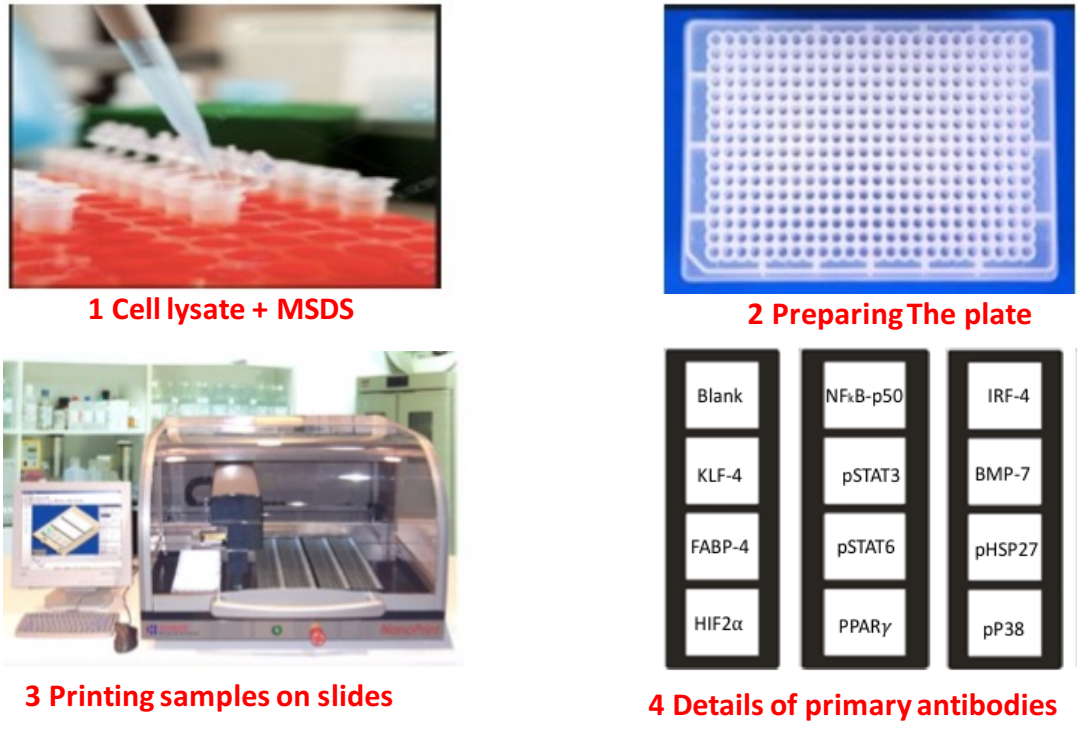


Figure 2.3: Summary of the stages of microarray set up.

First the samples are prepared by denaturation (1), then they are pipetted into a 384 plate (2), after being placed into the microarray machine for slide printing (3). The slides are then probed with different

primary antibodies (4) to find if there is any reaction, and detected with secondary antibodies and fluorescent markers.

2.2.14.1. Denaturation of the protein lysate samples ready for printing

A 1/3 volume of MSDS (β-mercaptoethanol sodium dodecyl sulphate) was added to the samples (for example, for a 50 µl sample, 16.6 µl MSDS was added), the tubes or plate were sealed and heated to 95 °C for 5-10 min, then cooled to room temperature. The plate was briefly centrifuged to recover all liquid before opening/peeling off the seal. In a class 2 safety cabinet 10 µl of each sample was added into a 380-well microarray plate. The plate was then sealed and covered with aluminium foil, then stored either at 4 °C if printing onto 4 pad nitrocellulose slides was taking place on the same day, or stored at -20 °C until printing was carried out.

2.2.14.2. Printing the samples onto the slides

The GenePix Array List (GAL) file was prepared before printing the slide, as it describes the samples on the slides based on their position on the plate. This file contains the information of each sample in a text file.

The plate was warmed to room temperature, placed in the microarray machine with the nitrocellulose slides and printing started. After printing, the slide and plate were removed from the microarray machine, the plate sealed, and the slide stored at -20 °C until the blocking was carried out. Samples were spotted in quadruplicate onto the nitrocellulose-coated glass slides at protein concentrations about 1-0.5 mg/ml.

2.2.14.3. Blocking the slide for microarray

The slide was warmed to room temperature and placed in the incubation chamber. Each array was blocked in 400 µl Super-G blocking buffer (Grace-BioLabs Inc) at room temperature for 4 hours or overnight on the shaker.

After incubation, the slide was removed from the shaker and the liquid discarded. Each array was blocked in Avidin solution, Life Technologies 1704221A (6-8 drops per array if using 4 arrays per slide) at room temperature for 10 minutes on the shaker, to allow binding of any Biotin sites present in the slides. The liquid was discarded, and the slide

washed quickly three times with PBS*T (Phosphate Buffered Saline and Tween 20), discarding the liquid each time. Blocking was then performed with Biotin solution, Life Technologies 1704221 B (6-8 drops per array if 4 arrays per slide) at room temperature for 10 minutes on a shaker, to saturate all the Biotin-binding sites left open on the Avidin. The liquid was discarded, and the slide washed quickly three times with PBS*T, then washed more thoroughly with three 2 minute incubations in PBS*T to remove all traces of Avidin and Biotin.

2.2.14.4. Application of primary and secondary antibodies to the slide

After blocking and washing, primary antibody was added to the slide (Figure 2.4) diluted appropriately in blocking buffer (Table 2.6) and incubated overnight at 4 °C. The plate was sealed to avoid loss of moisture by evaporation. The slide was washed four times at room temperature for 3 minutes in PBS*T, to remove any unbound primary antibody.

Table 2.6: Details of the antibodies used in microarray.

The volume needed per array is dependent on the size of the array, 100 µl is sufficient for wells of a 16-pad slide gasket.

Numbers	Antibody specificity	Isotype	Dilutions	Company	Cat- No
1	Pstat6	Rabbit	1/1000	Cell Signalling	9361S
2	IRF-4	Rabbit IgG	1/1000	Cell Signalling	4299S
3	KLF4	Rabbit IgG	1/1000	Cell Signalling	12173S
4	NFkB50	Rabbit IgG	1/1000	Cell Signalling	12540S
5	PPARγ	Rabbit IgG	1/1000	Cell Signalling	2435S
6	HIF-2α	Rabbit IgG	1/1000	Cell Signalling	7096S
7	BPM7	Rabbit	1/1000	Cell Signalling	4693S
8	FABP4	Rabbit IgG	1/1000	Cell Signalling	3544S
9	Pstat3	Rabbit	1/1000	Cell Signalling	9134S
10	Pp38	Rabbit IgG	1/1000	Cell Signalling	4511S

11	pHSP27	Rabbit IgG	1/1000	Cell Signalling	9709S

Biotinylated anti-rabbit secondary antibody (diluted 1:1000) and anti-mouse beta-actin (diluted 1:1000) in PBS*T were added (Figure 2.4), and incubated at room temperature with shaking for 1 hr. The slide was washed four times at room temperature for 3 minutes in PBS*T, to remove any unbound secondary antibody.

200 µl Streptavidin HRP (diluted 1:200 in Super G blocking buffer) was added to the slide and incubated at room temperature for 15 minutes with shaking. The slide was washed five times at room temperature for 3 minutes in PBS*T.

200µl amplification reagents premixed with a ratio 2:1:1 (Super G blocking buffer: amplification reagent: ultrapure water) was added and incubated for 10 minutes at room temperature with shaking. The slide was washed three times at room temperature for 3 minutes each in 20 % DMSO PBS*T, then washed three times at room temperature for 3 minutes in PBS*T.

Streptavidin-Licor 680 dye (diluted 1:5000 in Antibody Diluent) and anti-mouse-licor 780 (diluted 1:5000 in Antibody Diluent) were added to each block, the slides covered to protect from light, and incubated with shaking for 15 minutes at room temperature. The slide was washed five times at room temperature for 3 minutes in PBS*T, then washed once for 5 minutes with PBS.

The slides were dried by centrifugation at 1200g for 5 minutes or, if only one slide has been processed, dried with a nitrogen gun. The slide was then scanned with an Odiesy scanner to detect the interaction of the antibodies and the samples (Figure 2.4).

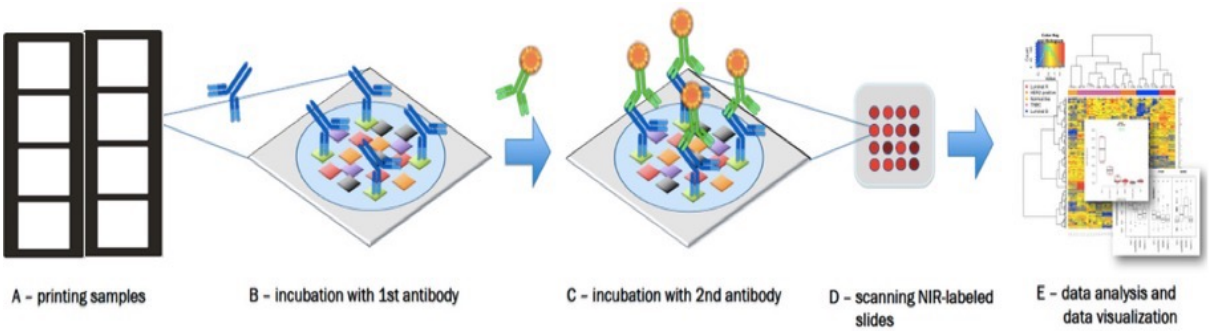


Figure 2.4: Summary of the microarray process of applying antibodies, detection and data analysis.

A) printing of the slides with samples, B) incubation with primary antibody, C) incubation with secondary antibody, D) scanning and detection of labelled slides, and E) data analysis and data visualisation.

2.2.15. Analysing microarray data

After scanning the microarray slides, there is quantitative information about each fluorescent spot on every slide which needs to be combined with the name and location of the antigens spotted on the slide (in the form of a GAL file) before statistical analysis can be carried out on the experiment. The GAL file is a text file with specific information about the location, size, and name of each antigen spot on the slide. This file includes information that GenePix uses to create an array list. The combined file can be created using the microarray GenePix software; the relevant wavelengths and colours can be assigned to the samples and controls, the spot sizes can be adjusted to make sure all of the signal is captured, and the pads can be lined up accurately to ensure all the samples are recorded. The combined data file can then be used to statistically analyse the results.

2.2.16. ELISA of IL-10 and TNF-α expression

The supernatants from the cell cultures that were used to generate the cell lysates for RPPA were used for the ELISA assay; the supernatants of the cultured cells that had been incubated for 24 or 48 h under the different test conditions were investigated by capture ELISA with antibodies specific for the cytokines IL- 10 and TNF-α using R&D Systems Duoset kits.

First, the 384-well ELISA plates were coated with 20 µl of the capture antibody (diluted with carbonate-bicarbonate buffer, pH 9.2) in triplicate and incubated overnight in the dark. The plates were then washed three times with PBS containing 0.05 % Tween and then 20 µl per well of reagent diluent containing 1 % BSA was added for 1 hour to block the plate. The plate was washed three times with PBS-0.05 % Tween then 20 µl per well of recombinant human TNF-alpha and IL-10 or the supernatant samples were added in triplicate and incubated for 2 hours. The plate was again washed three times with PBS-0.05 % Tween and 20 µl per well of the detection antibody was added to the

plate before incubating for 2 hours. The plate was washed three times with PBS-0.05 % Tween then 20 µl of diluted Streptavidin-HRP was added to each well and incubated for 20 minutes. The plate was washed three times with PBS-0.05 % Tween then 20 µl of substrate solution was added per well for 20 minutes, and finally 20 µl of stop solution (1M H_2SO_4) was added before the absorbance of the plate was read at 450 nm.

2.2.17. Statistical analysis

For flow cytometry, the mean fluorescence intensity (MFI) results for the different cell surface markers and treatment combinations were analysed and, to determine if any of the changes in fluorescence were statistically significant, the results were analysed by two-way ANOVA with Dunnett's multiple comparisons test.

Microarray data were analysed following subtraction of all the relevant blank values from the corresponding values for the signalling molecules expression. The median values were calculated for each signalling molecule for the 8 individual donors, then the changes across all signalling molecules were calculated, with CSE or nicotine treatments compared to no CSE or nicotine. Data were analysed for statistical significance by Wilcoxon matched pairs test.

For the ELISA data, as some of the data was not normally distributed so the analysis was non-parametric; the results were investigated for statistical significance using Wilcoxon matched pairs test with the Bonferroni correction to compare the sample to the ‘untreated’ relevant control. Any data values that were below the standard curve were replaced with zero values.

3. Investigation of the suitability of macrophages from the THP-1 cell line for use in studying COPD

3.1. Introduction

In COPD, macrophages recruit inflammatory cells to the lungs and release proteases that cause some of the damage seen in COPD (Tetley, 2002). To study the function of macrophages *in vitro* cell lines such as THP-1, a human pro-myeloid cell line developed by Tsuchiya *et al*. (1980), can be used.

THP-1 cells have significant morphology and differentiation similarities to primary monocytes and macrophages and can be differentiated into macrophage-like cells using reagents like phorbol 12-myristate 13-acetate (commonly known as PMA or TPA) or vitamin D3, which causes them to adhere to the bottom of the culture plates (Tsuchiya *et al*., 1980; Schwende *et al.,* 1996; Daigneault *et al*., 2010). Many studies have used PMA treated THP-1 cells to investigate macrophage functions, as their use has some advantages over human primary monocyte–macrophage cells in the lab, including ease of transformation and storage, and low genetic variability (Daigneault *et al.,* 2010).

Due to these benefits, the THP-1 cell line was evaluated for use in this study. THP-1 cells were cultured and differentiated with PMA, and stained for CD markers specific for monocytes and/or macrophages to confirm the identity of the cells before deciding whether to use them in further studies exposing them to CSE to investigate the changes to macrophages in COPD. Based on the recommendations in the guidelines produced by Becton-Dickinson Bioscience Company (B.D.) (Mittar, Paramban and McIntyre, 2011) the markers CD45RA, CD49a, HLA-DR and CD69 were chosen to stain THP-1 cell line to detect monocytes, CD44 and CD11b were chosen to detect macrophages, and CD14 and CD36 were chosen to detect both (Mittar, Paramban and McIntyre, 2011). The choice of CD markers is also consistent with other published data on THP-1 cells (reviewed by Chanput, Mes and Wichers, 2014). Forrester *et al*. (2018) have also very recently published a comprehensive analysis of CD marker expression by THP-1 monocytes and differentiated macrophages.

3.2. Materials and methods

3.2.1. Staining for flow cytometry analysis

After cells were harvested, 2 ml of samples were placed into FACS tubes, 1 ml of PBA was added, and tubes were centrifuged at 300 g, for 5 min. The pellets were re-suspended and Fc receptors were blocked by adding 1.5 µl recombinant human IgG (Sigma-Aldrich) to cells with incubation for 15 min at 4 °C prior to antibody staining. For detection of cell surface markers of monocytes, 5 µl of monoclonal mouse anti-human antibodies were added as follows: IgG1κ CD49a FITC (AbD Serotec), IgG1κ anti-CD45RA-PE (Beckman Coulter) and 2.5 µl anti-CD69-PC5 (Beckman Coulter) and IgG1κ anti-HLA-DR-ECD (Beckman Coulter). 5 µl of monoclonal mouse anti-human antibodies IgG2aκ anti-CD36-PE (Bio-Legend), IgG1κ anti-CD11b-FITC (eBioscience) and 2.5 µl IgG2aκ anti-CD14- ECD (Immuno-teck) and IgG2bκ anti-CD44-PC7 (eBioscience) were used as macrophage markers, together with relevant isotypes IgG1κ-FITC (BD Biosciences) and IgG2b-PE (BD Pharmingen). Incubation took place in the dark at 4^{o}C for 60 min. 1 ml PBA was added to wash off excess unbound antibodies, then samples were centrifuged at 300 g for 5 min. Finally, the cells were fixed using 400 µl of 0.5 % formaldehyde and stored at 4^{o}C until analysed.

3.3. Results

3.3.1. Phenotyping THP-1 cells

The presence of THP-1 monocyte surface markers (including CD49a, CD69, CD45RA and HLADR), the differentiated THP-1 macrophage marker CD44, and CD14 (which is found on both undifferentiated and differentiated THP-1 cells), were investigated by flow cytometry following cell surface staining. The results (a representative example of the five repeats is shown in Figure 3.1 and Figure 3.2) showed a healthy cell population as 92 % cells were gated. Macrophage markers CD11b and CD36 (which is found on both undifferentiated and differentiated THP-1 cells) were subsequently investigated (a representative example of the repeats is shown in Figure 3.3) where 96 % cells gated, and the results showed high levels of expression (based on MFI) of

these markers. Overall, the results show that the unstimulated THP-1 cells expressed the macrophage markers tested (CD11b and CD44), were virtually negative for the monocyte markers CD69 and CD49a, but expressed CD45RA, and were positive for HLA-DR, CD14 and CD36 (Table 3.1). All the antibodies employed gave good levels of staining of either the unstimulated or the stimulated THP-1 cells, thus providing an internal control of antibody efficacy.

Table 3.1: Summary of expression of selected cell surface markers on unstimulated and PMA-stimulated THP-1 cells.

+ = level of up-regulation, - = level of down regulation.

	Monocytes markers			Mono > Mac	Markers in common		Macrophages markers	
	CD45RA	CD49a	CD69	HLA-DR	CD14	CD36	CD11b	CD44
Unstimulated	+	+/-	-	+	+	++	+	+++
Stimulated	+/-	++	+	+	+	++	+	-

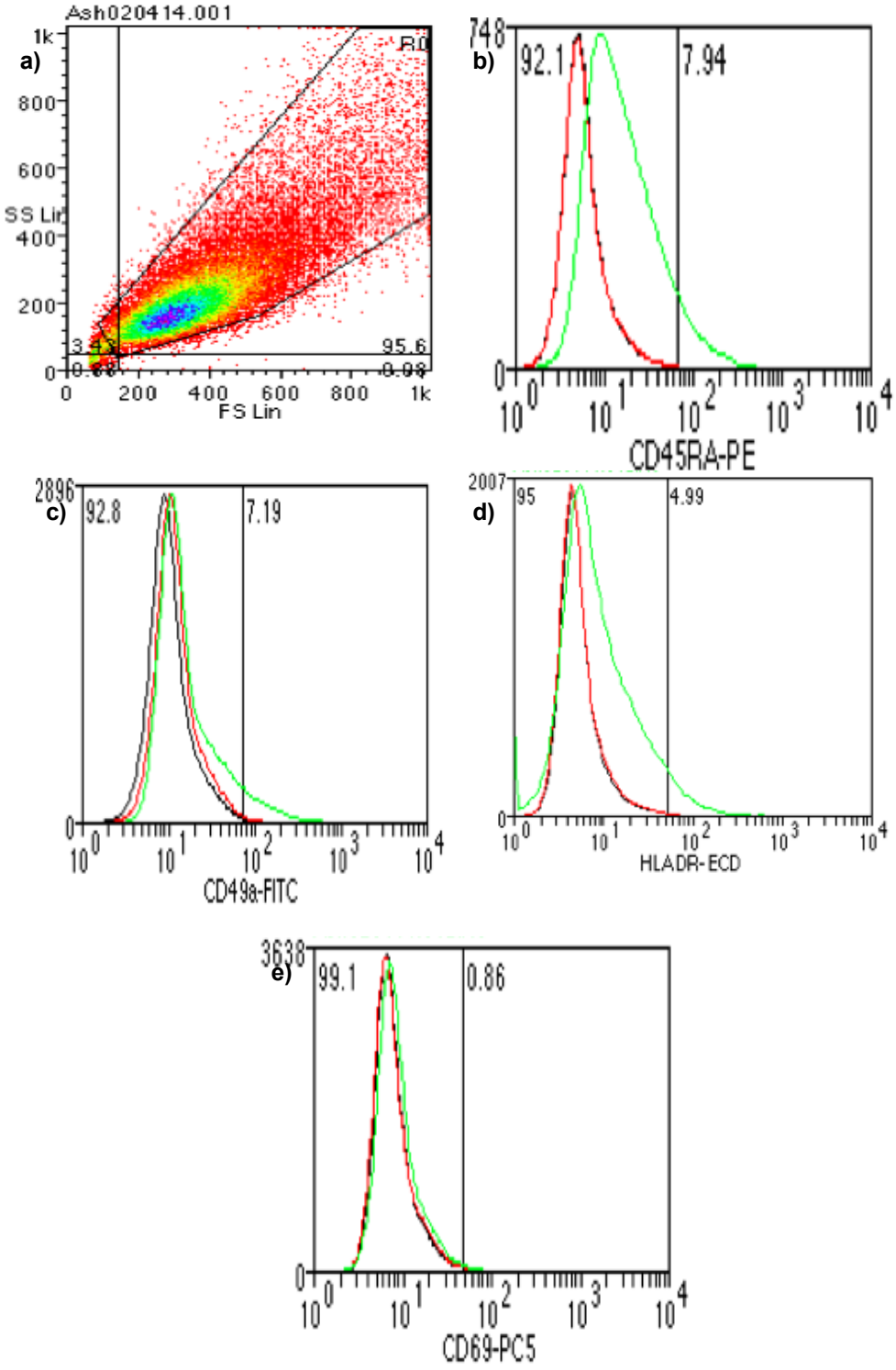


Figure 3.1: Flow cytometric analysis of surface Monocyte CD Markers on THP-1 cells.
a) gated scatter plot showing viability of cells. Flow cytometric analysis of surface markers on THP-1 cells, black curves represent un-stained cells; red curves the isotype control and the green curves the test antibodies b) CD45RA-PE c) CD49a-FITC, d) HLA DR-ECD, and e) CD69-PC5 data are representative of 5 repeats.

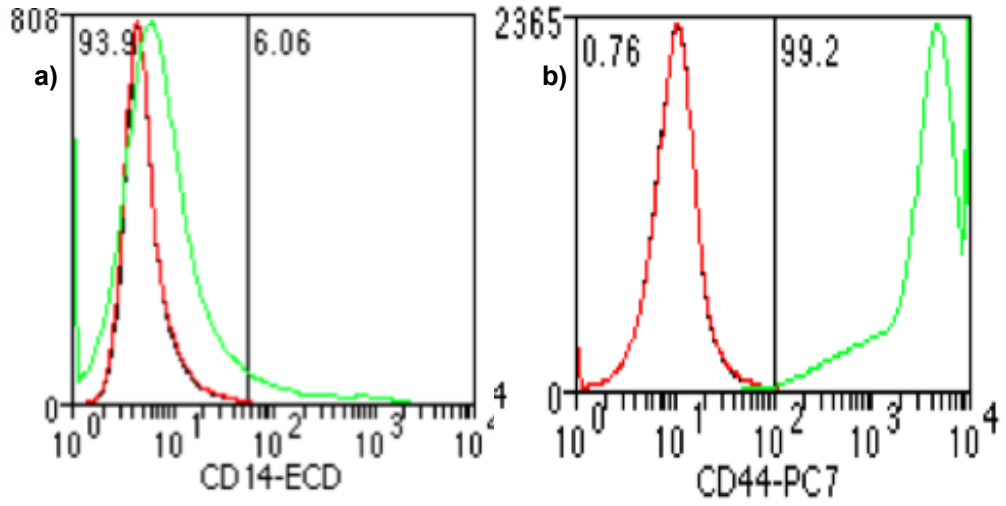


Figure 3.2: Flow cytometric analysis of surface cells macrophage CD14 and CD44 markers on THP-1 cells.

Flow cytometric analysis of surface markers on THP-1 cells black curves represent un-stained cells; red curves the isotype and the green curves represent the test antibodies a) CD14-ECD, and b) CD44-PC7 data are representative of 5 repeats.

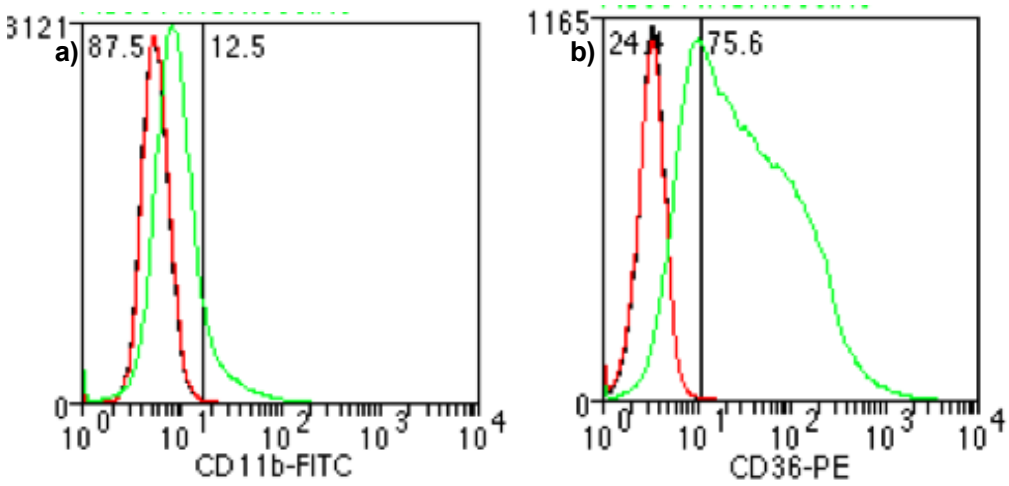


Figure 3.3: Flow cytometric analysis of surface cells macrophage CD11b and CD36 markers on THP-1 cells.

Flow cytometric analysis of surface markers on THP-1 cells black curves represent un-stained cells; red curves the isotype and the green curves represent the test antibodies a) CD11b-FITC and b) CD36-PE data are representative of 5 repeats**.**

By summarising the data from the 5 repeats (Figure 3.4) the actual change in the cell surface markers can be seen along with any differences between the repeats. There were

only small error bars for the un-stained and isotope control samples, which is as expected, and slightly larger error bars for the samples. The percent positive for each cell surface marker is quite low; CD69 has the lowest percent positive, then HLA, then CD49a and CD45 had the highest percent positive. The pattern for the mean sample MFI is similar to the percent positive, except here CD49a is the highest, not CD45. There were significant increases in expression in the samples for CD45a and CD49a ($P \leq 0.0001$), when compared to the isotype control.

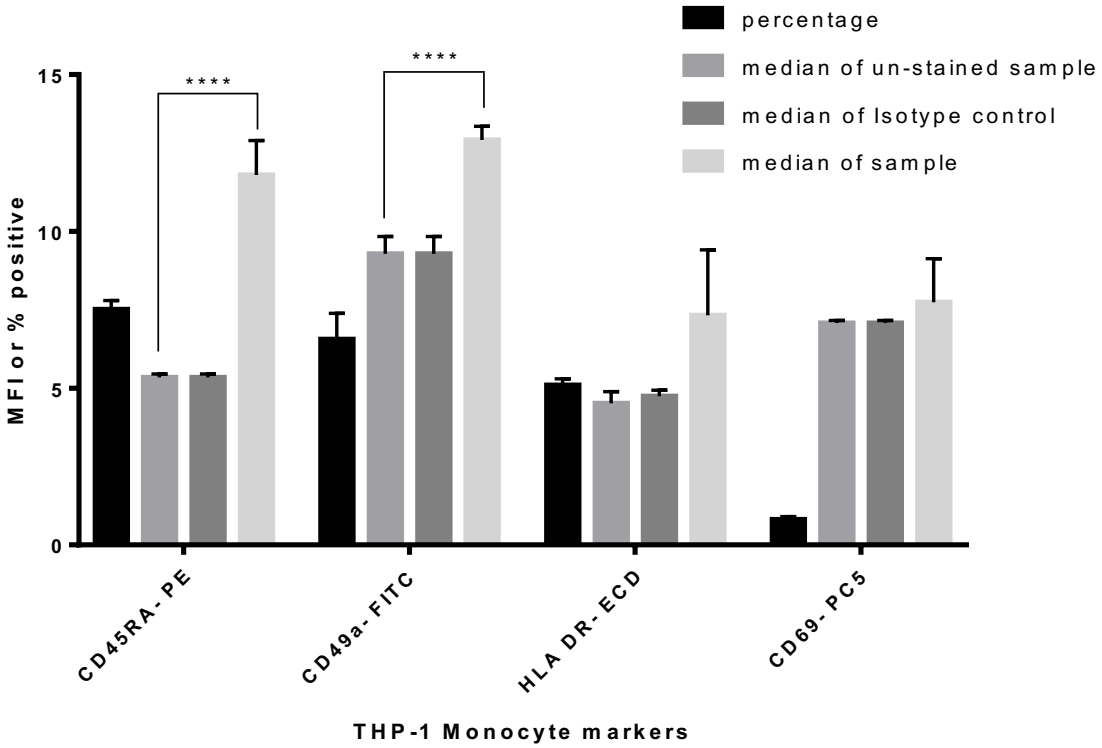


Figure 3.4: Mean MFI and percent positive of THP-1 monocyte markers.

Data plotted are mean (N = 5) and error bars are SD. The percentage bar shows the mean percent positive for each marker type. Statistical significance was determined using a t-test with Bonferroni-Dunn correction, P values < 0.05 were considered as significant, **** = P < 0.0001.

To ensure that the phenotype observed in the THP-1 cells was not a result of any errors in the tissue culture technique, THP-1 cells that were cultured by one of my more experienced colleagues were also investigated (Paul Radford). These were stained with selected antibodies for CD69-PC5, CD45RA-PE and CD14-ECD and analysed by flow cytometry. The results (a representative example of the repeats Figure 3.5) showed a

healthy cell population as 97 % cells were gated, and the same pattern of marker expression was observed, supporting my original results.

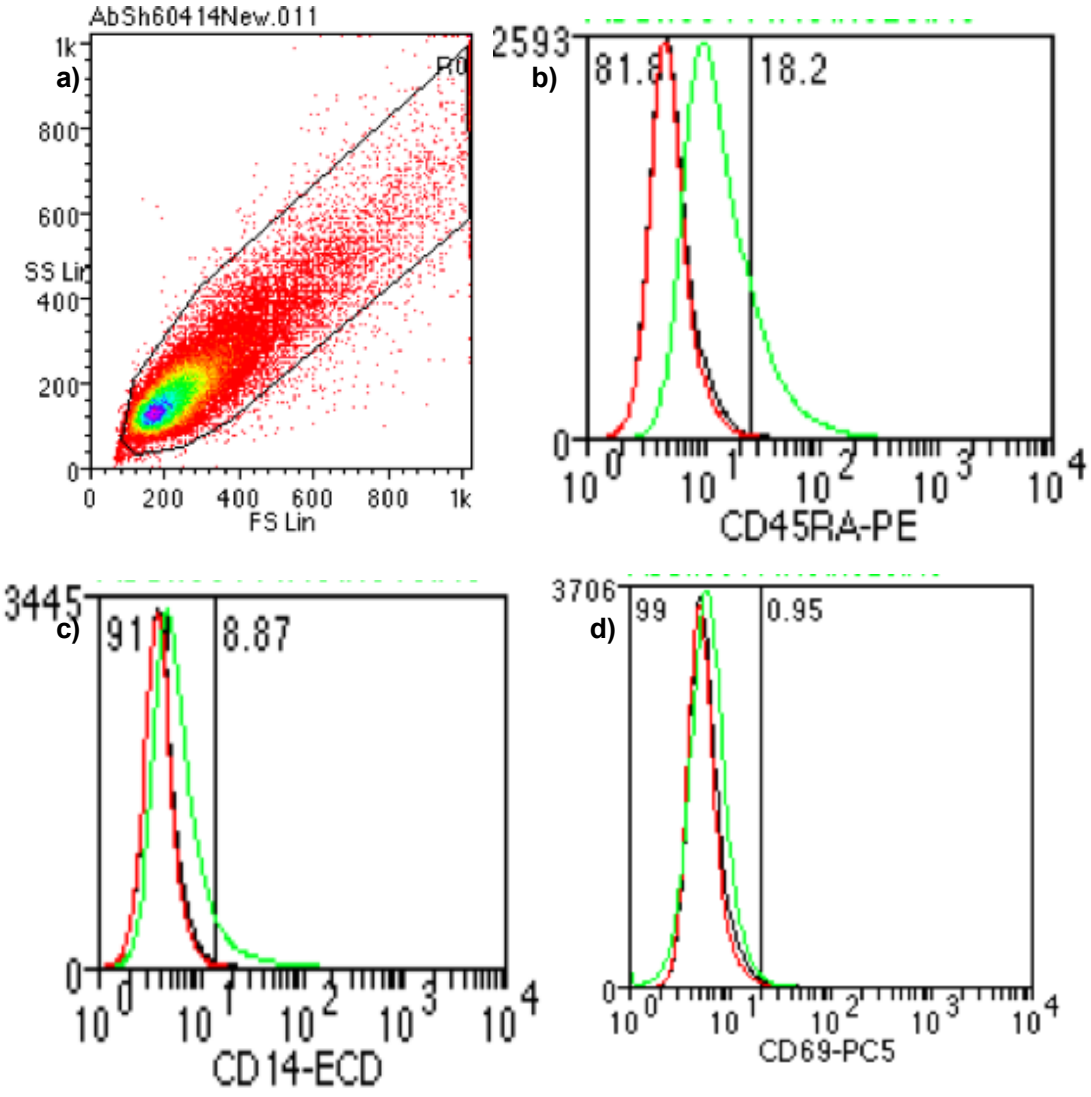


Figure 3.5: Flow cytometric analysis of surface markers CD69, CD45RA, and CD69 on THP-1 cells. a) gated scatter plot showing viability of cells. Flow cytometric analysis of surface markers on THP-1 cells, black curves represent un-stained cells; red curves the isotype and the green curves the test antibodies b) CD69-PC5, c) CD45RA-PE and d) CD14-ECD data are representative of 5 repeats.

The summary of the data for CD14, CD44, CD11b and CD36 are shown below (Figure 3.6). The percent positive for each cell surface marker is quite low for CD14 and CD11b, but CD44 and CD36 were 100 % positive. The pattern for the mean sample MFI shows low expression of CD14, and CD11b, with higher expression of CD36, and greatly up-

regulated expression of CD44. There were significant increases in expression in the samples for CD11b, CD36, CD44 ($P \leq 0.0001$ for all), and CD14 ($P = 0.0011$) when compared to the isotype control.

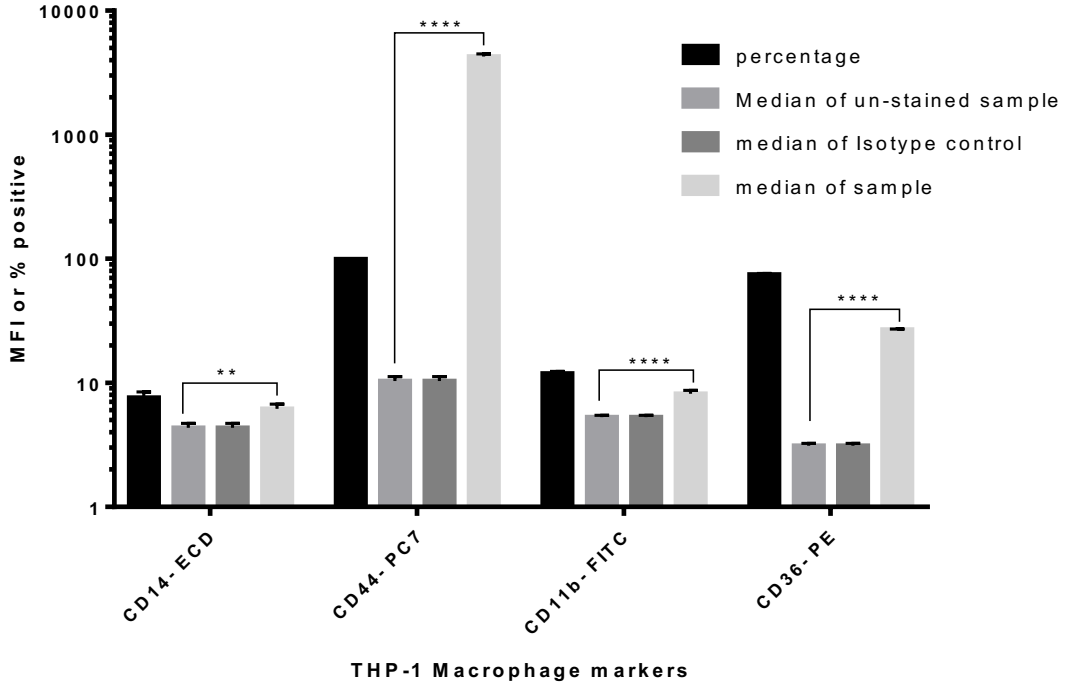


Figure 3.6: Mean MFI and percent positive of THP-1 macrophage markers.

Data plotted are mean (N = 5) and error bars are SD. The percentage bar shows the mean percent positive for each marker type. Statistical significance was determined using a t-test with Bonferroni-Dunn correction, P values < 0.05 were considered as significant, ** = P < 0.01, **** = P < 0.0001.

3.3.2. Stimulation of THP-1 cell line

It has been reported in the literature that stimulation of THP-1 cells results in a macrophage phenotype. To investigate this the cells were treated with PMA at different concentrations, initially using 50 pg/ml PMA, and then screened for cell surface markers using flow cytometry. The results (representative data for the repeats Figure 3.7 and Figure 3.8) indicated that CD49a and CD36 were highly expressed after stimulation, and the other markers were unchanged or down-regulated. Interestingly, CD49a (Figure 3.7c) had two populations with very different levels of expression.

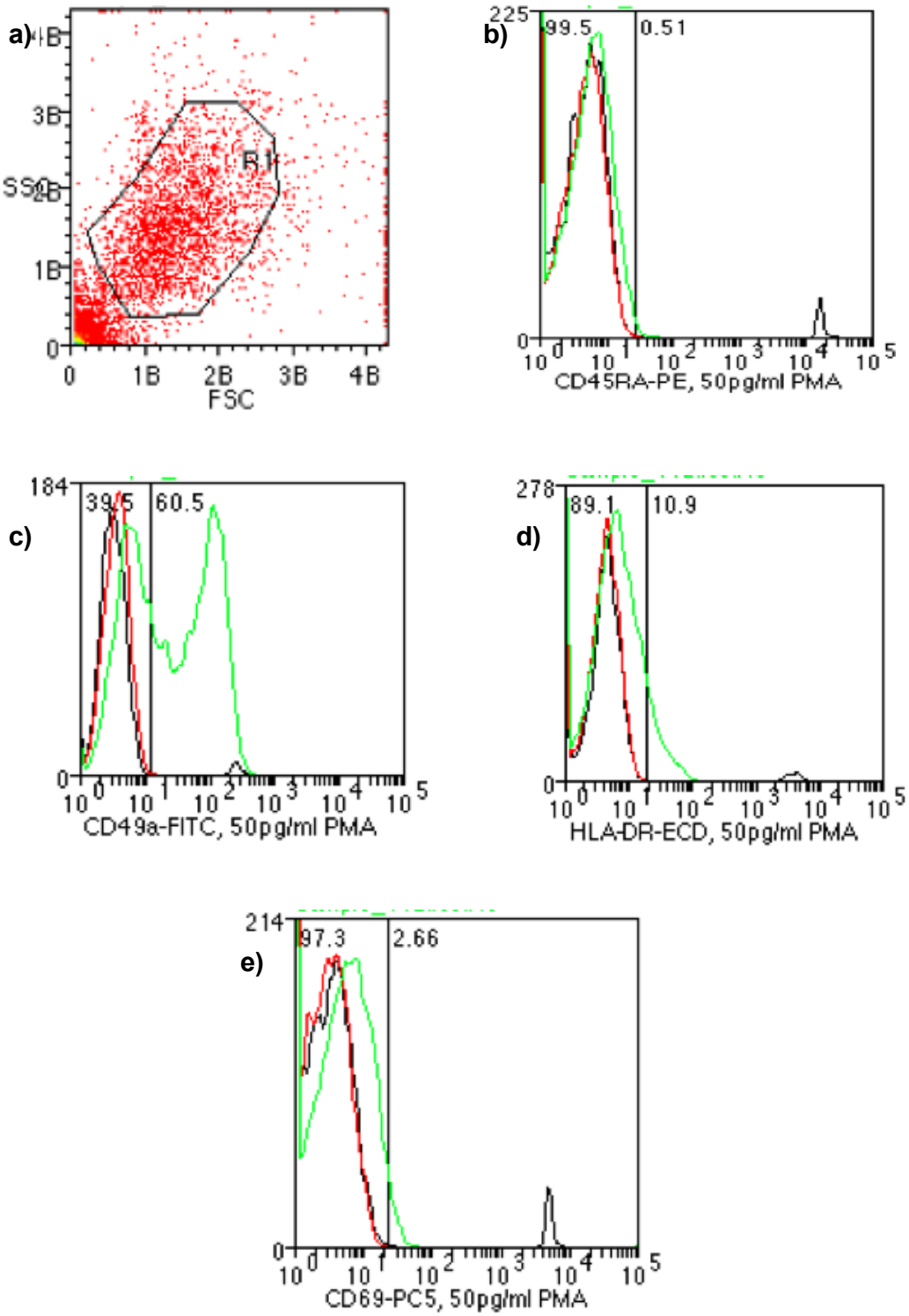


Figure 3.7: Flow cytometric analysis of surface monocyte CD markers after stimulation with 50 pg/ml PMA

a) gated scatter plot showing viability of cells. Flow cytometric analysis of surface markers on THP-1 cells: black curves represent un-stained cells; red curves the isotype and the green curves the test antibodies b) CD45RA-PE, c) CD49a-FITC, d) HLA DR-ECD, and e) CD69-PC5 data are representative of 5 repeats.

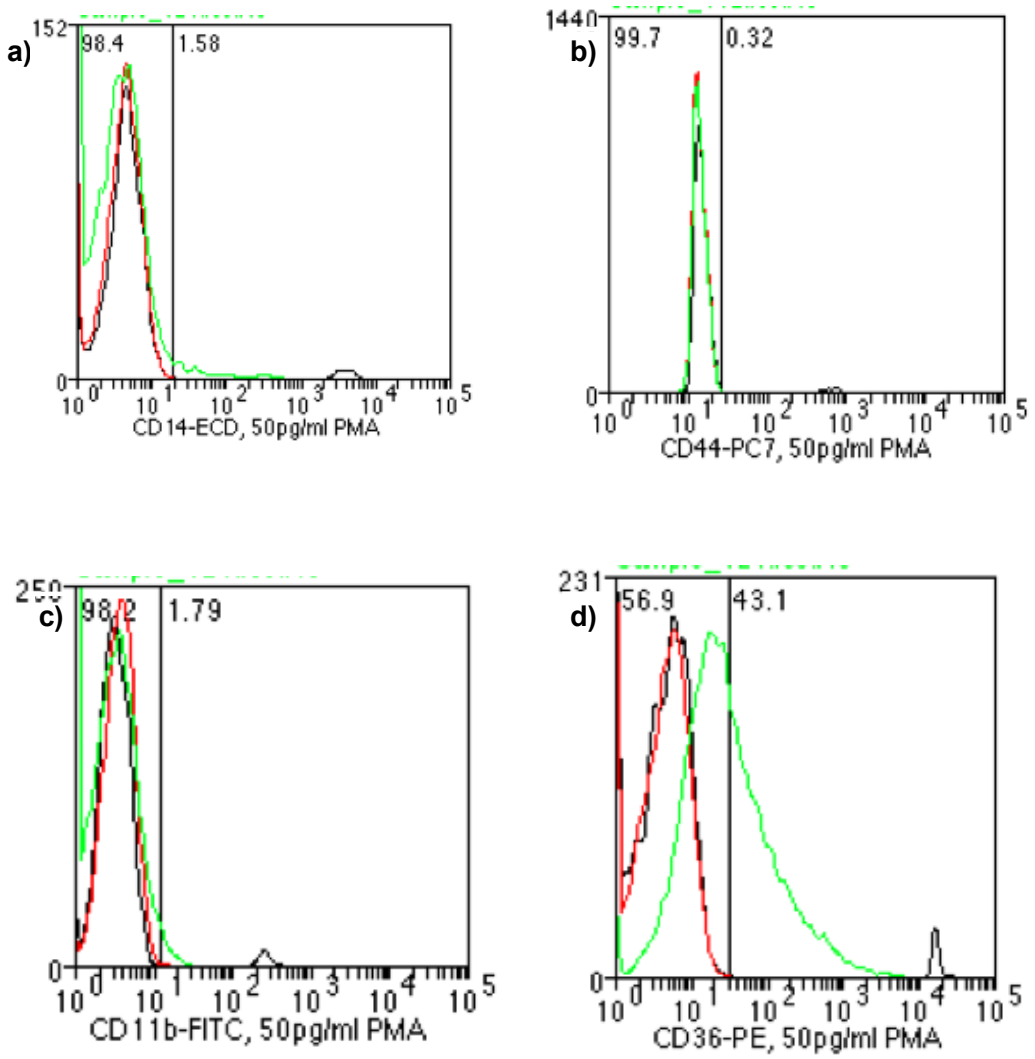


Figure 3.8: Flow cytometric analysis of surface macrophage CD markers stimulating with 50 pg/ml PMA

Flow cytometric analysis of surface markers on THP-1 cells: black curves represent un-stained cells; red curves the isotype and the green curves the test antibody a) CD14-ECD, b) CD44-PC7, c) CD11b-FITC, and d) CD36-PE data are representative of 5 repeats.

Subsequently, the cells were stimulated with different concentrations of PMA (100 pg/ml, 150 pg/ml, 250 pg/ml and 500 pg/ml), and staining was performed for only CD36, CD49a, CD14 and CD11b as they showed expression at 50 pg/ml PMA. The results for the different concentrations of PMA (representative plots of all repeats Figure 3.9 to Figure 3.12) showed CD36 and CD49a were highly expressed, CD14 had low levels of expression, and CD11b was moderately positive with the higher PMA concentrations. Increasing PMA concentrations above 100 pg/ml did not appear to further increase marker expression.

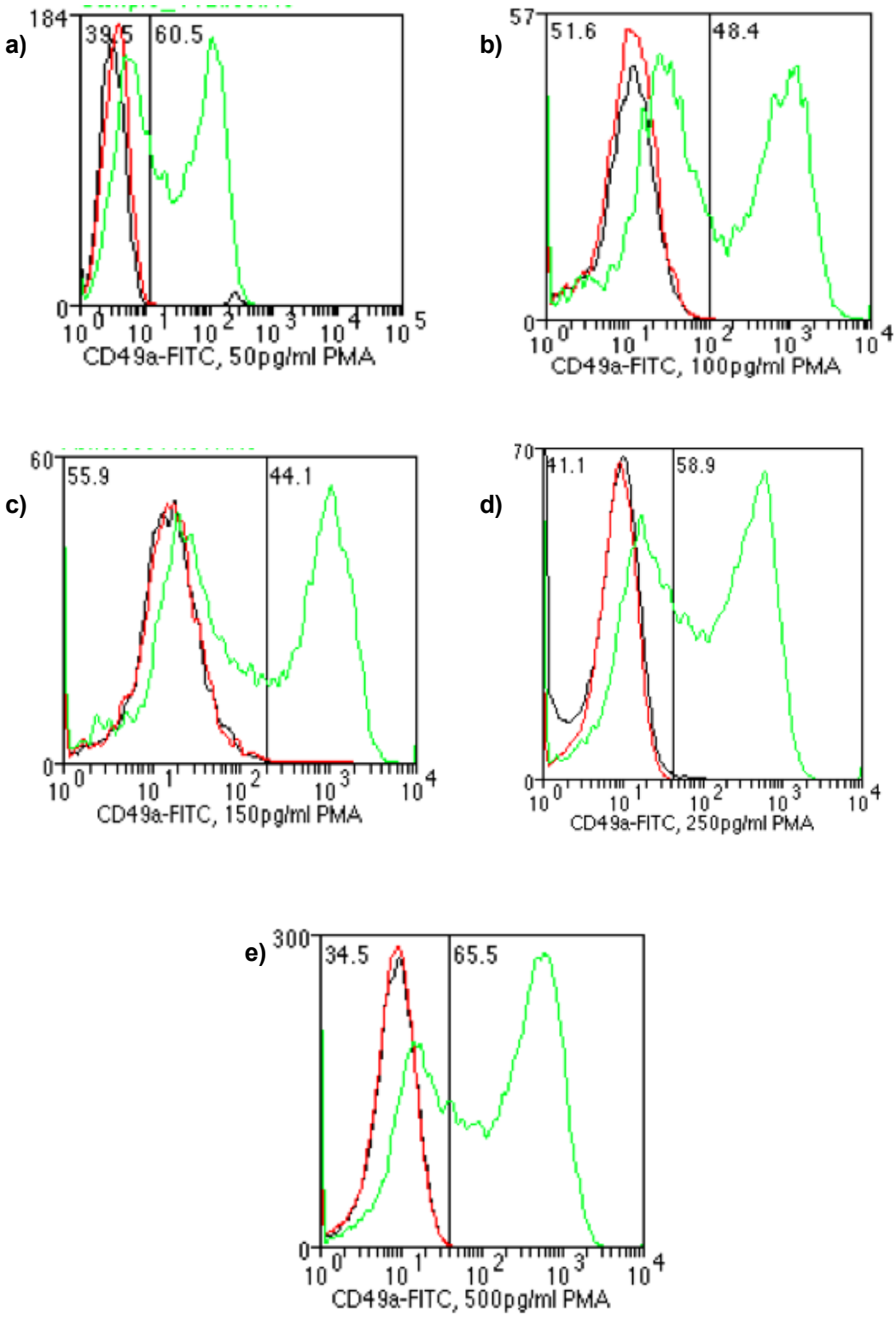


Figure 3.9: Flow cytometric analysis of CD49a expression after increasing PMA stimulation

Flow cytometric analysis of surface markers on THP-1 cells: black curves represent un-stained cells; red curves the isotype and the green curves the test antibody. Concentration of PMA a) 50 pg/ml, b) 100 pg/ml, c) 150 pg/ml, d) 250 pg/ml and e) 500 pg/ml data are representative of 5 repeats.

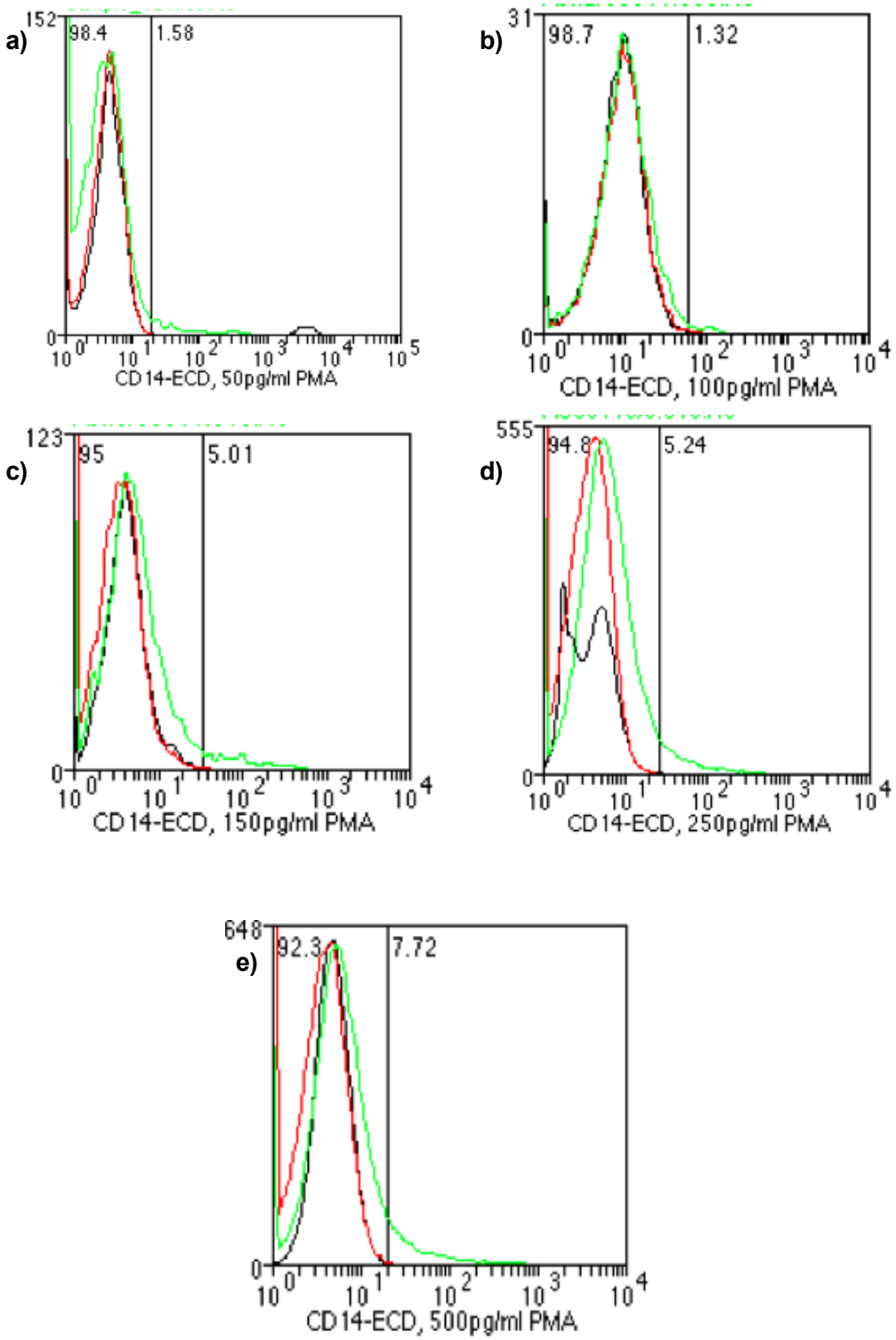


Figure 3.10: Flow cytometric analysis of CD14 expression after increasing PMA stimulation

Flow cytometric analysis of surface markers on THP-1 cells: black curves represent un-stained cells; red curves the isotype and the green curves the test antibody. Concentration of PMA a) 50 pg/ml, b) 100 pg/ml, c) 150 pg/ml, d) 250 pg/ml and e) 500 pg/ml data are representative of 5 repeats.

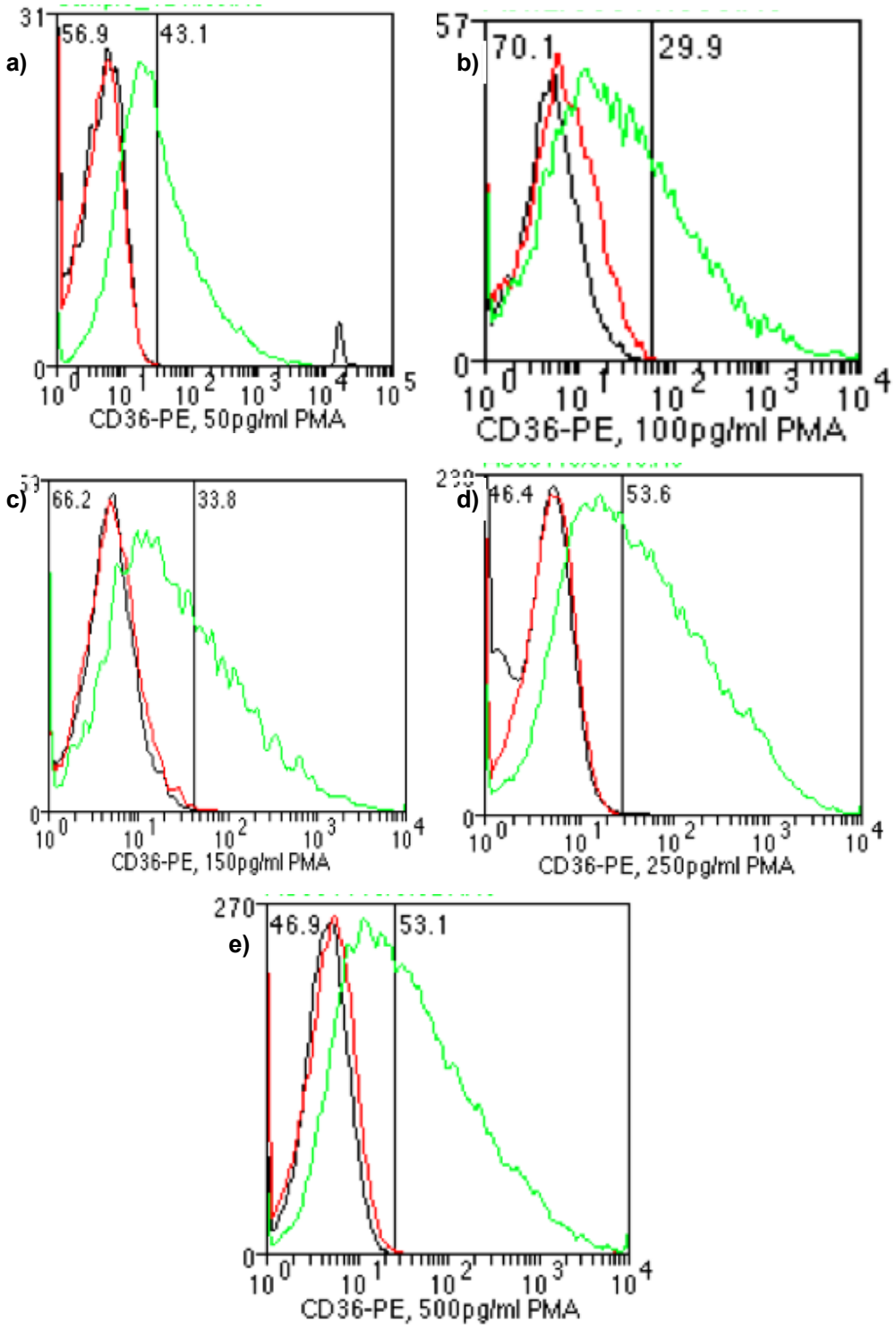


Figure 3.11: Flow cytometric analysis of CD36 expression after increasing PMA stimulation

Flow cytometric analysis of surface markers on THP-1 cells: black curves represent un-stained cells; red curves the isotype and the green curves the test antibody. Concentration of PMA a) 50 pg/ml, b) 100 pg/ml, c) 150 pg/ml, d) 250 pg/ml and e) 500 pg/ml data are representative of 5 repeats.

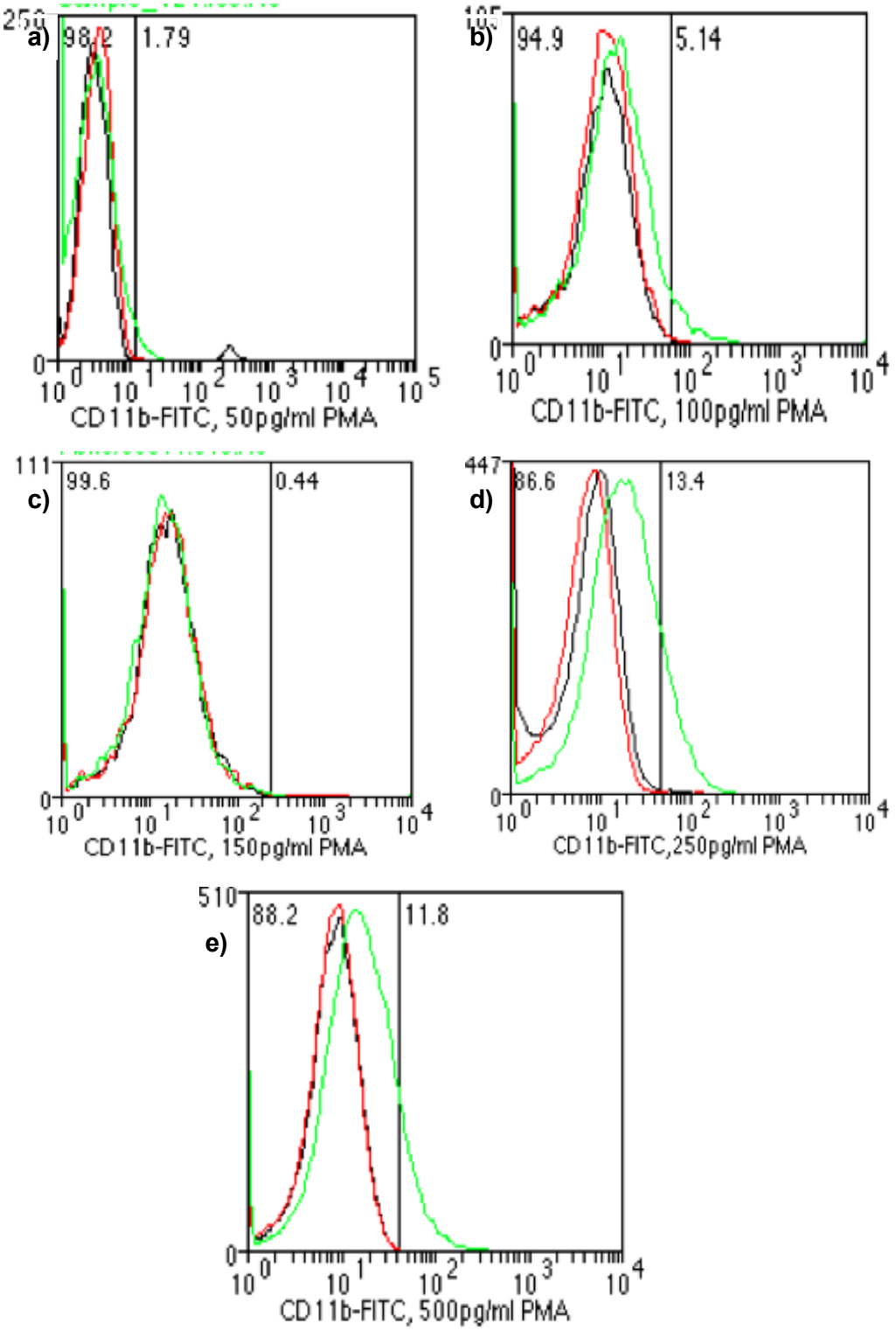


Figure 3.12: Flow cytometric analysis of CD11b expression after increasing PMA stimulation

Flow cytometric analysis of surface markers on THP-1 cells: black curves represent un-stained cells; red curves the isotype and the green curves the test antibody. Concentration of PMA a) 50 pg/ml, b) 100 pg/ml, c) 150 pg/ml, d) 250 pg/ml and e) 500 pg/ml data are representative of 5 repeats.

By summarising the data from the repeats for the different concentrations of PMA (Figure 3.13) the median change in the cell surface markers can be seen along with any differences between the repeats. All of the results had small error bars, it suggests the repeats were very similar. The percent positive for each cell surface marker is quite low; CD69 has the lowest percent positive, then HLA-DR followed by CD49a, and CD45 had the highest percent positive. The mean sample MFI was higher than the un-stained or isotype control cells for all concentrations of PMA for the markers CD49a ($P \leq 0.0001$ for all except 100 pg/ml which was $P \leq 0.001$) and CD36 ($P \leq 0.0001$ for all), whereas the concentration of PMA had a larger effect on the expression of CD14 and CD11b, with some of the conditions that used a lower PMA concentration resulting in a reduced expression ($P \leq 0.01$ for CD14 with 50 pg/ml PMA, $P \leq 0.05$ for CD11b with 50 pg/ml PMA) compared to the un-stained cells or isotope control. All but two of the treatments used produced significant differences in the sample when compared to the isotype control. The two conditions that did not show a difference were 100 pg/ml PMA with CD14, and 150 pg/ml PMA with CD11b: both of these gave abnormally high levels of fluorescence for both unstained cells and isotype control staining, suggestive of experimental error in these instances.

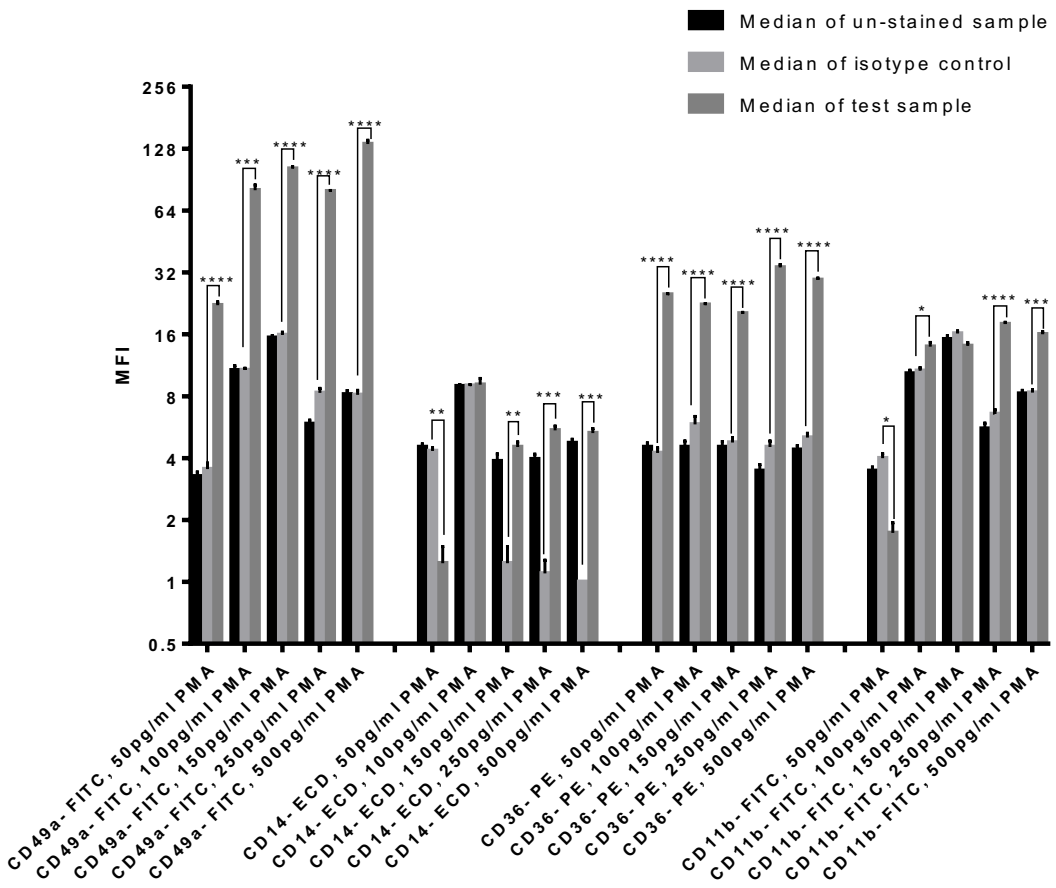


Figure 3.13: Mean MFI of THP-1 macrophage markers after treatment with PMA.

Data plotted are mean (N = 5) and error bars are SD. Statistical significance was determined using a t-test with Bonferroni-Dunn correction, P values < 0.05 were considered as significant, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$.

These results have shown there were significant increases in expression in the monocyte samples for CD11b, CD36, CD44 ($P \leq 0.0001$ for all), and CD14 ($P = 0.0011$) when compared to the isotype control, and in the macrophage samples for CD11b, CD36, CD44 ($P \leq 0.0001$ for all), and CD14 ($P = 0.0011$) when compared to the isotype control. In summary, as shown in table 3.1, PMA stimulation of THP-1 cells resulted in high expression of CD49a and CD36, lesser expression of CD69, HLA-DR, CD14 and CD11b, and little or no expression of CD45RA or CD44.

3.4. Discussion

COPD development is associated with several changes of the immune system (Sharma, Hanania and Shim, 2009); our interest is focused on the role of macrophages in this disease. As antigen presenting cells, macrophages play an important role not only in innate immunity, but also in the adaptive response of the immune system (Tetley, 2002). By understanding the relation of macrophages with other cells, this research might help to provide alternative and more effective plans for therapeutic approaches to limit the progressions of COPD or eliminate it.

The goal of this thesis was to study the effects of CS on macrophages *in vitro*, and in this chapter the suitability of the THP-1 cell line cultured in RPMI 1640 was investigated. As not all cell lines can produce similar results to the corresponding primary cells, our experiments were separated into two distinctive parts to investigate cell suitability for future experiments. The first part was to stain THP-1 cells for different markers of both monocytes and macrophages and detect their presence using flow cytometry, while the second part was to differentiate the THP-1 cells by using PMA at different concentrations to generate macrophages, then investigate the cell surface markers using flow cytometry.

Based on the recommendations in the guidelines produced by Becton-Dickinson Bioscience Company (B.D.) (Mittar, Paramban and McIntyre, 2011) the markers CD45RA, CD49a, HLA-DR and CD69 were chosen to stain THP-1 cell line to detect monocytes, CD44 and CD11b were chosen to detect macrophages, and CD14 and CD36 were chosen to detect both (Mittar, Paramban and McIntyre, 2011). The choice of CD markers was also consistent with other published data on THP-1 cells (reviewed by Chanput, Mes and Wichers, 2014; Forrester *et al.*, 2018).

Unexpectedly, there were differences detected between what has previously been reported and the results in the present study (Table 3.1), which demonstrated expression on cultured THP-1 cells of some macrophage markers even without the use PMA. For example, CD44 has been reported to be upregulated on THP-1 cells activated with PMA but (Zhang *et al.*, 2014; Mittar *et al.*, 2011), in the present study, it was found to be highly expressed on unstimulated THP-1 cells, and expression was lost on cells treated with PMA. Similarly, several studies have reported upregulation of expression of CD14, CD11b and CD36 on stimulated THP-1 cells (Daigneault *et al.*, 2010; Park *et al.*, 2007;

Schwende *et al.*, 1996; Zhou *et al.*, 2010; Forrester *et al.*, 2018), but no difference was observed in the present study for expression of these markers between stimulated and unstimulated cells. This may have been due to differences in the mode of stimulation of the cells, or abnormalities due to prolonged passaging of the cell line.

Some studies have suggested that the THP-1 cell line can be used successfully for studying more general immune mechanisms *in-vitro* (Park *et al*., 2007). However, our data showed that THP-1 cells did not produce the expected results, which agrees with the studies of Vaddi and Newton (1994) and Markotic *et al.* (2007), who found that they were not satisfied with THP-1 results. Vaddi and Newton (1994) described the biological responses of human monocytes and the THP-1 cell line by stimulating cells with members of the beta chemokine family, they found that human monocytes had a marked desensitisation to MCP-1, but there was no such desensitisation observed in THP-1 cells. Moreover, the THP-1 cells failed to show any response of aggregation and polarisation when stimulated with chemokines whereas, in monocytes, these two responses attained a peak magnitude after 10 min (Vaddi and Newton, 1994). Furthermore, there were some missing links in the downstream signal transduction pathways, and lack of consequent responses when the calcium response was prompted with chemokines in THP-1 cells (Vaddi and Newton, 1994). Markotić *et al.* (2007) compared several cytokines and their receptors in the THP-1 cell line and primary monocytes to understand whether hantaviruses induced the same immune reactions in these types of cells. They were interested in this question because monocytes are believed to be target cells for Hantaviruses, and also important sources and recipients for cytokine secretion (Markotić *et al.*, 2007). They found that infected primary monocytes expressed mostly beta- chemokines and their receptors, whereas THP-1 cells expressed receptors for CXC chemokines. They concluded that THP-1 cells are not a good model for *in vitro* research of the immune-pathogenesis of Hantaviruses in humans (Markotić *et al.*, 2007). Similarly, Tedesco *et al* (2018) have recently concluded that differentiated THP-1 cells do not fully reproduce the spectrum of responses of human monocyte-derived macrophages, such that THP-1 should be regarded as simplified model of the behaviour of primary macrophages.

Moreover, it is believed that THP-1 cells may be useful cells for generating macrophages but only in early passage (Schreier, 2009). Parallel analysis of primary

monocytes or others cell lines alongside THP-1 cells may yield interesting results and provide further insight into regulation in these cell types.

3.4.1. Conclusions

This study aimed to establish an *in vitro* experimental system to study the effects of CSE on macrophages. One of the ways to study this would be to use macrophage cell lines. However, our results showed that the THP-1 cell line used was not very efficient as some monocytes were still found among the differentiated macrophages, and some macrophage markers were expressed on THP-1 cells without stimulation. Furthermore, there was unexplained differences in marker expression between the THP-1 cells cultured in our laboratory and those reported in the literature. These findings, together with reports in the literature that THP-1 cells are of limited value as a model of primary human monocytes and macrophages, suggests that further study in this cell line would be of little use. Therefore, another type of cell, namely fresh peripheral blood derived monocytes, was investigated for their suitability for use in studying the effects of CSE (Chapter 4).

4. Investigating the suitability of peripheral blood mononuclear cells as a model to study COPD

4.1. Introduction

4.1.1. Investigating the role of macrophages in COPD using Peripheral Blood Mononuclear Cells

The results in the previous chapter showed that the THP-1 cells were not suitable for use in this study; thus, a different cell model was needed to evaluate the changes in expression of macrophage markers after exposure to CSE and nicotine in combination with Poly-IC or LPS. Another commonly used cell type for investigating the role that macrophages play in diseases are monocytes derived from Peripheral Blood Mononuclear Cells (PBMCs), as these can easily be manipulated and their interactions with the environment and other immune cells can easily be monitored.

Human PBMCs have been widely used as controls, both positive and negative, in different kinds of assays including ELISA and in flow cytometry assays (Slota *et al.*, 2011). These cells are characterised by relatively round nuclei, as seen in lymphocytes and monocytes, as distinct from the polymorphonuclear granulocytes. Such cells have a crucial role in the immune system in fighting infection. The population of lymphocytes contains different kinds of cells, including $CD4^+$ and $CD8^+$ T cells, B cells and Natural Killer (NK) cells; monocytes and dendritic cells are also included in the PBMC population. By using histopaque 1077 (ficoll) all these cells can be isolated from whole blood by centrifugation, forming a so-called 'buffy coat' of cells above the histopaque and underneath the layer of plasma (Vissers *et al.*, 2013).

In this study, the presence of different cell surface markers (CD14, CD16, CD206 and CD11b) on macrophages were investigated using flow cytometry. Each of these markers have a specific role and therefore any changes in their expression can be linked to changes in macrophage expression or activity. These markers were selected based

on previous studies, for example the studies in alveolar macrophages showed a positive correlation between the numbers of monocyte-like cells and expression of the monocyte-associated surface antigens CD11b and CD14, and an inverse relationship with the macrophage-associated surface antigens CD16 and CD206 (Krombach *et al*., 1996; Kapellos *et al*., 2018; Sica & Mantovani, 2012; Alkhattabi *et al*., 2018).

CD14 is known as the LPS receptor that binds to Endotoxin/LPS; it also associates with the other cellular proteins TLR4, LBP, LY96, and TLR2. It is a co-receptor of innate immunity that is strongly expressed on monocytes, macrophages, and weakly on neutrophils (Khazen *et al.*, 2005). CD16 is known as FcγRIIIA, Fc γ receptor, and is associated particularly with activated neutrophils. Generally, CD14 and CD16 are found together on secretory vesicles of neutrophils, but both of these markers are also expressed on macrophages, and NK cells (van de Velde, Mottram and Hogarth, 2006).

CD206, also known as the macrophage mannose receptor (MMR), is a scavenger receptor that is found principally on the surface of tissue macrophages, lymphatic and hepatic endothelial cells, in both humans and mice (Taylor, 2001; Stahl, 1992). This marker can be identified by a pattern of carbohydrate that it contains, and it plays an important role in phagocytosis of microorganisms in both host defence and homeostasis (McKenzie, 2002).

CD11b can be found on the surface of macrophages, neutrophils, monocytes, NK cells, and subclasses of lymphocytes (Khazen *et al.*, 2005). This marker plays a crucial role in the migration of leukocytes from peripheral blood to the sites of infection (Springer, 1990); it functions as an adhesion molecule as a subunit of the leucocyte integrin Mac-1 (Butcher, 1990).

LPS was selected to stimulate the cells as it is an activator of macrophages as it is a bacterial cell component of colonising bacteria, that could be present in COPD patients (Gordon and Taylor, 2005). Based on the work by Alkhattabi *et al*. (2018) who carried out similar studies in DCs, the stimulating effect was produced by addition of 100 ng/ml of LPS with 24 h incubation to help to generate mature macrophages. Poly-IC was selected as it is a synthetic analogue of double-stranded RNA (dsRNA), which has a molecular pattern associated with viral infection, another complication that could be

present in COPD patients (Gordon & Taylor, 2005). Again, based on the work by Alkhattabi (2015), we used 30 µg/ml of Poly-IC and incubated the cells for 24 h.

As PBMCs are a convenient source of human primary monocytes, they were used in this study. CD14 positive cells were isolated from human donor buffy coats by using CD14 MicroBeads, cultured in 24 well plates coated with 20 µl of 1 % HMDS (hexamethyldisilane) in 100 % propanol, and stimulated with M-CSF to form macrophages, which were then stained with antibodies to CD markers specific for macrophages to confirm the identity of the cells before exposing them to CSE and nicotine in combination with Poly-IC or LPS to investigate the changes to macrophages in COPD.

4.2. Methods

4.2.1. Coating culture plate with Hexamethyldisilazane (HMDS)

In order to minimise the adhesion of macrophages and increase the number of cells harvested, wells of a 24 well plate were coated with 20 µl of 1 %, 3 % or 5 % HMDS in ethanol [70 % or 100 %], or 100 % propanol, for 10 minutes, and washed with 1 ml ethanol 70 %. After the wells were dried, 750,000 $CD14^+$ cells were seeded per well and incubated at 37°C for 6-7 days. On day 0 all CD14 negative and positive cells were stained for the macrophage markers CD14, CD16, CD206, CD11b and CD36 in order to check the purity of cells. On Day 3 of culture, cells were fed with 500 µl per well of fresh RPMI complete medium containing M-CSF.

For collection of macrophages on day 7, the culture plate was placed on ice for 15 minutes; then the medium containing suspended cells was collected. The remaining adherent cells were washed once with cold EDTA in PBS (1.5 ml/ well) and placed on ice for 15 minutes. At the end of this time, medium has collected and placed into FACS tubes to assess the phenotype of the cells.

4.3. Results

4.3.1. Checking the phenotype of $CD14^-$ cells

PBMCs were fractionated using CD14 microbeads, as described in the Materials and Methods chapter (section 2.2.4). On day 0 both CD14 negative and CD14 positive cells were tested for macrophage cell surface markers (CD14, CD16, CD206, and CD11b) by flow-cytometry following cell surface staining. The results for the effluent ($CD14^-$) cells (Figure 4.1) indicated there was a good population of healthy cells, with the absence of expression of both the CD14 and CD206 markers (2 % or less of total cells), and the presence of CD16 and CD11b, which were expressed on up to 30 % of the cells. These results are as expected, and show the purity (lack of contamination) of CD14 negative cells.

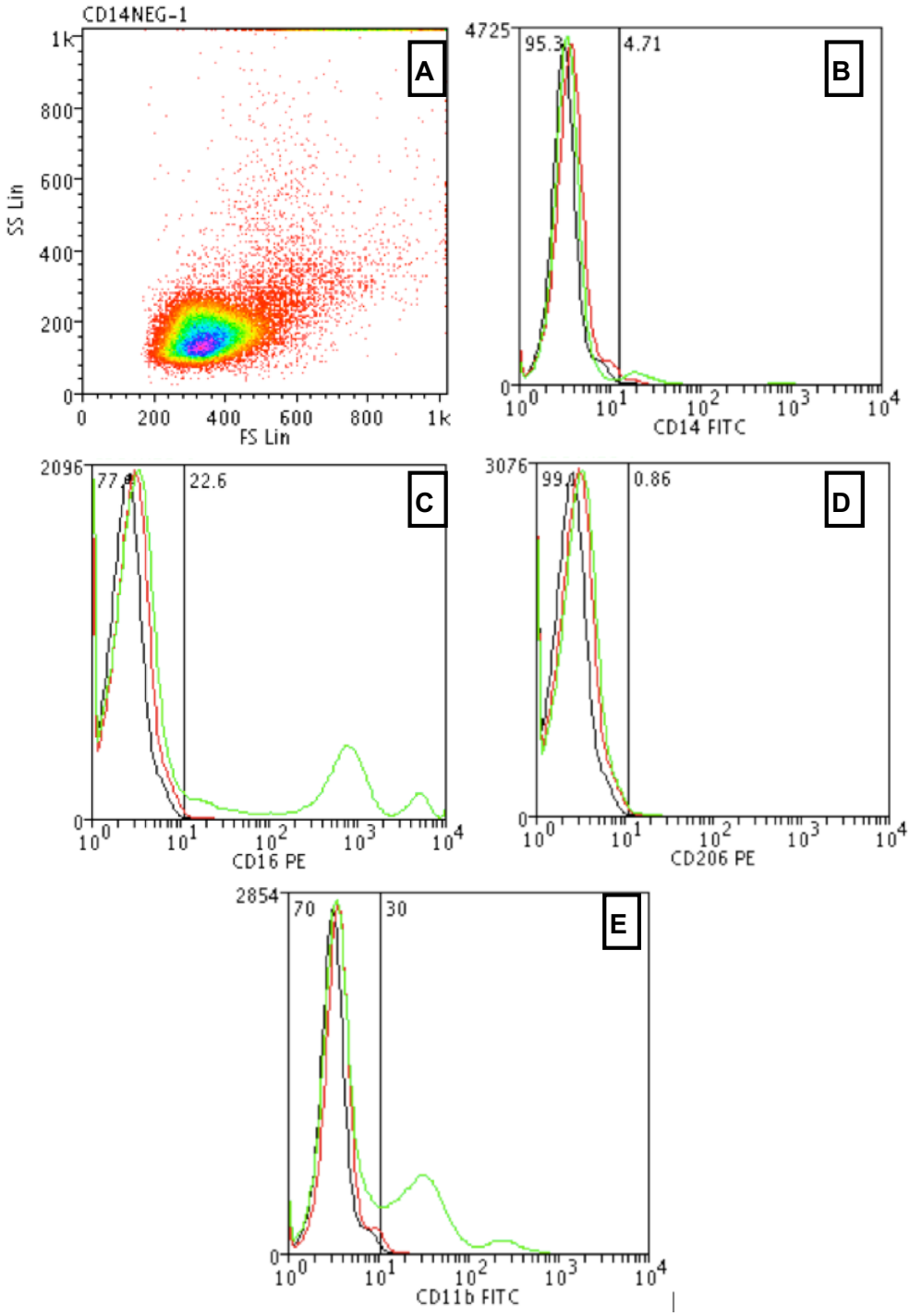


Figure 4.1: Testing the purity of CD14 negative cells on day 0

A) The plot of forward scatter and side scatter showing viability of cells. Flow cytometric analysis of surface markers on CD14 negative cells: black represents un-stained cells; red curves represent the isotype control and green curves represent test antibody, traces are representative of repeats (N = 4); B) CD14 FITC, C) CD16 PE, D) CD206 and E) CD11b FITC.

4.3.2. Checking the phenotype of $CD14^+$ cells

The set of markers used to stain CD14 negative cells was also used to investigate the purity of CD14 positive cells by flow-cytometry following cell surface staining. The results (Figure 4.2) indicated there was an absence of the mannose receptor CD206, with low expression of CD16 (~10 % of total cells), and high expression (up to 100 %) of CD14 and CD11b. These results are as expected and show the CD14 positive cells were 100 % without contamination. The scatter plot (Figure 4.1A) proved that these cells are a different size and shape from the $CD14^-$ cells, which is as expected for this type of cell.

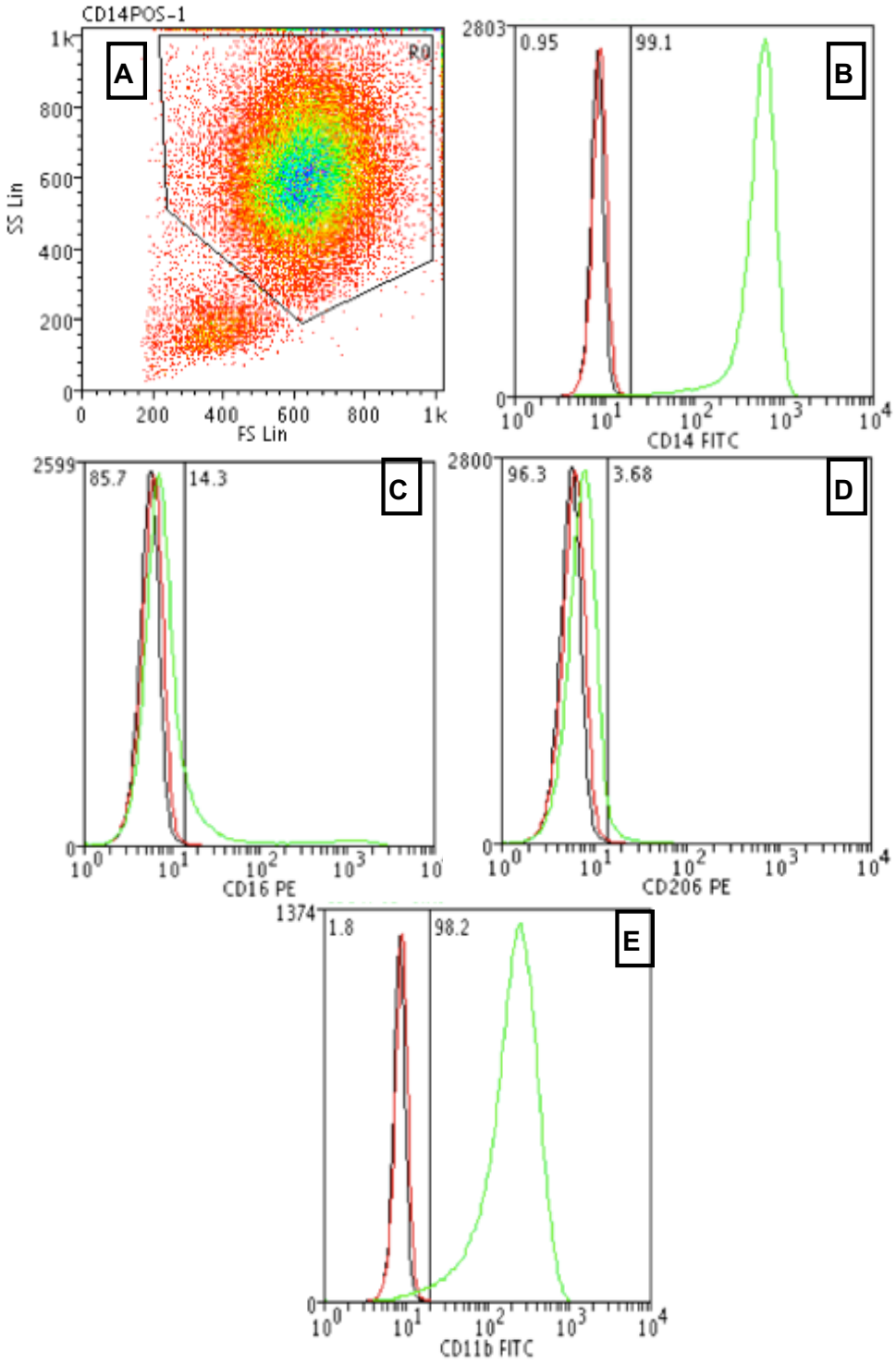


Figure 4.2: Testing the purity of CD14 positive cells on day 0

A) The plot of forward scatter and side scatter showing viability of cells. Flow cytometric analysis of surface markers on CD14 positive cells: black represents un-stained cells; red curves represent the isotype control and green curves represent test antibody, traces are representative of repeats (N = 4); B) CD14 FITC, C) CD16 PE, D) CD206 and E) CD11b FITC.

4.3.3. Culturing $CD14^+$ cells in different concentration of Hexamethyldisilazane (HMDS)

It has been reported in the literature that stimulating $CD14^+$ cells with M-CSF in complete medium for 7-10 days can lead to the production of mature M0-like macrophages (Gleissner, 2012). To investigate this, we treated cells with M-CSF in culture plates coated with different concentrations of HMDS, initially using 1 % and 3 % HMDS in 70 % ethanol, then expanding the conditions to include 5 % HMDS dissolved in 100 % ethanol or 100 % propanol. The reason for this was that the initial experiments without any coating produced a low number of recovered macrophages, so this experiment was carried out to determine whether coating with HMDS would reduce adhesion of the cells to the plastic surface and hence facilitate their recovery and yield from the wells of the tissue culture plates for use in further experimental procedures. To our knowledge, HMDS had not previously been used in this manner, so the ideal conditions for use needed to be identified. We investigated the macrophage phenotype by measuring the cell surface markers CD14, CD16, CD206 and CD11b by flow cytometry. Cells were gated according to size and granularity. R1 are considered dead cells and R0 are considered macrophages.

By comparing the results of the cells cultured without HMDS (Figure 4.3) to the different percentages of HMDS in different concentrations of alcohol, the effect of coating on cell recovery and viability, and expression of cell surface markers could be determined. The results for the different concentrations of HMDS in ethanol (Figure 4.4, Figure 4.5, Figure 4.6, Figure 4.7) show that generally there was variability in the quality of the cells, with some small deviations in the expression of the cell surface markers investigated. The results for the different concentrations of HMDS in propanol (Figure 4.8, Figure 4.9, Figure 4.10) showed generally a higher percentage of good quality cells and a similar level of expression of cell surface markers, which suggests that the coating does affect cell viability but does not significantly affect the expression of cell surface markers, making it a suitable medium with which to coat the plates.

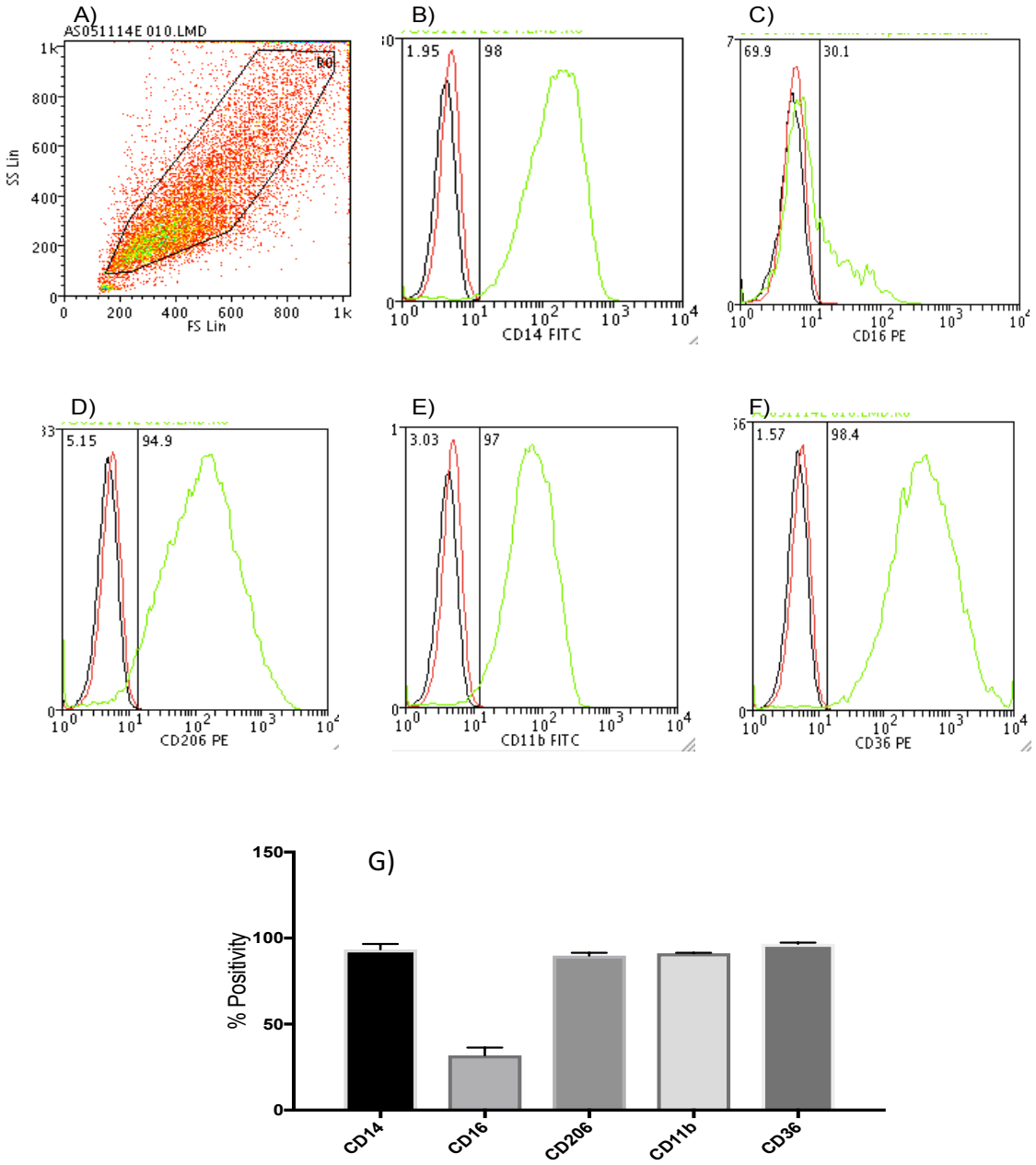


Figure 4.3: Macrophages CD markers on day 7, grown on plates not coated with HMDS

A) plot of forward scatter and side scatter. B) CD14 FITC, C) CD16 PE, D) CD206, E) CD11b FITC and F) CD36 PE histograms of flow-cytometric analysis of surface markers, black represents un-stained cells; green represents isotype control and red represents test antibody. G) Graph representation of percentage cell expressing CD14, CD16, CD206, CD11b and CD36. Error bars represent mean with standard deviation (N = 2).

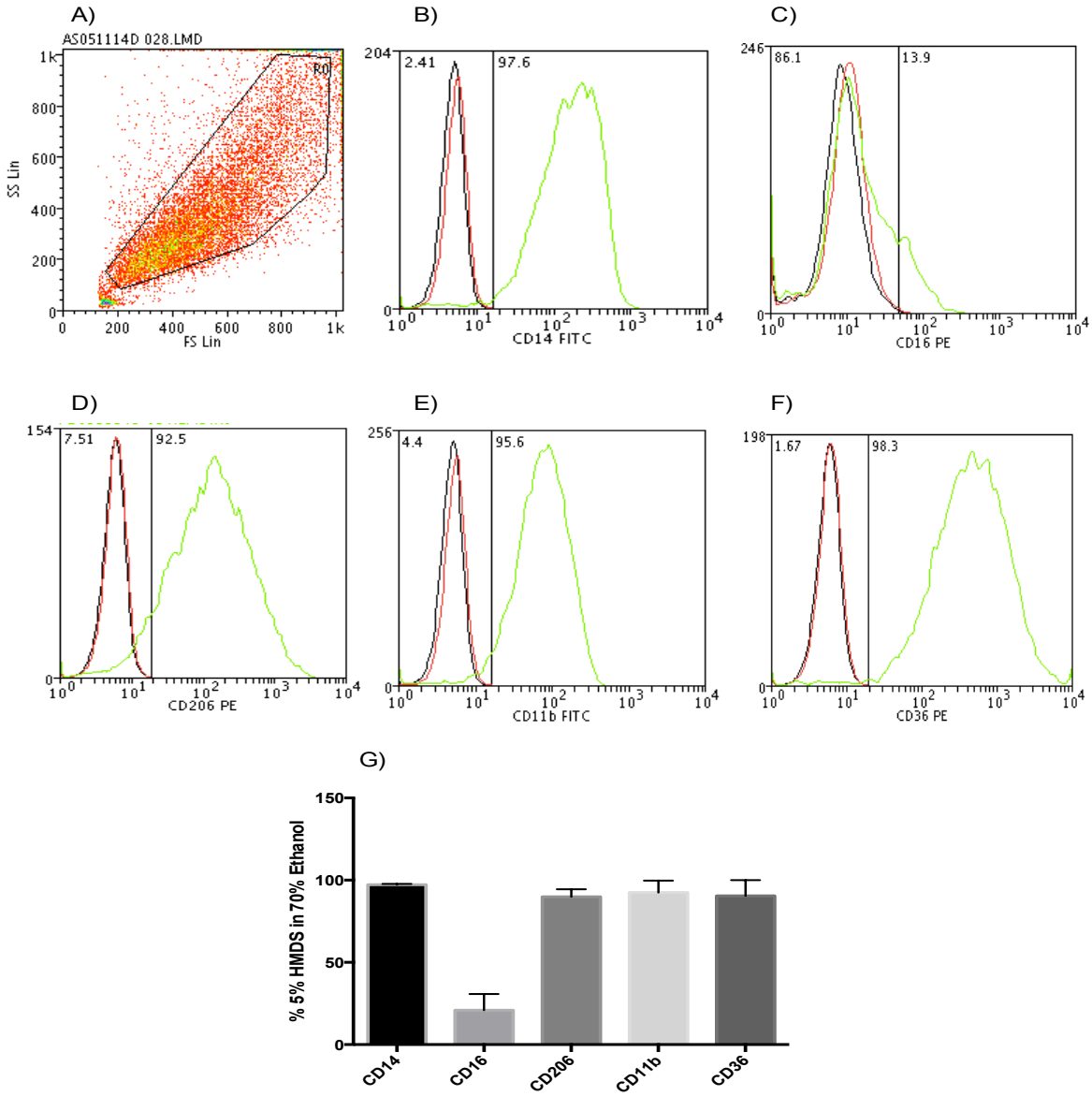


Figure 4.4: Macrophages CD markers on day 7, grown on plates coated with 5 % HMDS in 70 % ethanol

A) plot of forward scatter and side scatter. B) CD14 FITC, C) CD16 PE, D) CD206, E) CD11b FITC and F) CD36 PE histograms of flow-cytometric analysis of surface markers; black represents un-stained cells, green represents isotype control and red represents test antibody. G) Graph representation of percentage cell expressing CD14, CD16, CD206, CD11b and CD36. Error bars represent mean with standard deviation (N = 2).

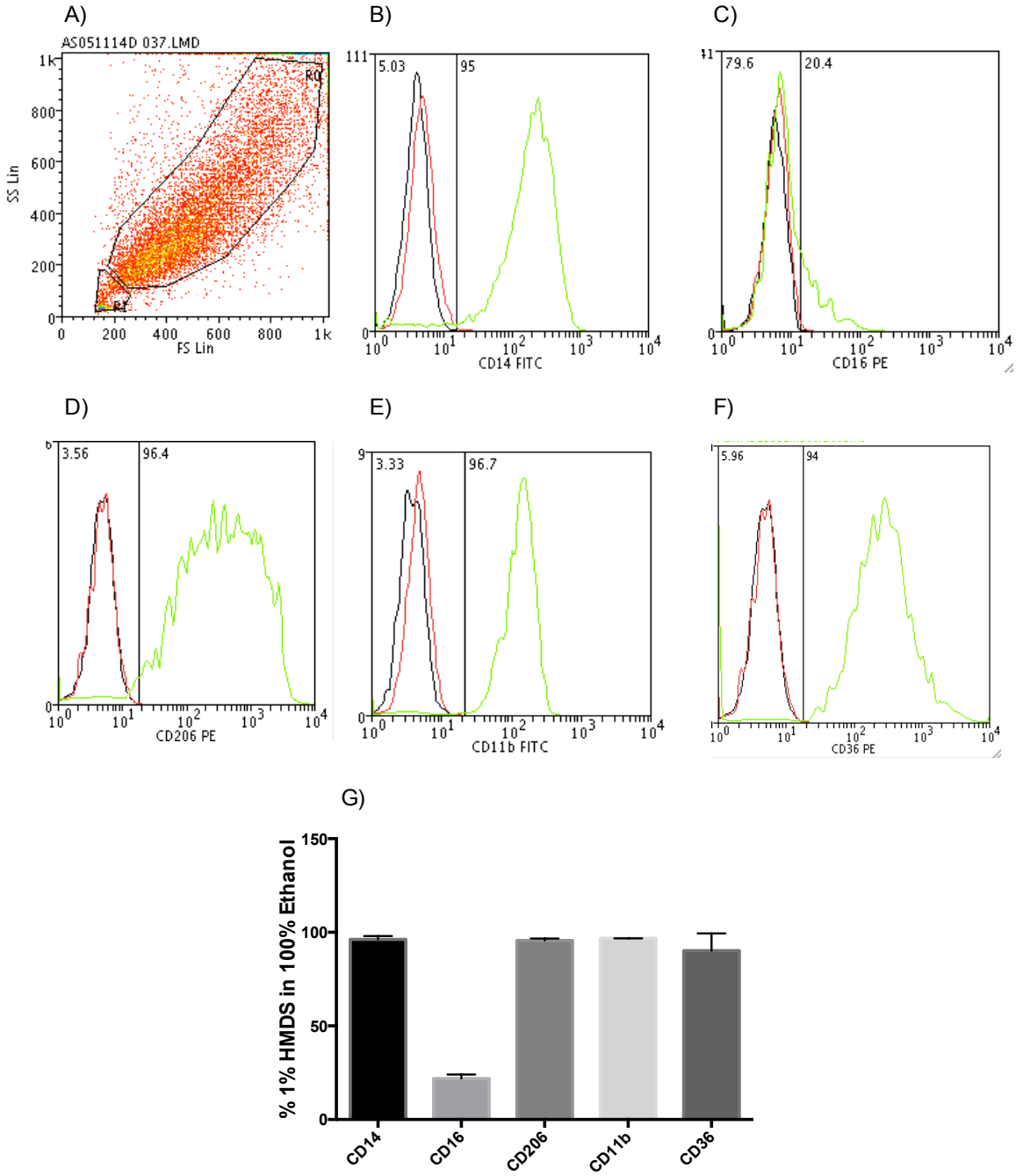


Figure 4.5: Macrophages CD markers on day 7, grown on plates coated with 1 % HMDS in 100 % ethanol

A) plot of forward scatter and side scatter. B) CD14 FITC, C) CD16 PE, D) CD206, E) CD11b FITC and F) CD36 PE histograms of flow-cytometric analysis of surface markers, black represents un-stained cells; green represents Isotype control and red represents test antibody. G) Graph representation of percentage cell expressing CD14, CD16, CD206, CD11b and CD36. Error bars represent mean with standard deviation (N = 2).

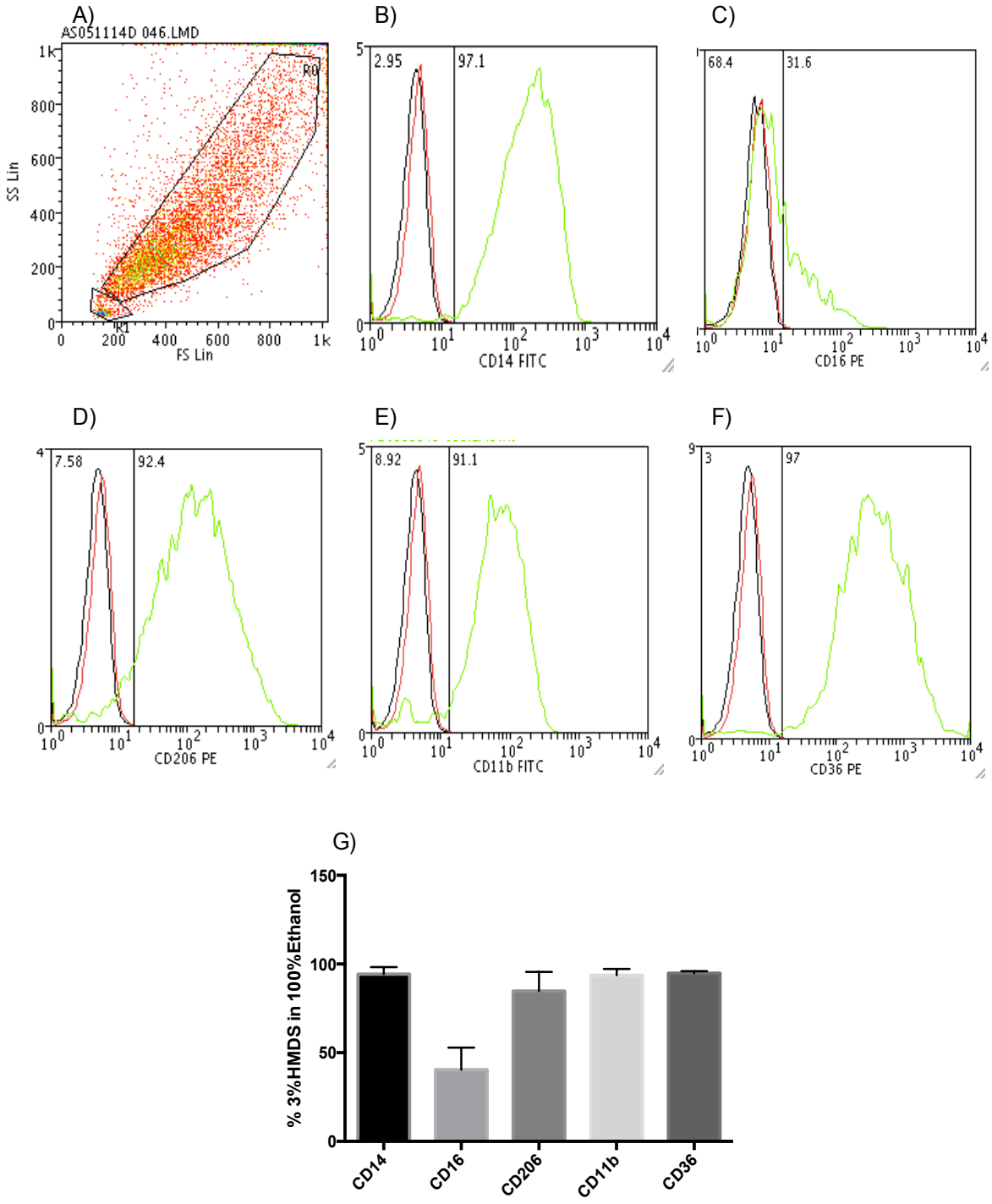


Figure 4.6: Macrophages CD markers on day 7, grown on plates coated with 3% HMDS in 100 % ethanol

A) plot of forward scatter and side scatter. B) CD14 FITC, C) CD16 PE, D) CD206, E) CD11b FITC and F) CD36 PE histograms of flow-cytometric analysis of surface markers, black represents un-stained cells; green represents Isotype control and red represents test antibody. G) Graph representation of percentage cell expressing CD14, CD16, CD206, CD11b and CD36. Error bars represent mean with standard deviation (N = 2).

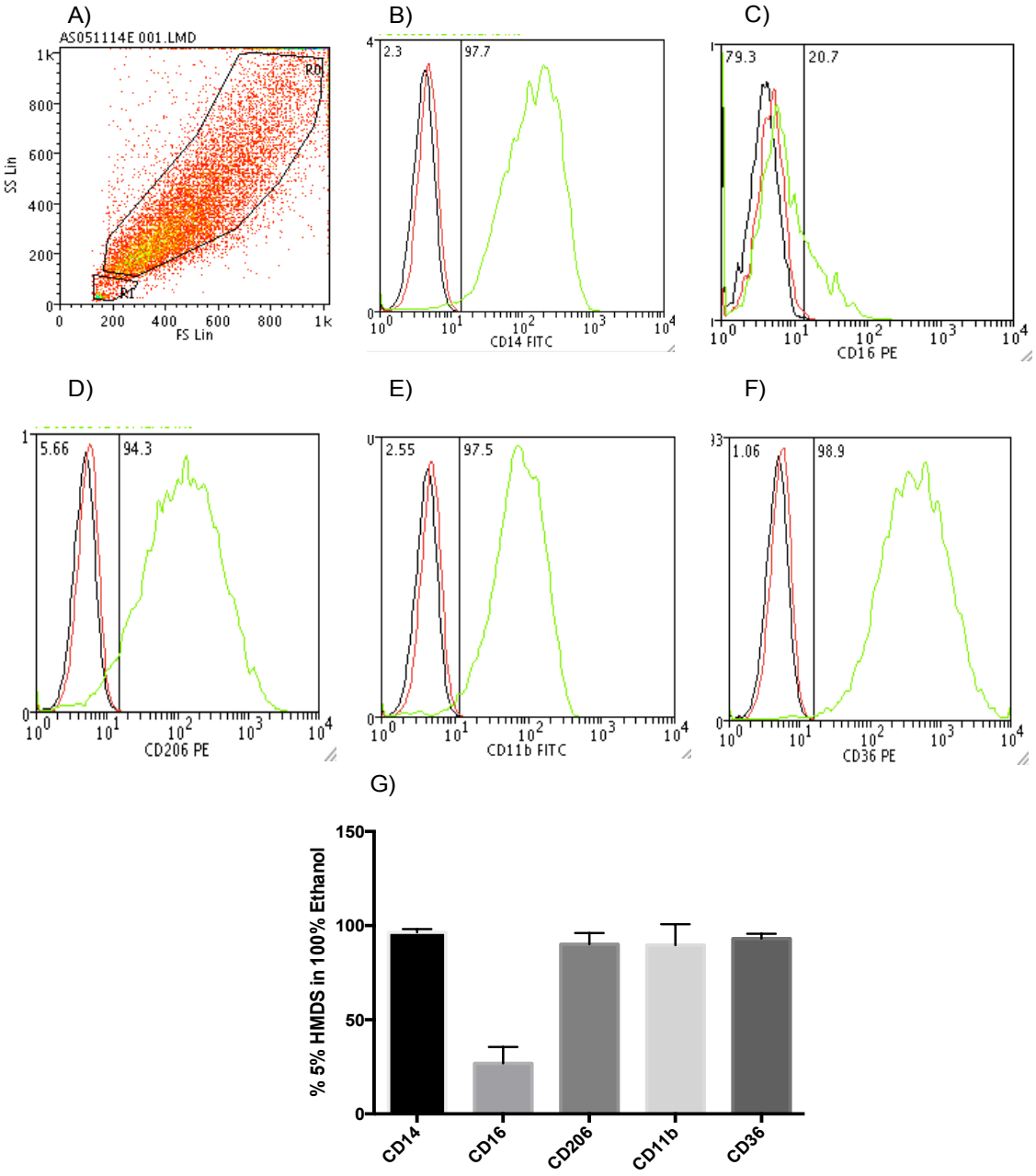


Figure 4.7: Macrophages CD markers on day 7, grown on plates coated with 5 % HMDS in 100 % ethanol

A) plot of forward scatter and side scatter. B) CD14 FITC, C) CD16 PE, D) CD206, E) CD11b FITC and F) CD36 PE histograms of flow-cytometric analysis of surface markers, black represents un-stained cells; green represents Isotype control and red represents test antibody. G) Graph representation of percentage cell expressing CD14, CD16, CD206, CD11b and CD36. Error bars represent mean with standard deviation (N = 2).

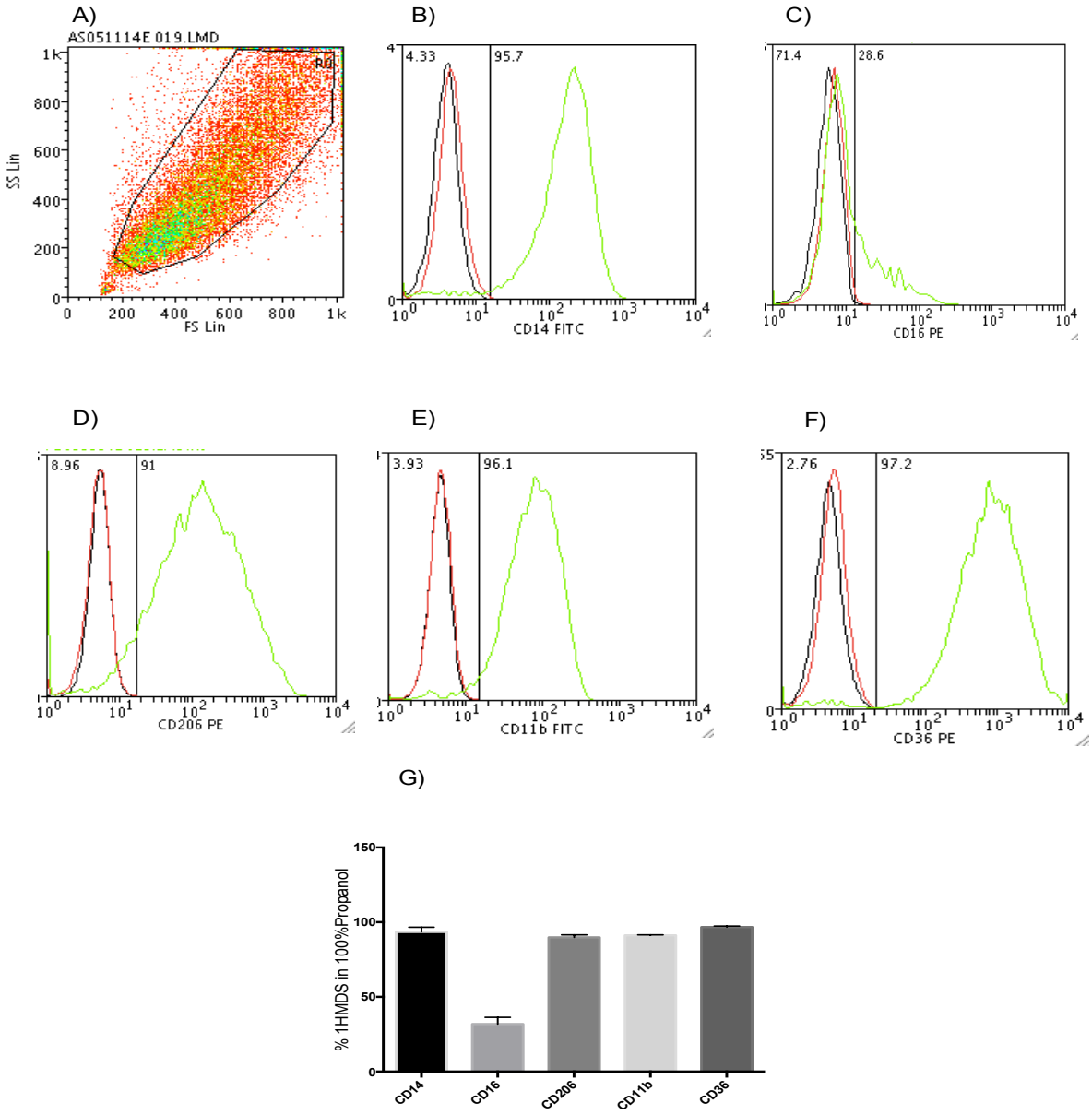


Figure 4.8: Macrophages CD markers on day 7, grown on plates coated with 1 % HMDS in 100 % propanol

A) plot of forward scatter and side scatter. B) CD14 FITC, C) CD16 PE, D) CD206, E) CD11b FITC and F) CD36 PE histograms of flow-cytometric analysis of surface markers, black -represents un-stained cells; green represents Isotype control and red represents test antibody. G) Graph representation of percentage cell expressing CD14, CD16, CD206, CD11b and CD36. Error bars represent mean with standard deviation (N = 2).

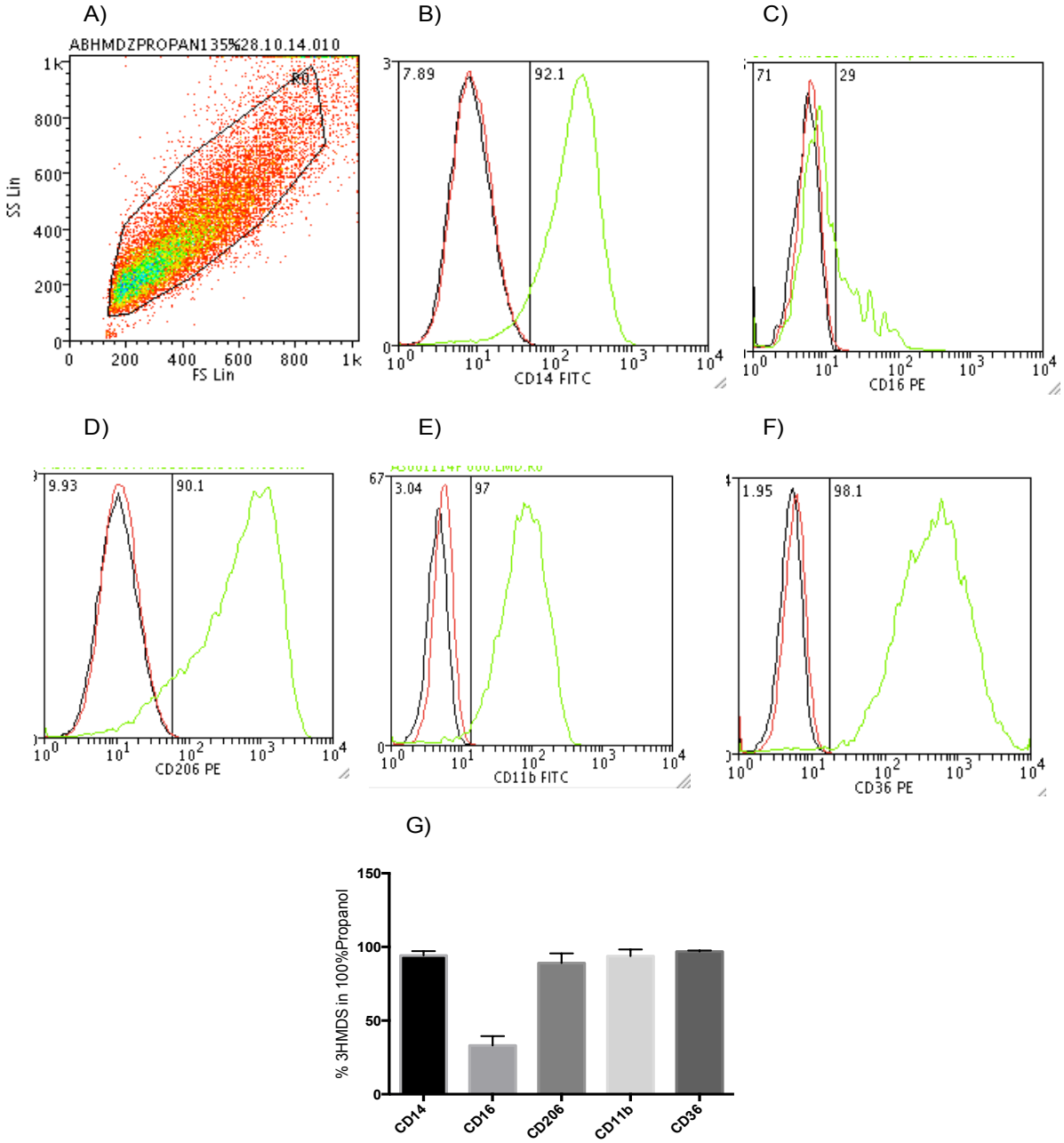


Figure 4.9: Macrophages CD markers on day 7, grown on plates coated with 3 % HMDS in 100 % propanol

A) plot of forward scatter and side scatter. B) CD14 FITC, C) CD16 PE, D) CD206, E) CD11b FITC and F) CD36 PE histograms of flow-cytometric analysis of surface markers, black represents un-stained cells; green represents Isotype control and red represents test antibody. G) Graph representation of percentage cell expressing CD14, CD16, CD206, CD11b and CD36. Error bars represent mean with standard deviation (N = 2).

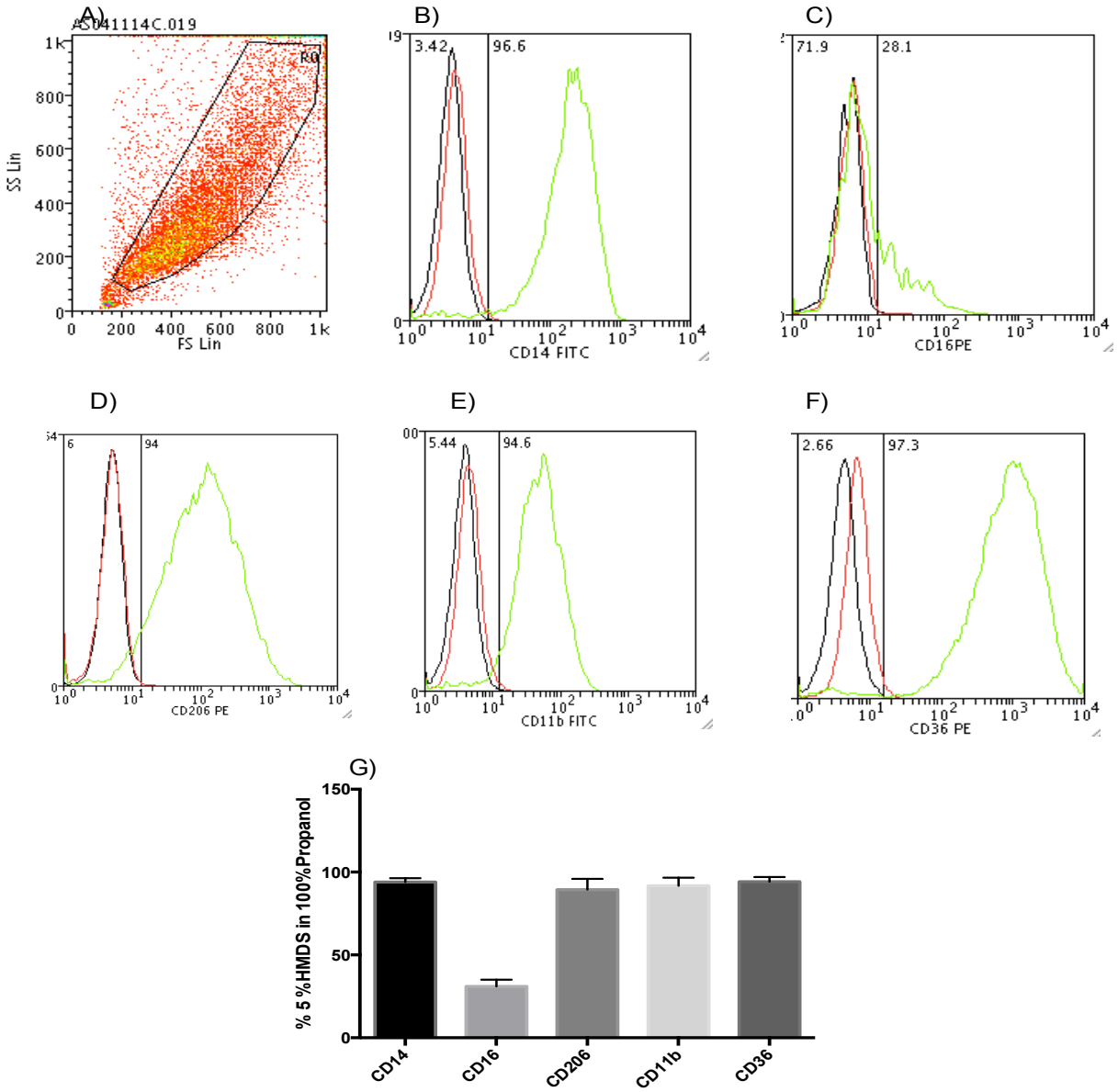


Figure 4.10: Macrophages CD markers on day 7, grown on plates coated with 5 % HMDS in 100 % propanol

A) plot of forward scatter and side scatter. B) CD14 FITC, C) CD16 PE, D) CD206, E) CD11b FITC and F) CD36 PE histograms of flow-cytometric analysis of surface markers, black represents un-stained cells; green represents Isotype control and red represents test antibody. G) Graph representation of percentage cell expressing CD14, CD16, CD206, CD11b and CD36. Error bars represent mean with standard deviation (N = 2).

The results shown above indicate that using plates coated with HMDS (in either ethanol or propanol) did not obviously affect the phenotype of the macrophages generated compared to cultures performed in the absence of HMDS.

To find the best conditions for harvesting a large number of good quality macrophages, the number of cells recovered for the different treatments were plotted. The results (Figure 4.11) showed that the conditions yielding the highest number of cells were 1 % HMDS in 100 % propanol (~25,000 cells), 3 % HMDS in 100 % propanol (~20,000 cells) and cells without any treatment (~15000 cells), respectively. Therefore 1 % HMDS in 100 % propanol was selected for use in future experiments.

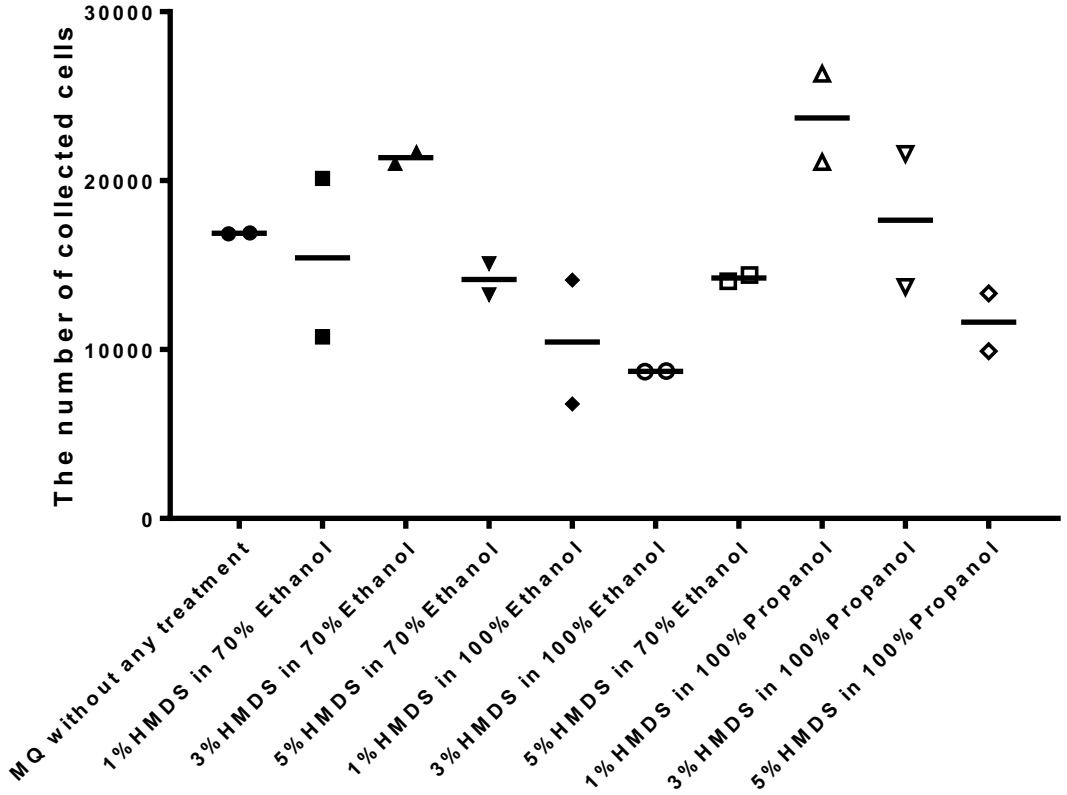


Figure 4.11: The number of macrophages collected under the different culture conditions
N = 2 for all treatments; bar represents the median value.

4.3.4. Phenotyping of macrophages on day 8

At day seven, macrophages were stimulated with 100 ng/ml LPS or 30 μg/ml Poly-IC and exposed to CSE (1 % or 3 %) or nicotine (50 or 150 μg/ml). Before the cells were treated, the cigarette extract was prepared (section 2.2.11), diluted to create 10 %, 20 % and 50 % CSE solutions, then the absorbance at 320 nm was measured, using phenol red free RPMI-1640 medium as a blank, to create a CSE standard curve (Figure 4.12).

The CSE extract was then diluted to create 1 and 3 % solutions for use in the experiments.

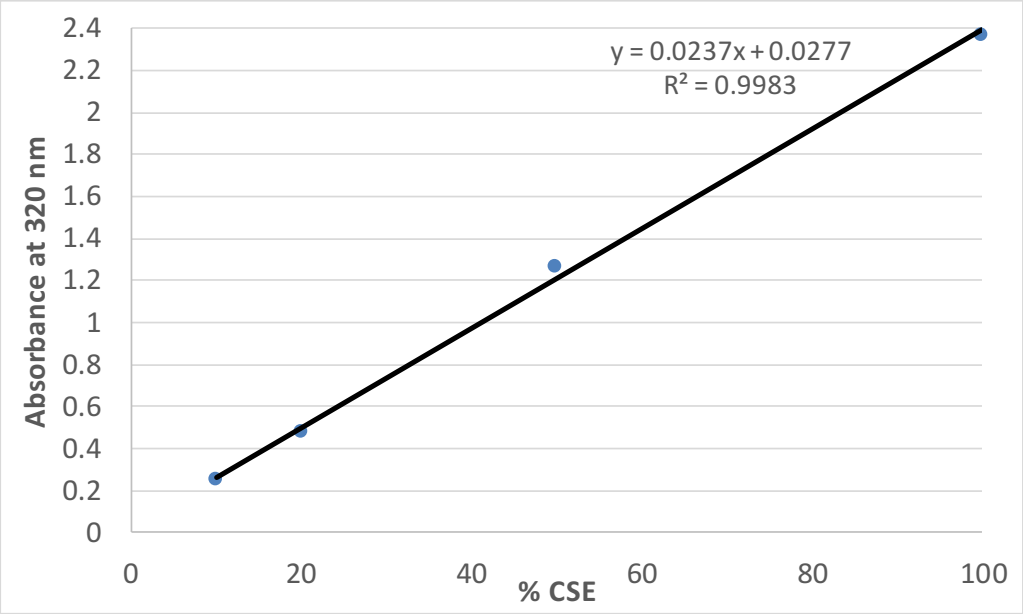


Figure 4.12: CSE extract standard curve.

After 24 h of incubation, cells were harvested and used to detect the expression of the cell surface markers used previously, by flow cytometry. Examples of the results for the controls without CSE or nicotine for all the cell markers investigated are shown below (Figure 4.13 – representative of the 4 repeats). Examples of the flow cytometry results produced after exposure to CSE and nicotine for CD14 (Figure 4.14), CD16 (Figure 4.15), CD206 (Figure 4.16) and CD11b (Figure 4.17), show that some of the treatment combinations produced changes in expression when compared to the controls (Figure 4.13). The analysis of the combined data is given in the next section.

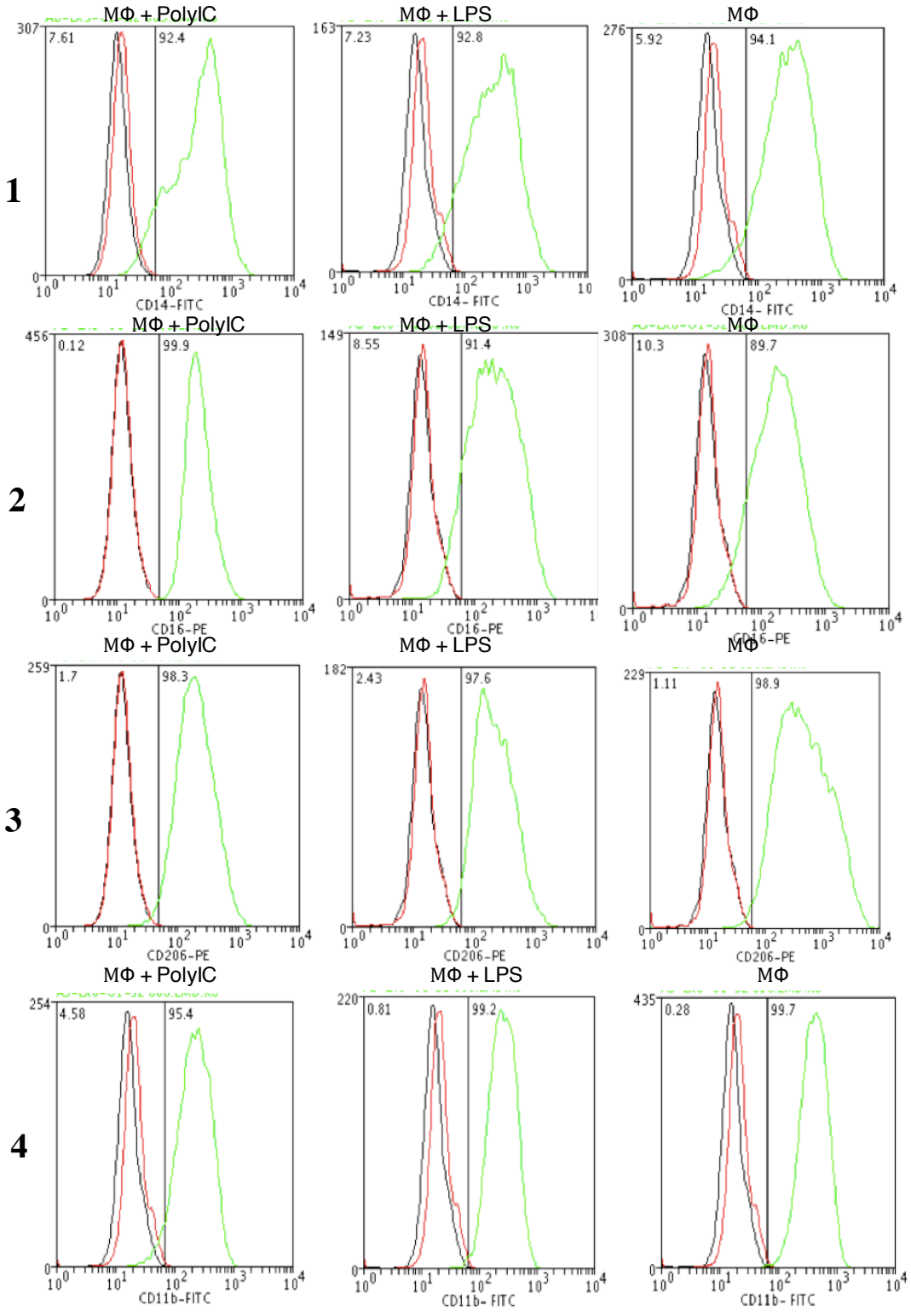


Figure 4.13: Analysis of the controls for expression of all the markers on day 8 after stimulation

Flow cytometric analysis of MΦ surface markers: black represents un-stained cells; red curves represent the isotype control and green curves represent test antibody, traces are representative of repeats (N = 4). Left = macrophages (MΦ) treated with Poly-IC, middle = MΦ treated with LPS, and right = MΦ only control, for 1) CD14, 2) CD16, 3) CD206 and 4) CD11b.

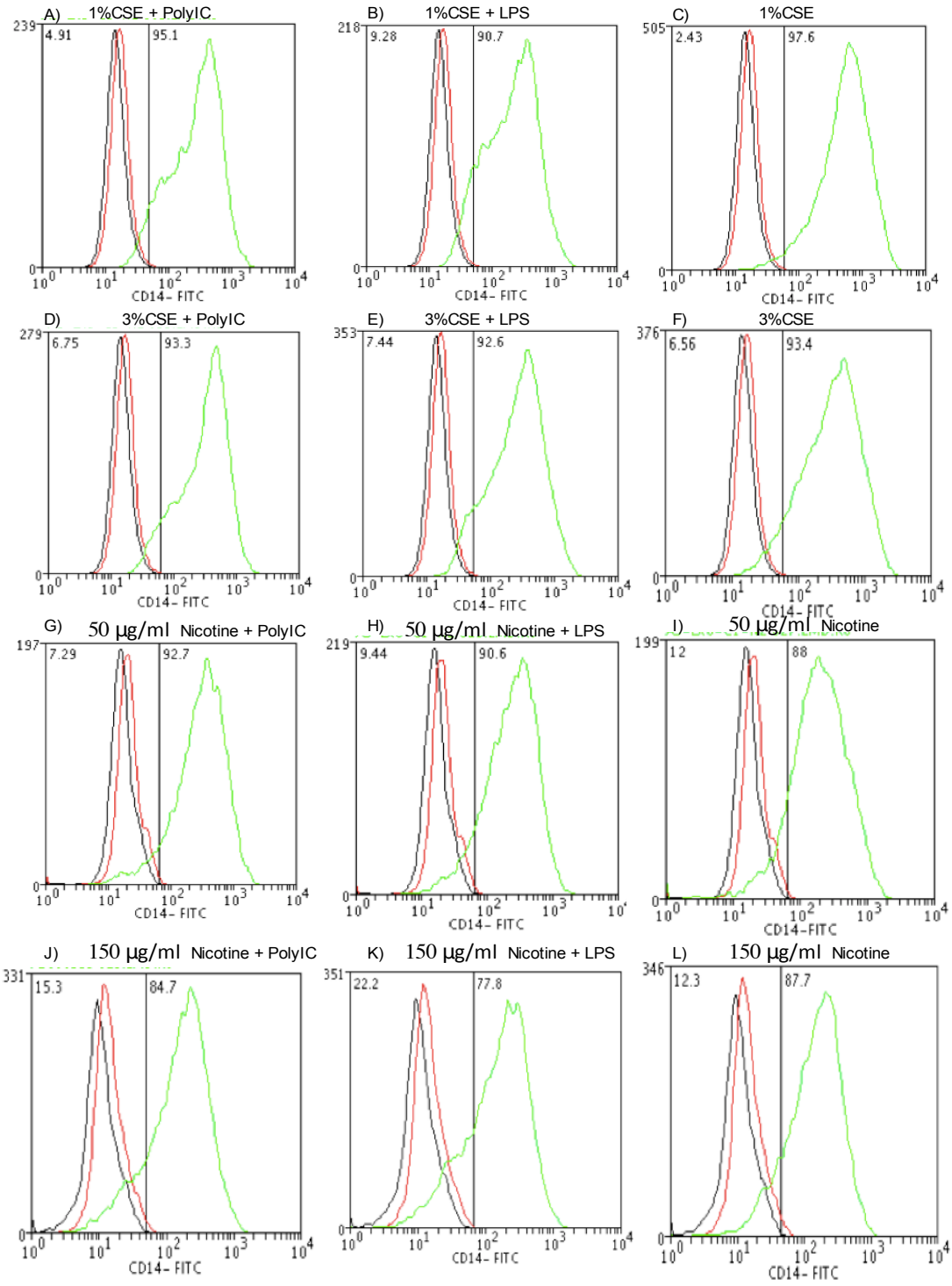


Figure 4.14: Expression of CD14 measured by flow cytometry for all treatments on day 8

Flow cytometric analysis of MΦ surface markers, black represents un-stained cells; red curves represent the isotype control and green curves represent test antibody, traces are representative of repeats (N = 4). A) 1 % CSE with Poly-IC, B) 1 % CSE with LPS and C) 1 % CSE. D) 3 % CSE with Poly-IC, E) 3 % CSE with LPS and F) 3 % CSE, G) 50 µg/ml Nicotine with Poly-IC, H) 50 µg/ml Nicotine with LPS, I) 50 µg/ml Nicotine, J) 150 µg/ml Nicotine with Poly-IC, K) 150 µg/ml Nicotine with LPS and L) 150 µg/ml Nicotine.

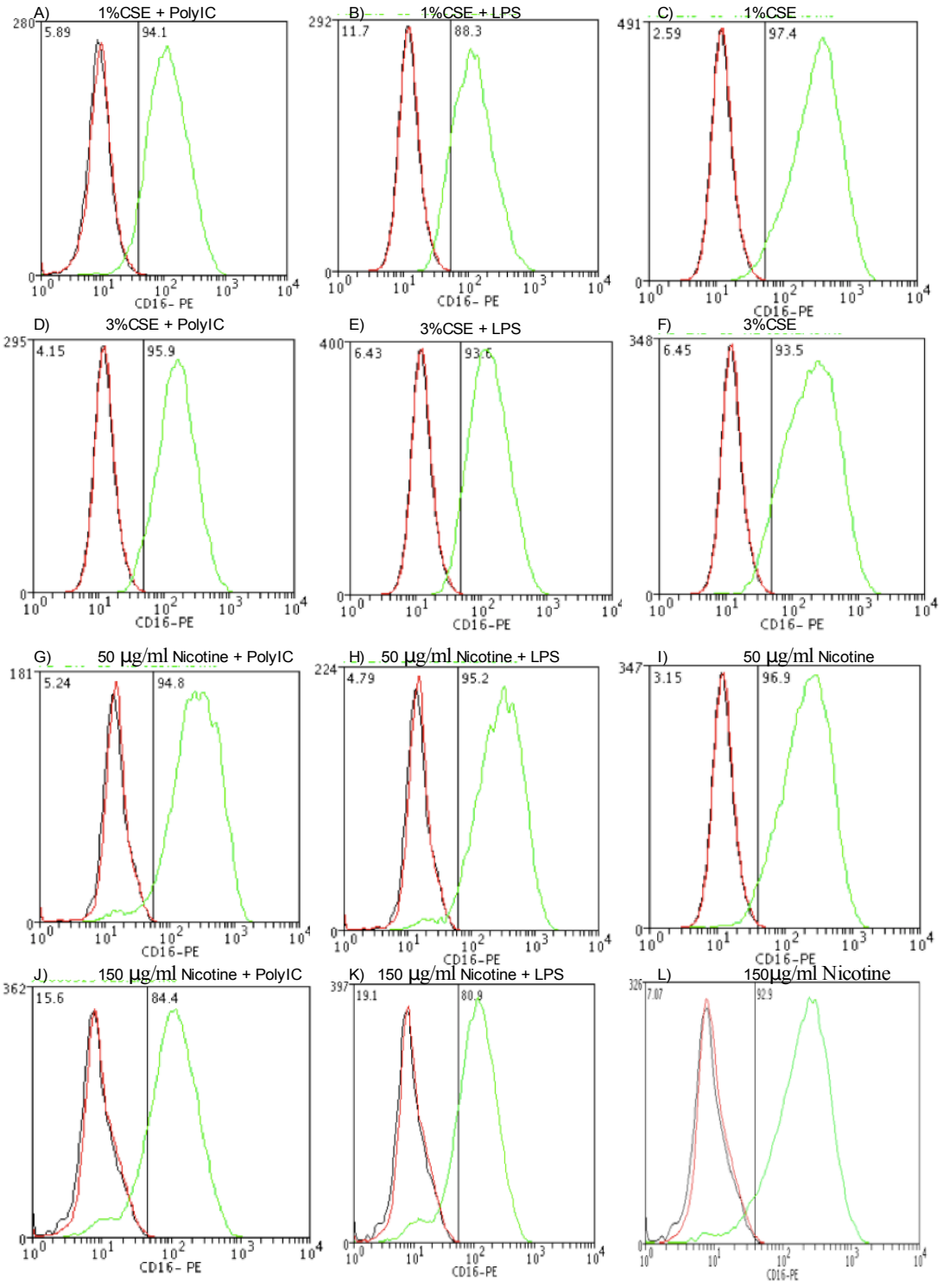


Figure 4.15: Expression of CD16 measured by flow cytometry for all treatments on day 8

Flow cytometric analysis of MΦ surface markers, black represents un-stained cells; red curves represent the isotype control and green curves represent test antibody, traces are representative of repeats (N = 4). A) 1 % CSE with Poly-IC, B) 1 % CSE with LPS and C) 1 % CSE. D) 3 % CSE with Poly-IC, E) 3 % CSE with LPS and F) 3 % CSE, G) 50 µg/ml Nicotine with Poly-IC, H) 50 µg/ml Nicotine with LPS, I) 50 µg/ml Nicotine, J) 150 µg/ml Nicotine with Poly-IC, K) 150 µg/ml Nicotine with LPS and L) 150 µg/ml Nicotine.

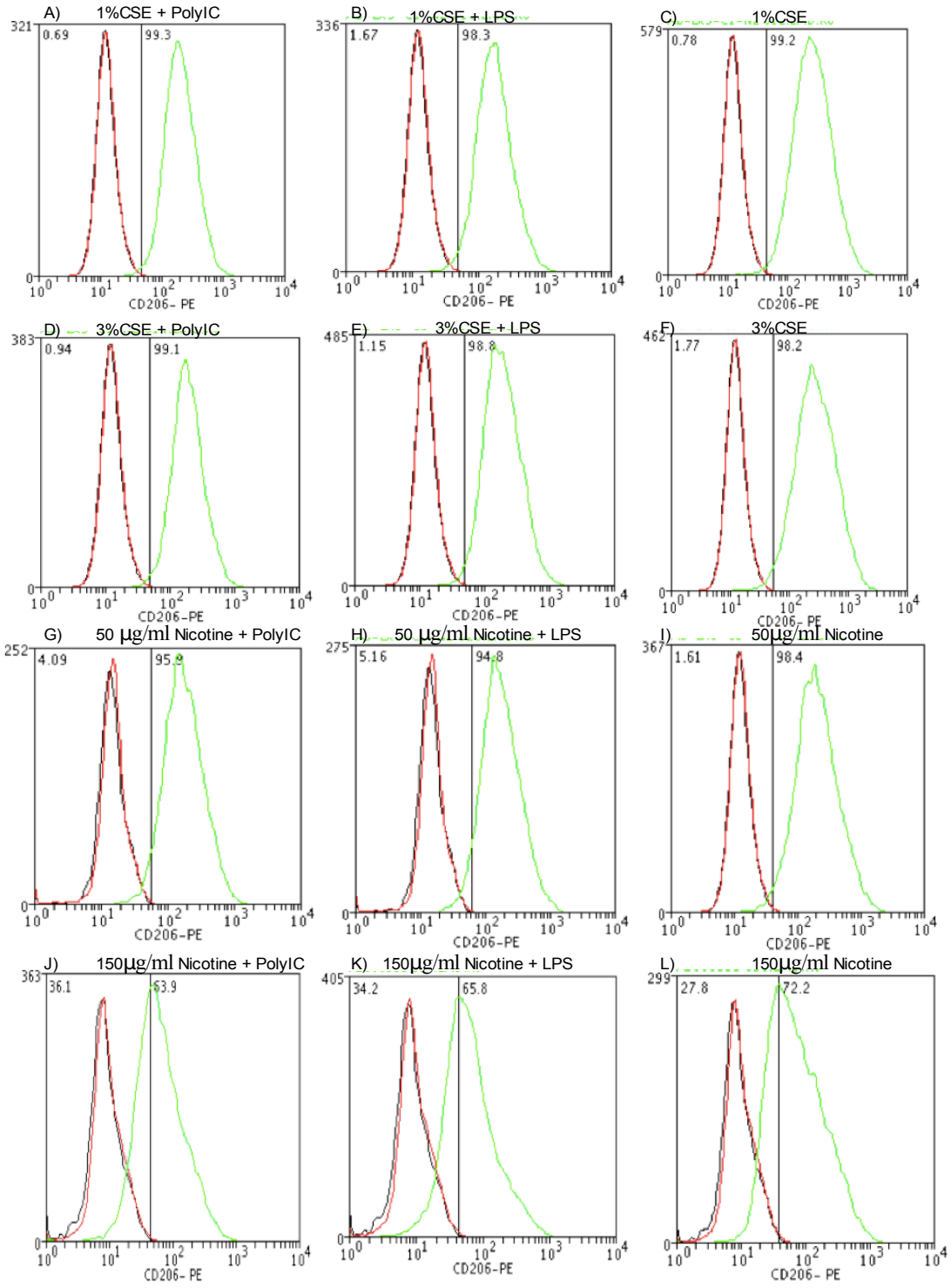


Figure 4.16 Expression of CD206 measured by flow cytometry for all treatments on day 8

Flow cytometric analysis of MΦ surface markers, black represents un-stained cells; red curves represent the isotype control and green curves represent test antibody, traces are representative of repeats (N = 4). A) 1 % CSE with Poly-IC, B) 1 % CSE with LPS and C) 1 % CSE. D) 3 % CSE with Poly-IC, E) 3 % CSE with LPS and F) 3 % CSE, G) 50 µg/ml Nicotine with Poly-IC, H) 50 µg/ml Nicotine with LPS, I) 50 µg/ml Nicotine, J) 150 µg/ml Nicotine with Poly-IC, K) 150 µg/ml Nicotine with LPS and L) 150 µg/ml Nicotine.

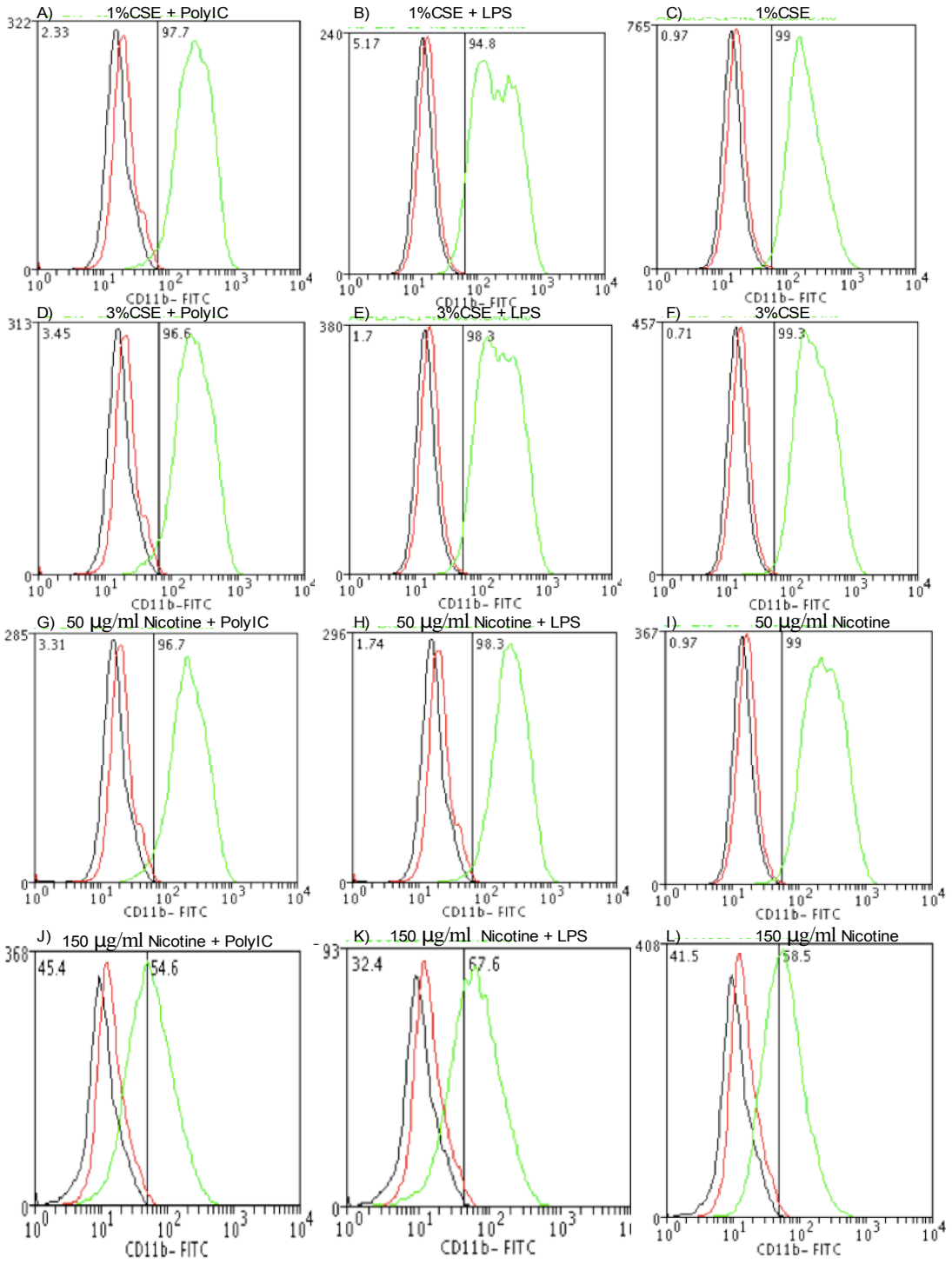


Figure 4.17: Expression of CD11b measured by flow cytometry for all treatments on day 8

Flow cytometric analysis of MΦ surface markers, black represents un-stained cells; red curves represent the isotype control and green curves represent test antibody, traces are representative of repeats (N = 4). A) 1 % CSE with Poly-IC, B) 1 % CSE with LPS and C) 1 % CSE. D) 3 % CSE with Poly-IC, E) 3 % CSE with LPS and F) 3 % CSE, G) 50 µg/ml Nicotine with Poly-IC, H) 50 µg/ml Nicotine with LPS and I) 50 µg/ml Nicotine. J) 150 µg/ml Nicotine with Poly-IC, K) 150 µg/ml Nicotine with LPS, L) 150 µg/ml Nicotine.

4.3.5. Median fluorescence intensity (MFI) and statistical analysis

By analysing the results of the MFI for the different cell surface markers and treatment combinations, the actual differences between the sample sets can be seen more clearly. To investigate if any of the changes in fluorescence observed were statistically significant, the results were analysed by two-way ANOVA with Dunnett's multiple comparisons test.

For the macrophage controls without CSE or nicotine, the results (Figure 4.18) show that there was variability between the samples measured for CD14 expression, resulting in large error bars, and there were no significant differences detected, possibly due to differences between the four donors used to isolate PBMCs. For CD16 the results again showed no significant differences. The results for CD206 and CD11b showed a reduction in expression with Poly-IC and LPS treatment compared to the untreated control, which was significant for CD206 ($P = 0.0156$ for Poly-IC, $P = 0.0498$ for LPS) but not for CD11b. Overall these results suggest that there are some differences between the treatment groups but the low reproducibility between the repeats meant the differences were mainly not statistically significant.

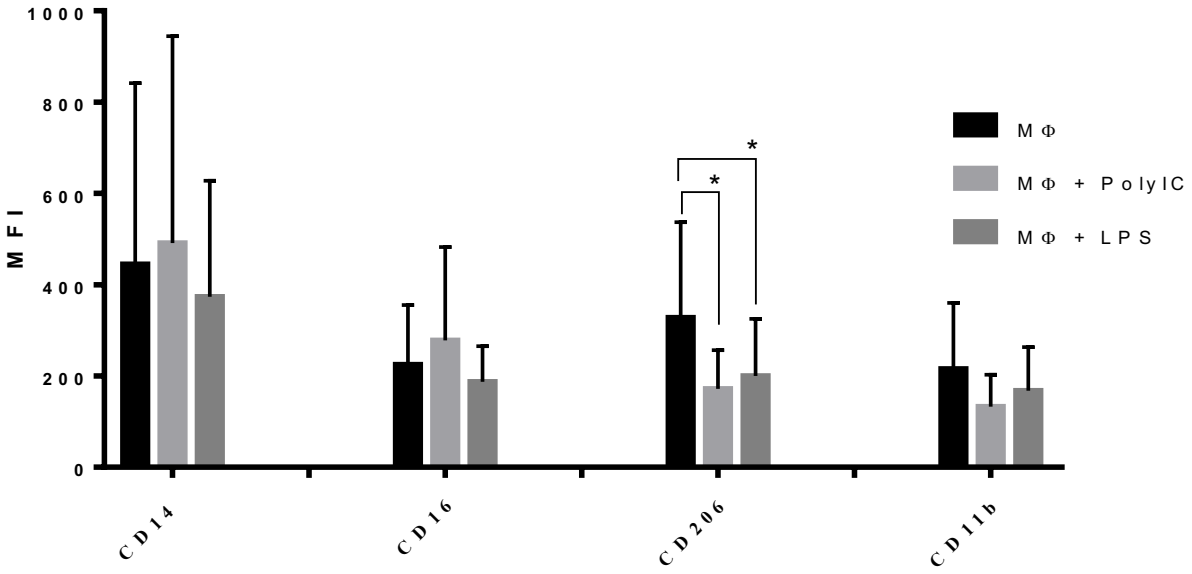


Figure 4.18: The MFI of macrophages for all the cell surface markers on day 8, with Poly-IC and LPS only

MΦ = macrophages, LPS = lipopolysaccharide. The mean values were calculated from the 4 different donors and plotted (N = 4), error bars represent SD. Data were analysed for statistical significance by a two-way ANOVA with Dunnett's multiple comparisons test, P values < 0.05 were considered as significant, * = $P < 0.05$.

By comparing the MFI for CD14 for the different treatments, the results (Figure 4.19) show that there was a large variability between the samples, resulting in large error bars, but some significant differences were observed. For the cells treated with Poly-IC, there was a significant reduction in CD14 with 50 μg/ml nicotine when compared to the control without CSE/nicotine (just macrophages) (P = 0.0170), and a significant reduction in expression for 150 μg/ml nicotine when compared to the control (just macrophages) (P = 0.0003). This was also seen for the cells without Poly-IC or LPS treatment; there was a significant reduction in CD14 expression after treatment with both concentrations of nicotine (P = 0.0374 for 50 μg/ml, P = 0.0157 for 150 μg/ml nicotine). Overall these results suggest that the nicotine treatments, with either Poly-IC or no co-treatment, produced a significant reduction in CD14 expression, but there was no significant effect of CSE.

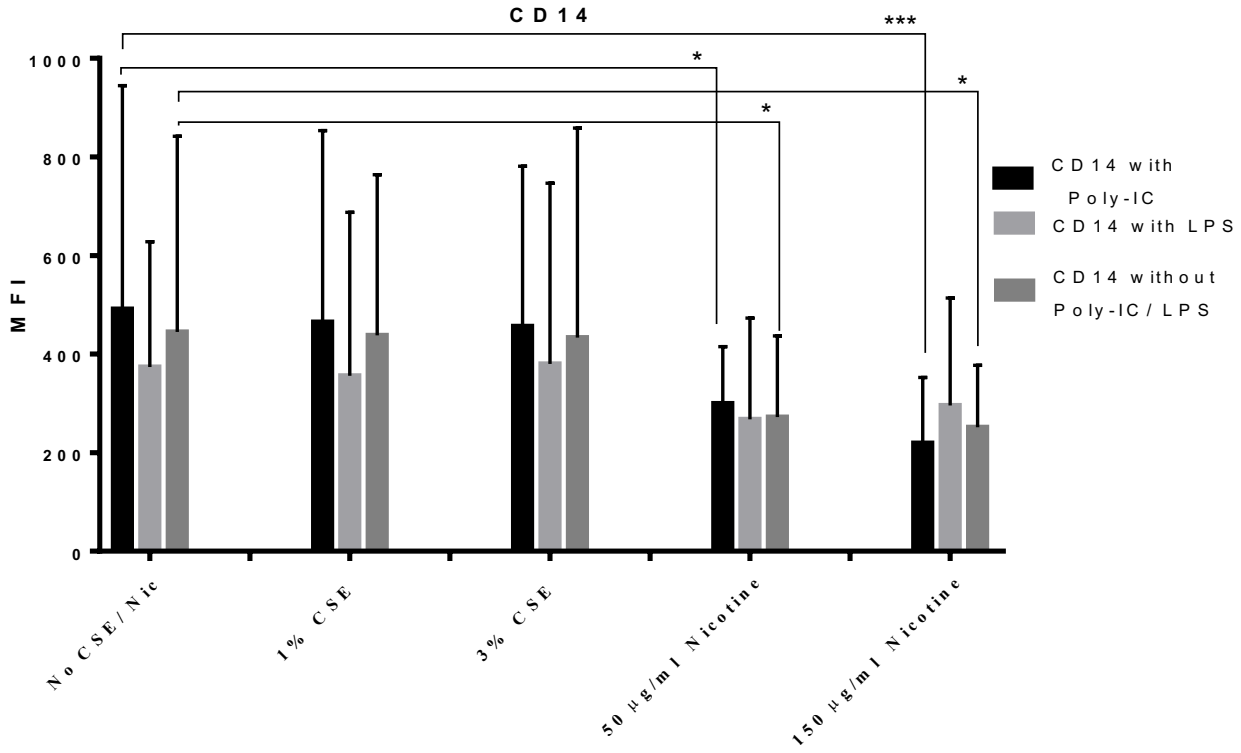


Figure 4.19: The mean MFI of the marker CD14 for all the treatments on day 8

Nic = nicotine, LPS = lipopolysaccharide. The mean values were calculated from the 4 different donors and plotted (N = 4), error bars represent SD. Data were analysed for statistical significance by a two-way ANOVA with Dunnett's multiple comparisons test, P values ≤ 0.05 were considered as significant, * = P ≤ 0.05, *** = P ≤ 0.001.

The CD16 results for all treatments (Figure 4.20) show that there was a large variability between some of the samples, which means any actual change may have been masked

by the large error bars. For the Poly-IC treated cells, there was a trend of a decreased expression with increasing concentrations of CSE when compared to the untreated cells (macrophages only), and this was seen to a greater extent for the nicotine treatments, but the difference was not significant. For the LPS and untreated cells, there were only minor differences between all the different treatments. Overall, the results for CD16 expression show no significant differences between the treatments.

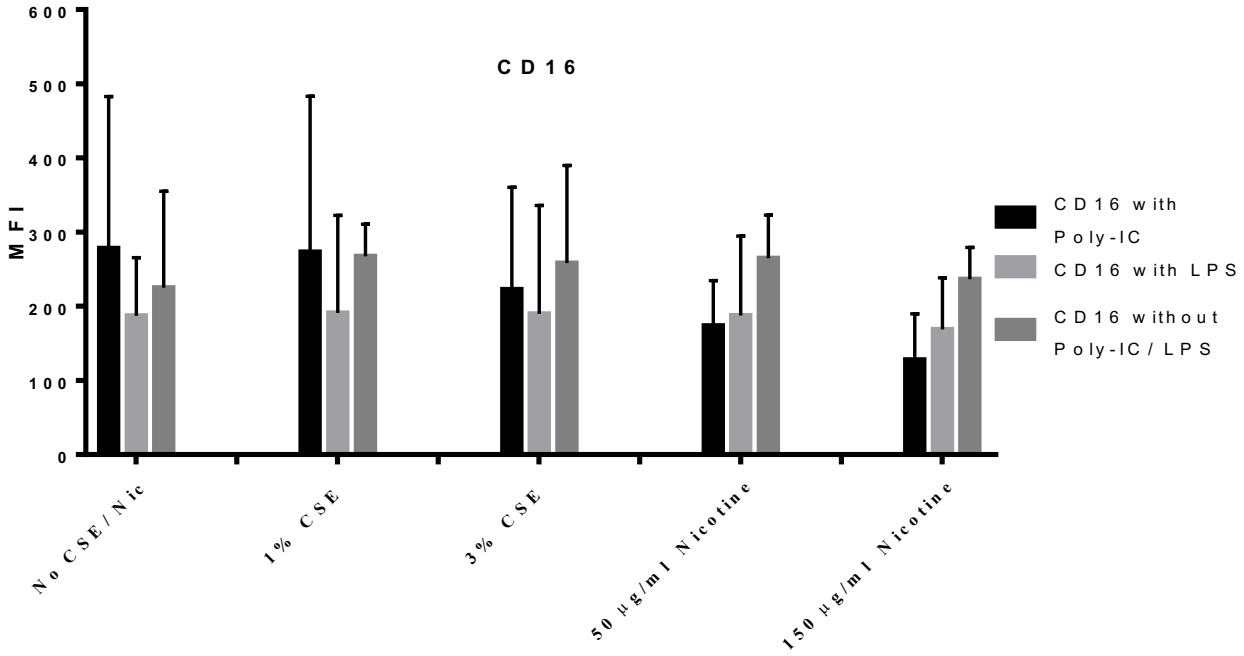


Figure 4.20: The mean MFI of the marker CD16 for all the treatments on day 8

Nic = nicotine, LPS = lipopolysaccharide. The mean values were calculated from the 4 different donors and plotted (N = 4), error bars represent SD. Data were analysed for statistical significance by a two-way ANOVA with Dunnett's multiple comparisons test, P values ≤ 0.05 were considered as significant.

The CD206 results for all treatments (Figure 4.21) show that there was a large variability between some the samples, which means the pattern of decreasing fluorescence observed for the Poly-IC and LPS cells after treatment with increasing doses of CSE and nicotine were not significant differences. For the cells without Poly-IC or LPS, there was also a trend for decreasing fluorescence with increasing concentrations of CSE and nicotine, and the difference for 150 µg/ml nicotine was a significant decrease ($P \leq 0.05$). Overall these results suggest that even though some differences could be seen for CD206 expression for the different treatments, only the 150 µg/ml nicotine in the cells without Poly-IC or LPS produced a significant result.

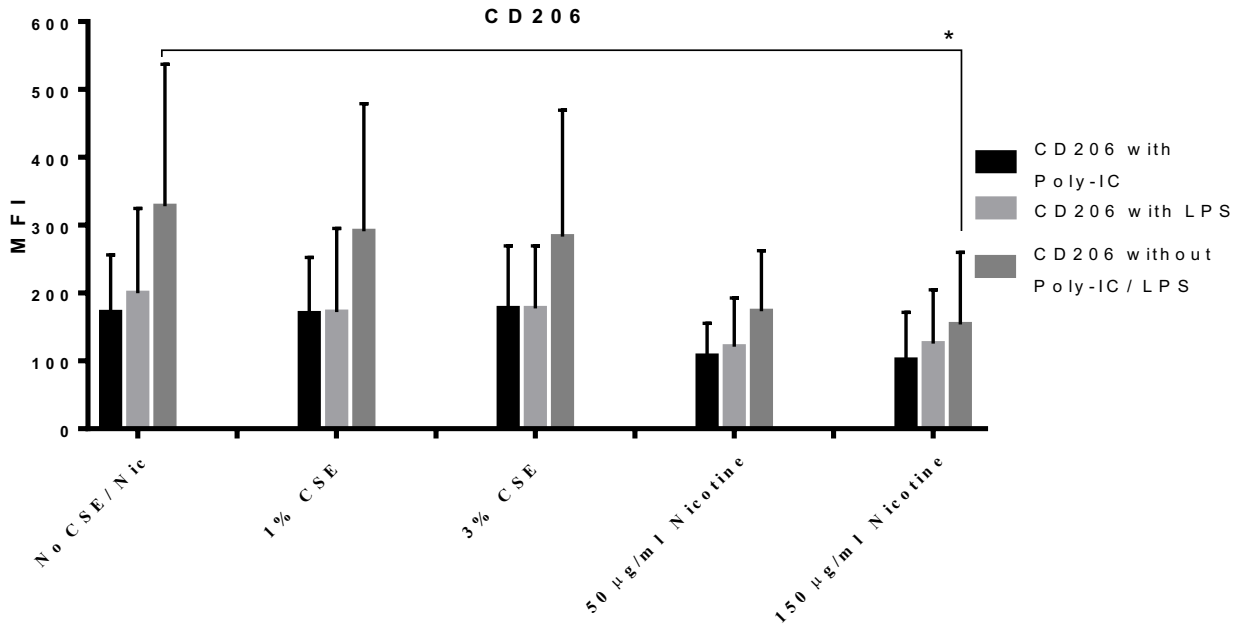


Figure 4.21: The mean MFI of the marker CD206 for all the treatments on day 8

Nic = nicotine, LPS = lipopolysaccharide. The mean values were calculated from the 4 different donors and plotted (N = 4), error bars represent SD. Data were analysed for statistical significance by a two-way ANOVA with Dunnett's multiple comparisons test, P values ≤ 0.05 were considered as significant, * = $P < 0.05$.

The CD11b results for all treatments (Figure 4.22) show that there was a large variability in expression measured, meaning the small changes in fluorescence due to the treatments were hidden by the large error bars. As with the other cell surface markers, the largest decrease in CD11b expression for Poly-IC, LPS and untreated cells was seen with the 150 µg/ml nicotine co-treatment, but the differences were not significant. Overall these results suggest there is general decrease in CD11b expression after co-treatment with 3 % CSE and both nicotine treatments. However, the changes are only of the order of 20-30 % and the variability of the independent experimental replicates is large (standard deviation 50-60 %): on this basis a power calculation indicates that about 10 experimental replicates would be needed to show a statistically significant difference, whereas only four replicates were performed.

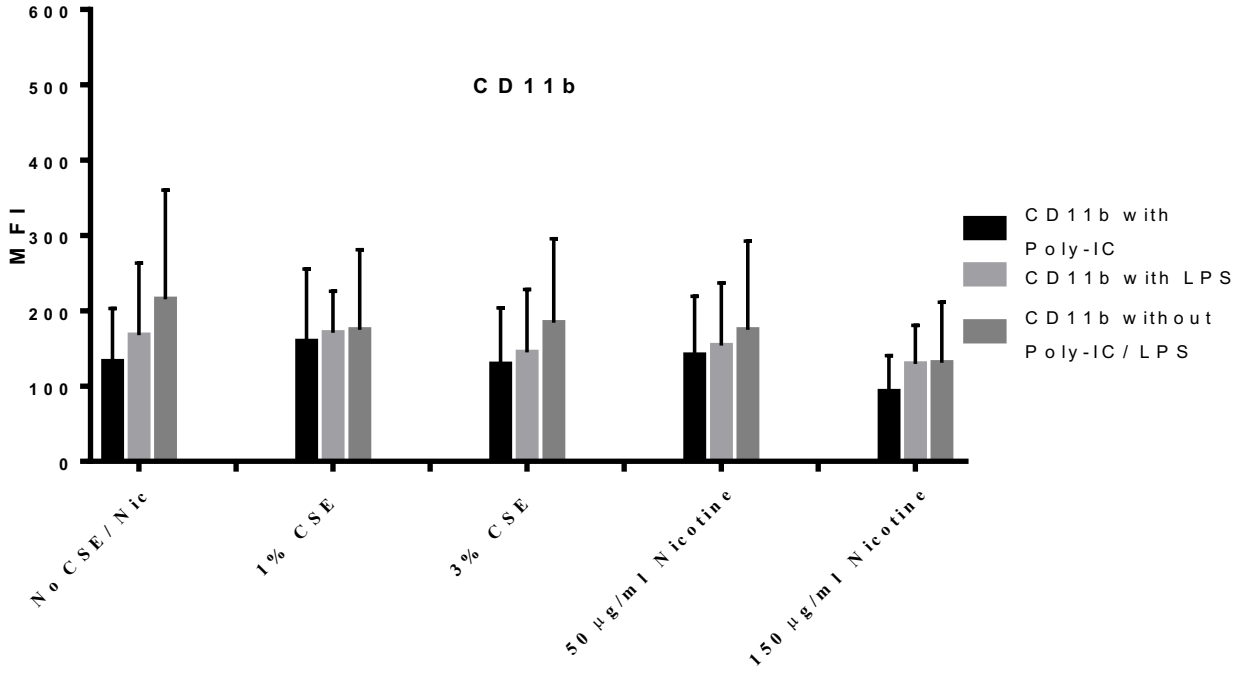


Figure 4.22: The mean MFI of the marker CD11b for all the treatments on day 8

Nic = nicotine, LPS = lipopolysaccharide. The mean values were calculated from the 4 different donors and plotted (N = 4), error bars represent SD. Data were analysed for statistical significance by a two-way ANOVA with Dunnett's multiple comparisons test, P values ≤ 0.05 were considered as significant.

By combining all the results for the different markers (Figure 4.23), the extent of the changes for the different conditions can be seen. There are large error bars for the majority of the treatment conditions which may have masked some of the actual differences, but it is clear from the results that the treatments used affect the expression of the cell surface markers investigated.

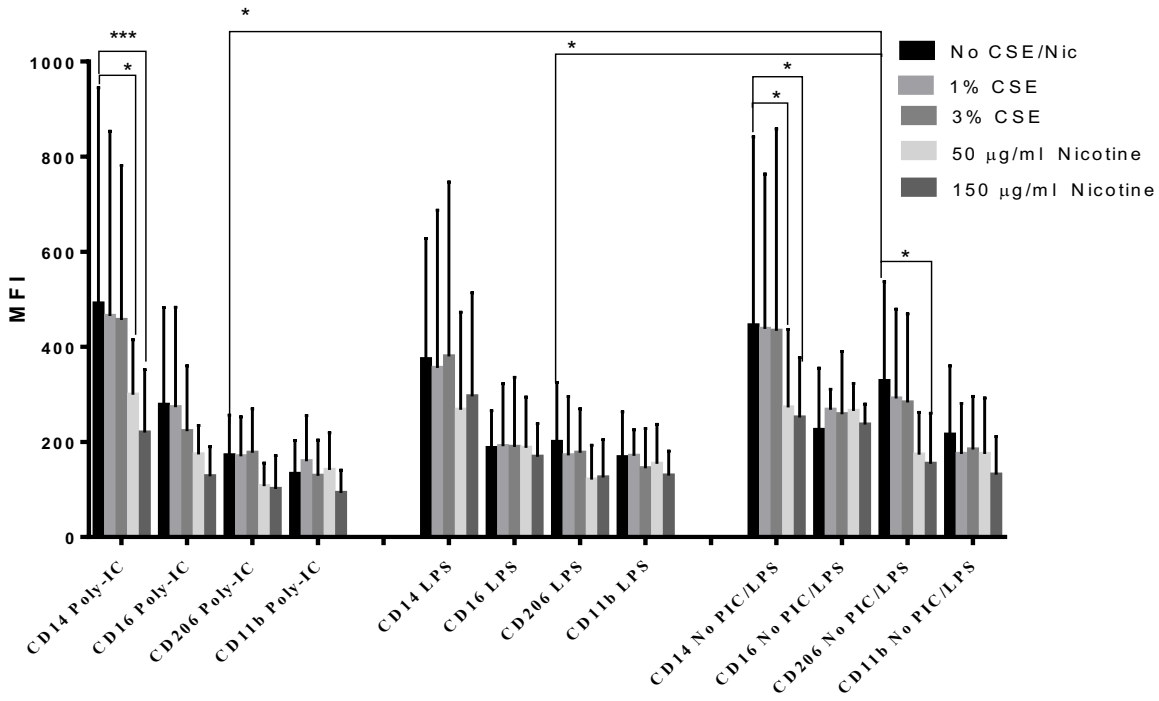


Figure 4.23: The mean MFI of CD14, CD16, CD206 and CD11b for all the treatments on day 8

CSE = cigarette smoke extract, Nic = nicotine, PIC = Poly-IC, LPS = lipopolysaccharide. The mean values were calculated from the 4 different donors and plotted (N=4), error bars represent SD. Data were analysed for statistical significance by a two-way ANOVA with Dunnett's multiple comparisons test, P values ≤ 0.05 were considered as significant, * = P ≤ 0.05, and *** = P ≤ 0.001.

Overall by comparing the data for the flow cytometry traces, the MFI and the standardised data, the results show that there were some changes in expression produced by the different treatments. By analysing the results of the MFI, the actual differences between the data sets could be observed. The results showed there was significant suppression of CD14 expression by Poly-IC-treated macrophages with 50 and 150 µg/ml nicotine (P = 0.0170 and P = 0.0003, respectively), and for nicotine without Poly-IC or LPS treatment (P = 0.0374 for 50 µg/ml, P = 0.0157 for 150 µg/ml nicotine) when compared to the control (only macrophages). For CD206, there was a significant decrease in expression after treatment with only 150 µg/ml nicotine (P ≤ 0.05), with Poly-IC alone (P = 0.0156) or with LPS alone (P = 0.0498) when compared to the relevant controls. These results show that the treatments affect the cell surface markers, depending on the combination of treatments used. Unlike nicotine alone, CSE was not observed to suppress expression of CD14 or CD206, although CSE will contain nicotine. The likely reason for this is that the concentration of nicotine in 1 % and 3 % CSE will be substantially lower than concentrations of pure nicotine employed in the

cultures (i.e. 50 µg/ml and 150 µg/ml). Based on an average cigarette containing about 1.2 mg of nicotine, 1 % and 3 % CSE will contain 1.2 µg/ml and 3.6 µg/ml of nicotine, at most.

4.4. Discussion

Constituents of cigarette smoke are the main cause of COPD, and the progression of COPD is related to numerous changes in the immune system (Sharma *et al.,* 2009). Our ultimate goal is to determine the role of macrophages in this disease and its progression. Due to the function of macrophages as APCs, they can play a crucial role, not only in innate immunity, but also in the adaptive response of the immune system (Pappas *et al*., 2013). By understanding the relationship of macrophages with other immune cells, this research could help to provide alternative and more effective therapeutic approaches to limit the progression of COPD.

The direct aim of this study was to understand the effect of cigarette smoke and nicotine on macrophages *in vitro*. To achieve this goal, PBMCs were used and CD14 positive cells were isolated from the buffy-coat by using histopaque 1077. The CD14 positive cells were cultured in RPMI 1640 complete medium with M-CSF on tissue culture plates coated with 1 % HMDS in 100 % propanol. HMDS is a silicon support (Osthoff *et al.*, 1957); this is the first study that has used this type of support to try and increase the number of mature macrophages recovered by reducing their adherence to the plate.

Our experiments investigated two approaches; the first was exposing cells to different concentrations of CSE (1 % and 3 %) in the presence and absence of Poly-IC and LPS. The second was similar and involved exposing the macrophages to nicotine (50 and 150 µg/ml) in the presence and absence of Poly-IC and LPS. Nicotine is the major addictive constituent of tobacco, which is why it was also examined on its own.

In our study, the isolated $CD14^+$ cells were 99 % pure. Subsequently, these monocytes were cultured for 7 days in the presence of M-CSF (Gleissner, 2012) and on day 7 macrophages were treated with the relevant combination of LPS, Poly-IC, CSE and nicotine, and then they were incubated for a further 24 h. After 24 h, the plates were incubated on ice for 30 minutes, and cells were harvested before staining for expression of CD14, CD16, CD206 and CD11b, and analysis by flow cytometry. This experiment

was repeated four times. These four CD markers were selected as ones widely used in studies of macrophage differentiation; it should be noted that a variety of other markers could have been employed, and should be considered for future studies. This includes markers closely associated with the antigen presentation function of macrophages (e.g. MHC class II, CD80 and CD86).

This study also investigated the effect of using the different treatments of HDMS on the number of collected cells and maintenance of the phenotype of differentiated macrophages. Macrophages were grown on plates containing the different concentrations of HDMS, harvested on day 7 and cells were stained with CD14, CD16, CD206, CD11b and CD36 antibodies, and this was repeated twice.

For the macrophages on day 7, for all concentrations (5 % HDMS in 70 % ethanol; 5 %, 3 %, and 1 % HDMS in 100 % ethanol; 5 %, 3 %, and 1 % HDMS in 100 % propanol; and un-coated), the results showed high expression of CD14, CD11b and CD36 (up to 100 % of total cells) and lower expression of CD16 (~30 % of total cells), confirming the presence of mature macrophages. Overall the highest yield of cells was found after using 1 % HMDS in 100 % Propanol (~25,000 cells), which shows HMDS can be successfully used to increase the number of recovered macrophages.

For the investigation of the effects of CSE and nicotine on macrophages in the presence and absence of Poly-IC and LPS, the results for the flow cytometry traces showed that the only statistically significant effect of Poly-IC and LPS was to cause a reduction in CD206 expression, which is consistent with these stimulators pushing the phenotype of the day 8 M0 macrophages towards a M1 phenotype, which would have reduced CD206 expression.

The results of the flow cytometry showed suppression of CD14 expression by nicotine in the absence of poly-IC or LPS, and in combination with Poly-IC, and suppression of CD206 expression by 150 μg/ml nicotine in the absence of poly-IC or LPS. It is unlikely that the observed reduction in CD14 and CD206 expression is simply due to reduction in macrophage viability by CSE or nicotine by non-specific toxicity, otherwise a reduction in expression of all the markers examined might have been expected. Ideally, the possible effects of CSE and nicotine on cell viability should be assessed more specifically by examining treated cells for markers of apoptosis, e.g. increased staining with propidium iodide, increased cell surface expression of annexin V.

The results of this study fit with other studies in the literature that show CSE and nicotine can affect macrophages. For example, studies have shown that pure nicotine and nicotine in CSE can inhibit macrophage action against *Mycobacterium tuberculosis* (MTB) by increasing the intracellular MTB burden and inhibiting autophagosome formation in primary murine alveolar macrophages and THP-1 cells (Bai *et al*., 2017). Nicotine has also been shown to induce Treg TFG-β and IL-10 production and increase migration of THP-1 cells, which reflects the increased numbers of macrophages found in the lungs of smokers (Bai *et al*., 2017). Nicotine can reduce the T-cell population and macrophage response through restriction of receptor and antigen mediated signal transduction in the lymphoid system, which reduces the overall immunological response and can result in an increased incidence of tuberculosis (Mishra *et al*., 2015).

Further evidence of the effects of nicotine in macrophages comes from studies into the effect of nicotine on the cytokine response and antimicrobial activity to *Legionella pneumophila* in the murine MH-S alveolar macrophage cell line. These studies have suggested that nicotinic acetylcholine receptors (nAChRs) play an important role in the alteration of alveolar macrophage activity as they are involved in macrophage immune function regulation by nicotine, and this could contribute to cigarette-induced risk factors in smokers (Matsunaga *et al*., 2001). This study found that nicotine down-regulated TNF-α, IL-12 and IL-6 production but not IL-10 that had been induced by *L. pneumophila* infection, which led to increased bacterial replication, but when nAChRs were blocked with a non-selective antagonist these effects were no longer seen (Matsunaga *et al*., 2001). Other studies in a wide variety of cells (including cancer cells, embryonic and adult stem cells) have shown that nicotine binding to nAChRs can de-regulate cell-mediated immunity, apoptosis, cell proliferation, inflammation, invasion, migration, and angiogenesis (Cardinale *et al*., 2012; Schaal *et al*., 2014). For example, studies using *in vitro* and *in vivo* methods to evaluate the effect of nicotine on lung cancer cells have suggested that nicotine may influence cancer development and treatment effectiveness, as it can suppress apoptosis, promote angiogenesis, migration, proliferation, and invasion of cancer cells (Czyżykowski *et al*., 2016).

Studies into the effects of Poly-IC and CSE in a mouse model to investigate viral exacerbations of lung disorders showed that Poly-IC induced lung inflammation, enhanced CSE related inflammation, with increased caspase-3 expression in areas of

alveolar inflammation, but without any effect on lung tissue caspase-3/-7 activity with or without CSE (Murray *et al*. 2017). These results are supported by the work of Kang *et al*. (2008) who found that Poly-IC in combination with CSE induced IL-18, type I IFN and IFN-γ, which agrees with our results that showed Poly-IC increased the number of macrophages with a pro-inflammatory M1 phenotype (Kang *et al*., 2008).

The results from this study have shown that nicotine produced a greater suppression of some of the markers investigated than CSE, this may be due to the dose of nicotine within the CSE, or the differences between the donors, but could also be due to the other components in CSE itself exerting an effect. A study on the effect of complete CSE, CSE without nicotine (CSE-O) and the nicotine component of CSE (CSE-N) on CYP2A13 (a key enzyme in cigarette toxicity and metabolism) in immortalized human bronchial epithelial cells found that CSE-O and CSE were toxic to the cells, whereas the CSE-N produced less cytotoxicity (Ji *et al*., 2017). The study also found that CSE-O increased apoptosis by changing the expression of three pairs of pro- and anti-apoptotic proteins: cleaved-poly adenosine diphosphate-ribose polymerase/poly adenosine diphosphate-ribose polymerase, cleaved-caspase-3/caspase-3, and Bcl-2 associated X protein/B cell lymphoma-2, and CSE-N decreased the metabolic activation of CYP2A13 to CSE (Ji *et al*., 2017). Overall, these results show that the other components of CSE can exert a different effect to nicotine, which could mask the activity of nicotine within the CSE. As nicotine works as an agonist of nAChRs, it would be possible to investigate the effects of nicotine within the CSE by blocking this receptor using a competitive inhibitor to nAChR such as mecamylamine, or by using macrophages from mice with genetic disruption of nAChR specific subunits (Egleton *et al*., 2008; Wen *et al*., 2016; Bai *et al*., 2017).

Overall the results of the current suggest that, as expected based on previous studies, CSE and nicotine can cause changes in the cell surface markers of the macrophages, suggesting further investigation into the changes produced in the macrophages should be carried out to determine the extent of the effects on these cells. One major factor to be considered in future experiments is how to reduce the experimental variation between the repeats to make the results of the experiments more significant. This could be achieved by including more repeats to reduce the effect of outliers, or by altering the experimental design to reduce sources of error and variation between the repeats.

4.4.1. Conclusions

Despite generally having a protective role in infectious diseases, macrophages have been shown to have a deleterious effect in COPD and other smoking related diseases (Tetley, 2002). Part of the problem could be because more alveolar macrophages with an M2 expression profile exist in the disease state, which results in increased levels of tissue damage and fibrosis (Vlahos and Bozinovski, 2014). This study aimed to establish an *in vitro* model to study the effect of CSE on macrophage function in COPD. Here, we established the procedure to generate macrophages from $CD14^+$ monocytes isolated by positive selection and stimulated them to be mature using M-CSF. Our results showed that the monocytes isolated from buffy coats expressed the appropriate cell surface markers of differentiated macrophages and treatment with HMDS for coating tissue culture plates achieved satisfactory results. The results of this study suggest that the treatments investigated can have an effect on the expression of cell surface markers, there was significant suppression of CD14 expression by Poly-IC with 50 and 150 μg/ml nicotine ($P = 0.0170$ and $P = 0.0003$, respectively), and for nicotine without Poly-IC or LPS treatment ($P = 0.0374$ for 50 μg/ml, $P = 0.0157$ for 150 μg/ml nicotine) when compared to the control. For CD206, there was a significant decrease in expression after treatment with only 150 μg/ml nicotine ($P \leq 0.05$), Poly-IC ($P = 0.0156$) or LPS ($P = 0.0498$) when compared to the control.

Overall this study suggests that nicotine and CSE had detrimental effects on the metabolism and function of these innate immune cells, which agrees with the effects of nicotine and CSE seen in other studies in alveolar macrophages and other cell lines. Therefore, further studies were undertaken using RPPA to investigate the effect of CSE and nicotine on the pathways involved in macrophage signalling, including the LPS-induced response, the IFNγ pathway, the cytokine pathway and TNFα, as this will further elucidate the effects of CSE on macrophages and provide insight into how these cells can affect the progression of COPD.

5. Analysis of differences in cell surface markers by RPPA

5.1. Introduction

The results of the previous chapters indicate that CSE and nicotine used in combination with or without Poly-IC and LPS can alter the expression of key macrophage cell surface makers; therefore, they should be suitable for use to investigate the effect of CSE and nicotine on the regulation of macrophage related signalling proteins. To clarify the effect of CSE/nicotine and how they relate to COPD progression, several cell-signalling and macrophage-related proteins (pSTAT6, pSTAT3, IRF4, KLF-4, BMP-7, FABP-4, pHSP27, NFκB-P50, PPAR-γ, HIF2α, pP38 MAPK) were selected for analysis by RPPA. The selected proteins have a variety of functions that can help to determine how CSE, nicotine, LPS and Poly-IC affect macrophages and therefore could help to explain the changes seen in COPD.

Signal transducer and activator of transcription 6 (STAT6) regulates cytokine-induced gene expression; STAT6, PPARγ, KLF-4, and IRF4 may also coordinate the M2 polarisation of macrophages (Kapoor *et al.*, 2015).

STAT3 is constitutively activated in many human tumours; it possesses oncogenic potential and anti-apoptotic activities, and there is evidence to suggest cytokine-activated STAT3 and STAT6 cooperate in macrophages to promote a secretory phenotype that enhances tumour progression in a cathepsin dependent manner (Bromberg *et al.*, 1999).

IRF4 is a member of Interferon Regulatory Factors (IRF) family; it is involved in M2 polarisation and functions as a negative regulator of TLR signalling by associating with the MyD88 adaptor molecule (Günthner and Anders, 2013).

Krüppel-like factor 4 (KLF4) is a critical regulator of macrophage polarisation, it was found to cooperate with STAT6 to induce an M2 genetic program and inhibit M1 targets via sequestration of co-activators required for NF-κB activation (Liao *et al.*, 2011).

Bone morphogenetic protein 7 (BMP-7) is induced in M2 polarisation *in vitro* via activation of the SMAD-PI3K-Akt-mTOR pathway. BMP-7 has the potential to mediate cellular plasticity and mitigate the inflammatory immune response, which resulted in decreased plaque formation and improved blood velocity (Singla, Singla and Wang, 2016).

Fatty acid binding protein 4 (FABP4) is involved in the regulation of glucose and lipid metabolism in relation to inflammatory and metabolic processes in target cells, especially adipocytes and macrophages (Tuncman *et al.*, 2006).

Extracellular heat shock protein 27 (HSP27) acts as a signalling molecule to activate nuclear factor-κB (NF-κB) in macrophages. Activation of NF-κB signalling by rHSP27 is associated with up-regulated expression and secretion of key pro- and anti-inflammatory cytokines. Moreover, it is the balance in expression of these mediators and antagonists of inflammation, and hence atherogenesis, that yields a favourable net effect of HSP27 on the vessel wall (Salari *et al.*, 2013).

NF-κB1 (p50) homodimers differentially regulate pro- and anti-inflammatory cytokines, such as interleukin-10 (IL-10) in macrophages (Cao *et al.*, 2006).

Peroxisome proliferator-activated receptor γ (PPARγ) alters macrophage trafficking, increases efferocytosis and phagocytosis, and promotes M2 macrophage activation. In COPD, PPARγ has been shown to regulate genes involved in oxidative metabolism and activation of the M2 phenotype (Croasdell *et al.*, 2015).

The hypoxia inducible factors (HIF) HIF-1α and HIF-2α play a role in regulating M1/M2 polarisation with HIF-1α promoting NOS2 expression and the M1 state and HIF-2α arginase 1 expression and the M2 state (Dehn *et al.*, 2016).

P38 MAP kinases (p38 MAPKs) are key MAPKs involved in the production of inflammatory mediators, including TNF-α and cyclooxygenase-2 (COX-2). p38 MAPK signalling plays an essential role in regulating cellular processes, especially inflammation (Yang *et al.*, 2014).

Previously, the most common methods for detecting changes in protein expression were ELISA or Western blot, which have disadvantages (as well as advantages) as they are labour-intensive techniques that can detect only a limited number of proteins simultaneously and need a relatively large sample size. The newer technique of RPPA has certain advantages over the previously used techniques; it is a more powerful method that is highly sensitive and quantitative, allowing many samples to be analysed simultaneously, different sample types can be analysed, such as cell lines, tissues, plasma, or serum, and it requires a much smaller sample volume as only nanolitre quantities are required (Tibes *et al.*, 2006; Mueller, Liotta and Espina, 2010). These advantages make RPPA a suitable tool for detecting changes in protein expression in the different pathways of interest.

The method for RPPA starts with spotting the samples on the microarray slide, followed by incubation with the specific antibodies for the proteins of interest, with each antibody incubated on a separate array to enable the detection of a single protein in a mixture of other proteins. This is followed by incubation with a secondary, tagged antibody, for detection of the protein by the microarray scanners (Sheehan *et al*., 2005).

The optimisation of the microarray is important as several factors may affect the performance of this method, such as the sample preparation, the type of solid support, or slides used, and the printing and blocking buffers selected, as all these factors can affect the stability and binding of the proteins and the intensity of the background signal (Aguilar-Mahecha *et al.*, 2009; Akbani *et al.*, 2014).

Two cytokines were investigated using ELISA to detect how the different treatments affected their expression, these were TNF-α and IL-10. Tumour necrosis factor-α (TNFα) has been shown to play a pivotal role in orchestrating the cytokine cascade in many inflammatory diseases and because of this role as a "master-regulator" of inflammatory cytokine production, it has been identified as a therapeutic target in a

number of diseases. Macrophages are major producers of TNFα and are also highly responsive to TNFα (Salari *et al.*, 2013), particularly M1 macrophages.

Interleukin-10 (IL-10) is a major immunosuppressive cytokine that is produced by M2 macrophages. It is a critical component in the maintenance of the fine balance between swift and potent immune responses against invading pathogens and the control of detrimental pathological injury. Many cells of the innate and adaptive arms of the immune system can produce IL-10, including DCs, macrophages, mast cells, NK cells, eosinophils, neutrophils, B cells, $CD8^+$ T cells, $CD4^+$ Th2 cells, and regulatory T cells. A major role of IL-10 is to limit the extent of the activation of both the innate and the adaptive immune cells to maintain a homeostatic state. This role of IL-10 is vitally important in protecting the host from infection-associated immunopathology, autoimmunity, and allergy, such as sepsis, arthritis, insulitis, inflammatory bowel disease (IBD), and so on. In addition to these activities, IL-10 regulates growth and differentiation of B cells, NK cells, cytotoxic and helper T cells, mast cells, granulocytes, dendritic cells, keratinocytes, and endothelial cells (Cao *et al.*, 2006).

The aim of the work described in this chapter was to use the previously optimised RPPA to detect differences in expression of the selected proteins in macrophages isolated from PBMCs after they had been treated with the different combinations of CSE or nicotine with LPS or Poly-IC. Based on the results of the RPPA, an ELISA of the supernatants used for the RPPA was carried out to measure TNF-α and IL-10 levels to differentiate between the activity of M1 and M2 macrophages, respectively.

5.2. Results

5.2.1. Analysis of RPPA after 2 hours of incubation

The preparation of cell lysates for RPPA from the monocyte-derived macrophages of eight different donors (section 2.2.12) occurred on days 7, 8 and 9 of cell culture; purified monocytes were initially grown for one week with M-CSF in a 24-well tissue culture plate coated with 1 % HMDS in 100 % propanol then, on day 7 of cell growth, the macrophages were harvested and re-suspended in complete RPMI-1640 medium

for cell counting. Cells were then plated and treated with different concentrations of CSE and nicotine in the presence or absence of Poly-IC or LPS.

After 2 hours of incubation with the different combinations of LPS, Poly-IC, CSE and nicotine, samples were taken, and lysates were prepared for analysis of changes in protein expression by RPPA (sections 2.2.14 and 2.2.15).

Results were initially analysed for statistical significance using a paired t-test with Bonferroni correction to compare the treated and untreated samples, with the data grouped into the different markers analysed (or blank, i.e. blocking buffer only) to allow changes due to the different treatments to be seen.

The initial analyses of the RPPA data for the individual signalling molecules did not show any clearly significant changes associated with CSE or nicotine treatment. Also, another issue was that some of the expression values for the signalling molecules were not very different from the blank controls (blocking buffer only), which suggests that there was either low expression of these signalling molecules, or there were technical issues with the RPPA giving high background staining. Work by other people in the lab examining signalling molecules by RPPA has indicated that effects of modulating agents on individual signalling molecules are likely to be quite subtle and not show statistically significant differences unless very large numbers of independent replicates were performed, which was not practically possible to achieve. Therefore, it was decided it would be beneficial to examine the global effect of the modulating agents on all signalling molecules collectively. To achieve this, further analysis of the RPPA data was performed following subtraction of all the relevant blank values from the corresponding values for the signalling molecules expressed so that only truly positive values were included in the analysis. The median values were then calculated for each signalling molecule for the 8 individual donors, then the changes across all signalling molecules were calculated, with CSE or nicotine treatments compared to no CSE or nicotine, and LPS and Poly-IC treatments compared to no LPS or Poly-IC. Data were analysed for statistical significance by Wilcoxon matched pairs test and values of $P < 0.05$ were considered significant.

Treatment of macrophages without or with only Poly-IC or LPS for 2 hours (Figure 5.1) showed that these treatments generally caused a significant suppression of the

median values of the signalling molecules investigated. Poly-IC produced a borderline significant reduction (P = 0.054), and LPS produced a significant reduction (P = 0.024). One marker (P50) was upregulated after treatment with Poly-IC, and KLF-4 and PPAR-γ were up-regulated after treatment with LPS.

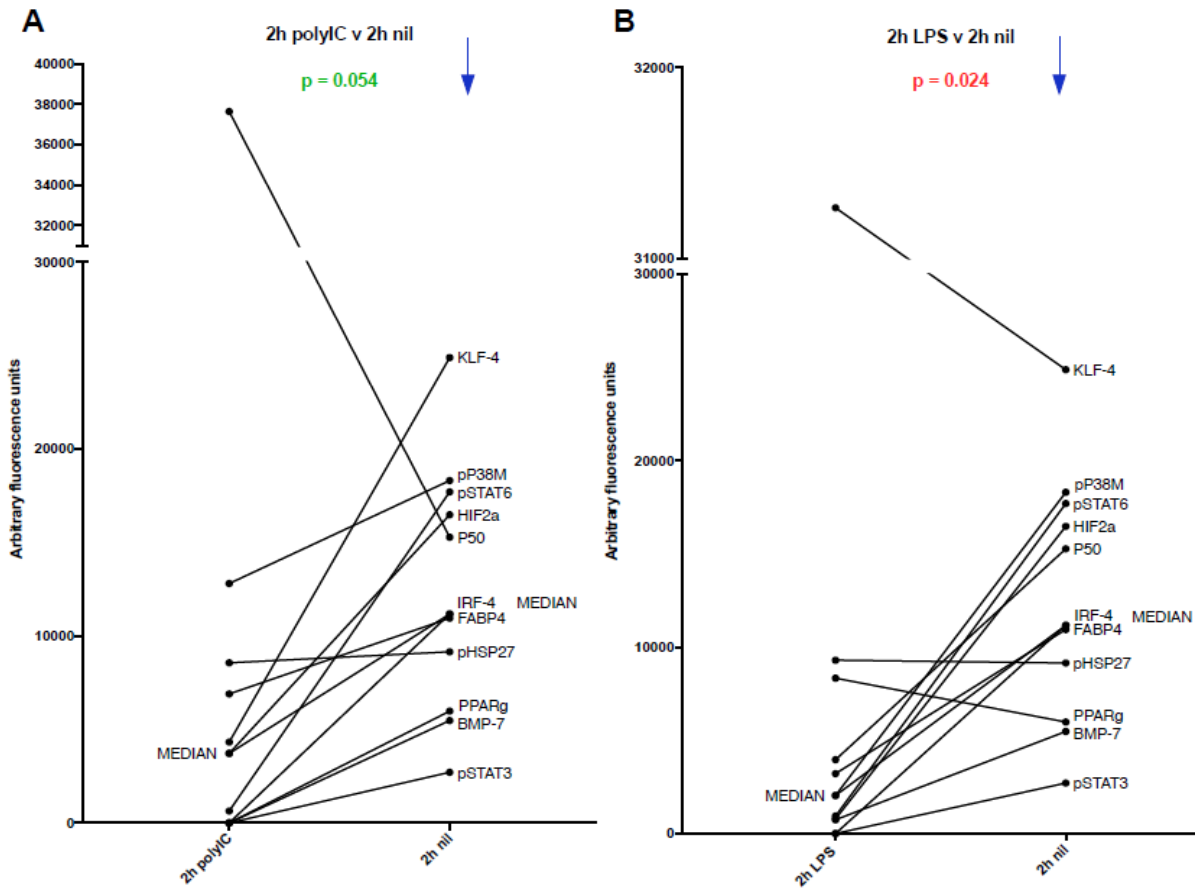
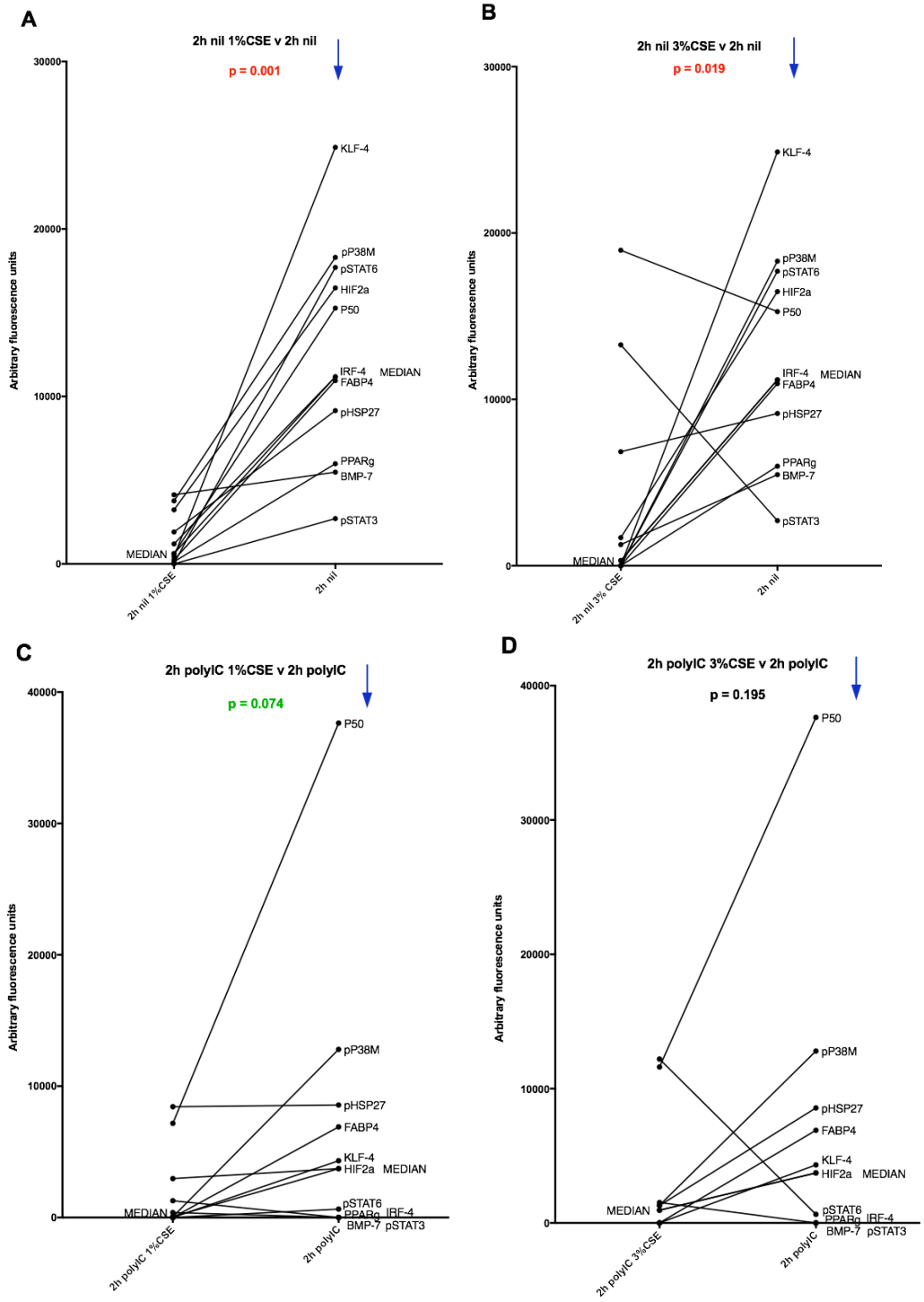


Figure 5.1: RPPA of median values of signalling molecule expression after 2 h incubation without or with Poly-IC or LPS only.

A) graph showing the change in the median value expression for all signalling molecules investigated after treatment with Poly-IC compared to untreated macrophages (nil). B) graph showing the change in the median value expression for all signalling molecules investigated after treatment with lipopolysaccharide (LPS) compared to untreated macrophages (nil). MEDIAN = the point for the median value for all the signalling molecules combined. Blue arrow at the top indicates whether the median of the values goes down or goes up with the treatment. Data were analysed for statistical significance using by Wilcoxon matched pairs test and values of $P < 0.05$ were considered significant: p values in red show significant differences, p values in green show 'borderline significant' (between 0.05 and 0.1), and p values in black are not near significance (> 0.1).

The results for the macrophages treated with CSE in the absence or presence of Poly-IC and LPS for 2 hours (Figure 5.2) showed that the median expression was

significantly suppressed for the majority of the markers investigated (1 % CSE P = 0.001, 3 % CSE P = 0.019, 1 % CSE with LPS P = 0.027, 3 % CSE with LPS P = 0.004, and 1 % CSE with Poly-IC was borderline significant). Only 2 markers showed up-regulation for 3 % CSE only (P50 and pSTAT3), and 1 marker (pSTAT6) showed up-regulation for 3 % CSE with Poly-IC and 1 % CSE with LPS.



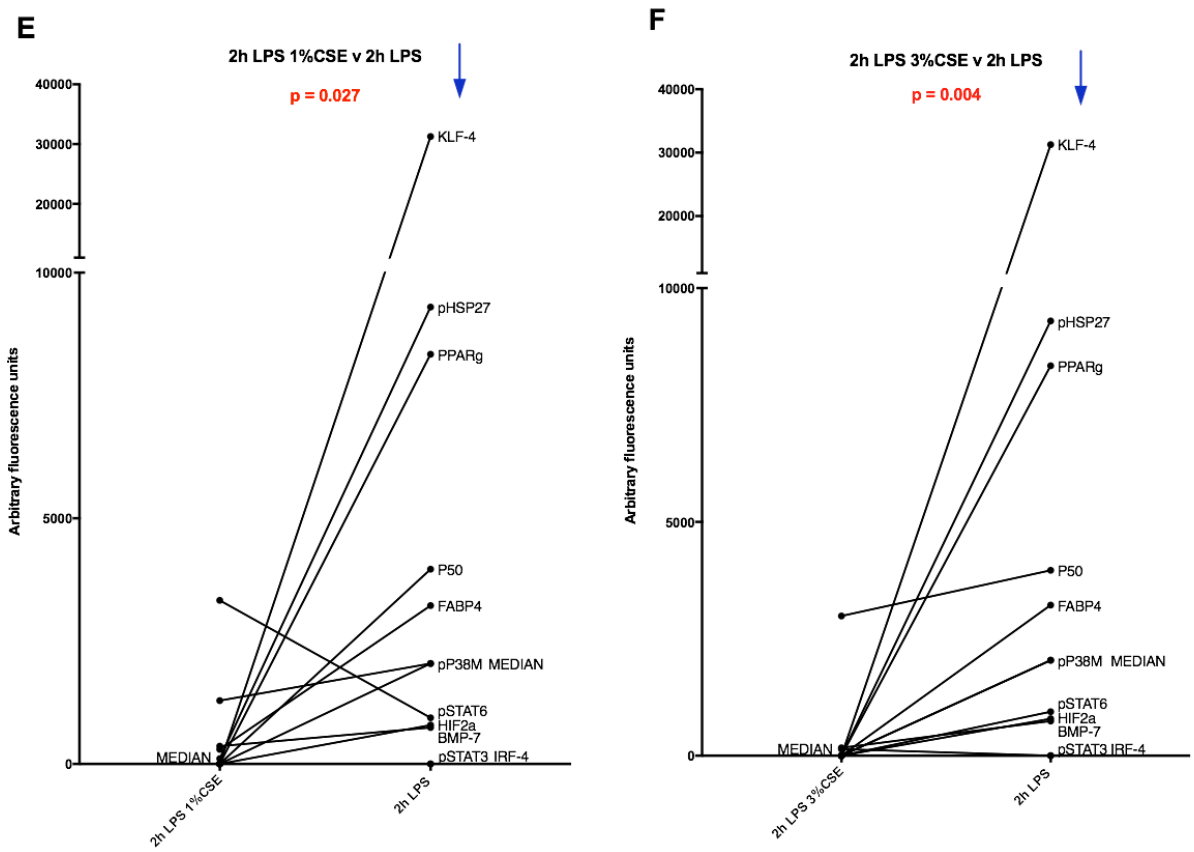
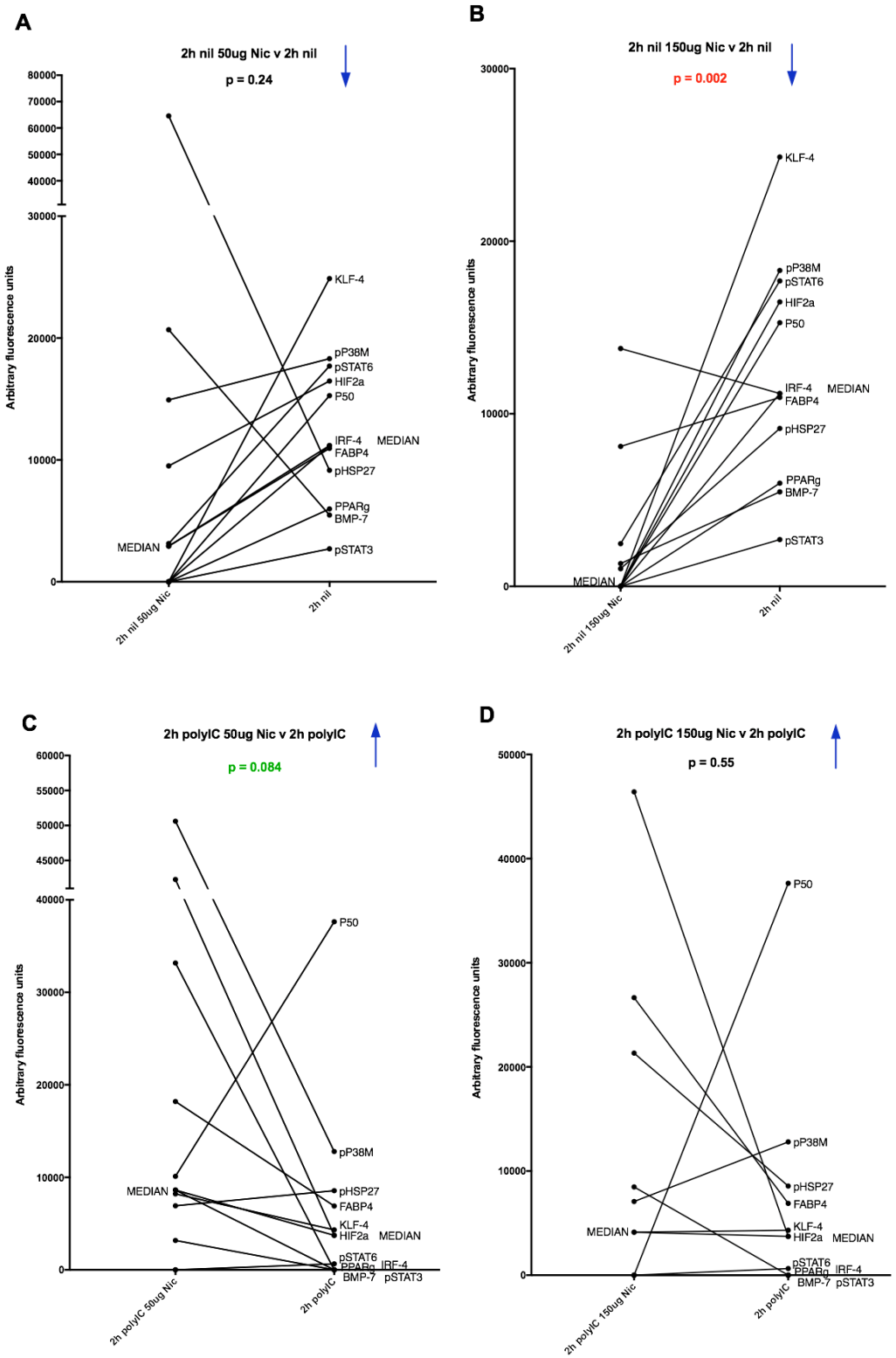


Figure 5.2: RPPA of median values of signalling molecule expression after 2 h incubation with CSE +/- Poly-IC or LPS.
Graphs showing the change in the median value expression for all signalling molecules investigated after treatment with A) 1 % CSE, B) 3 % CSE, C) 1 % CSE+ Poly-IC, D) 3% CSE+ Poly-IC, E) 1 % CSE + lipopolysaccharide (LPS), and F) 3 % CSE + LPS compared to the relevant controls. MEDIAN = the point for the median value for all the signalling molecules combined. Blue arrow at the top indicates whether the median of the values goes down or goes up with the treatment. Data were analysed for statistical significance using by Wilcoxon matched pairs test and values of $P < 0.05$ were considered significant: p values in red show significant differences, p values in green show 'borderline significant' (between 0.05 and 0.1), and p values in black are not near significance (> 0.1).

The results for the macrophages treated with nicotine in the absence or presence of Poly-IC or LPS for 2 hours showed that the nicotine only treatments generally caused a suppression of the median values for the signalling molecules investigated, but this was significant only with 150 µg/ml nicotine ($P = 0.002$), with only 2 markers (pHSP27 and BMP-7) showing an increase in expression after treatment with 50 µg/ml nicotine, and 1 after treatment with 150 µg/ml nicotine (IRF-4). By contrast, treatment with nicotine in combination with Poly-IC or LPS generally caused an increase in the

expression of the signalling molecules investigated, but none of these were statistically significant (50 µg/ml nicotine with Poly-IC gave borderline significant up-regulation, P = 0.084).



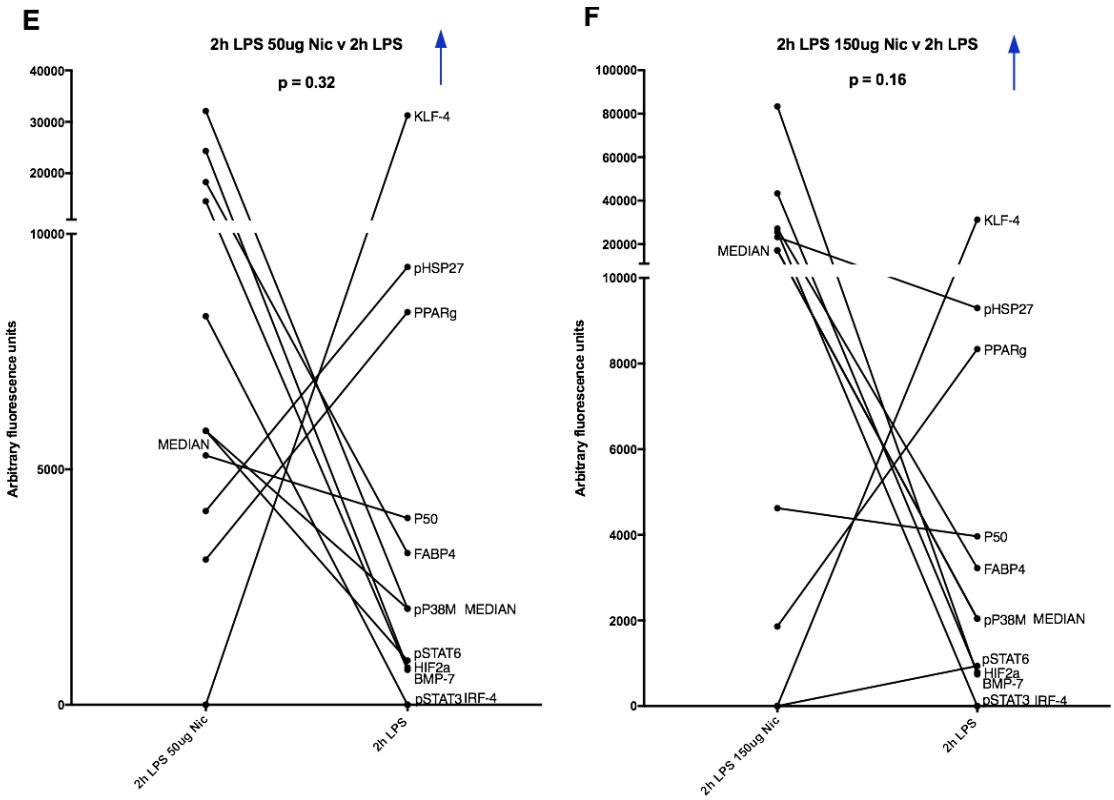


Figure 5.3: RPPA of median values of signalling molecule expression after 2 h incubation with nicotine +/- Poly-IC or LPS.

Graphs showing the change in the median value expression for all the signalling molecules investigated after treatment with A) 50 µg/ml nicotine (nic), B) 150 µg/ml nic, C) 50 µg/ml nic + Poly-IC, D) 150 µg/ml nic + Poly-IC, E) 50 µg/ml nic + lipopolysaccharide (LPS), and F) 150 µg/ml nic + LPS compared to the relevant controls. MEDIAN = the point for the median value for all the signalling molecules combined. Blue arrow at the top indicates whether the median of the values goes down or goes up with the treatment. Data were analysed for statistical significance using by Wilcoxon matched pairs test and values of $P < 0.05$ were considered significant: p values in red show significant differences, p values in green show 'borderline significant' (between 0.05 and 0.1), and p values in black are not near significance (> 0.1).

5.2.2. Analysis of RPPA after 24 hours of incubation

After 24 hours of incubation with the different combinations of LPS, Poly-IC, CSE and nicotine, another set of samples were taken for analysis of changes in protein expression by RPPA. The culture supernatants were also stored for analysis of secreted cytokines by ELISA (section 2.2.16).

As with the 2-hour results, the 24-hour RPPA results were initially analysed for statistical significance using a paired t-test with Bonferroni correction to compare the

treated and untreated samples, but due to the previously mentioned problems, the data were then re-analysed to evaluate the global changes across all signalling molecules, with CSE or nicotine treatments compared to no CSE or nicotine. Data were analysed for statistical significance by Wilcoxon matched pairs test and values of $P < 0.05$ were considered significant and values with $0.05 < P < 0.1$ were considered to be borderline significant. The results for the macrophages treated with Poly-IC or LPS only for 24 h show that both treatments generally caused an increase in expression of the signaling molecules, but the overall changes were not statistically significant.

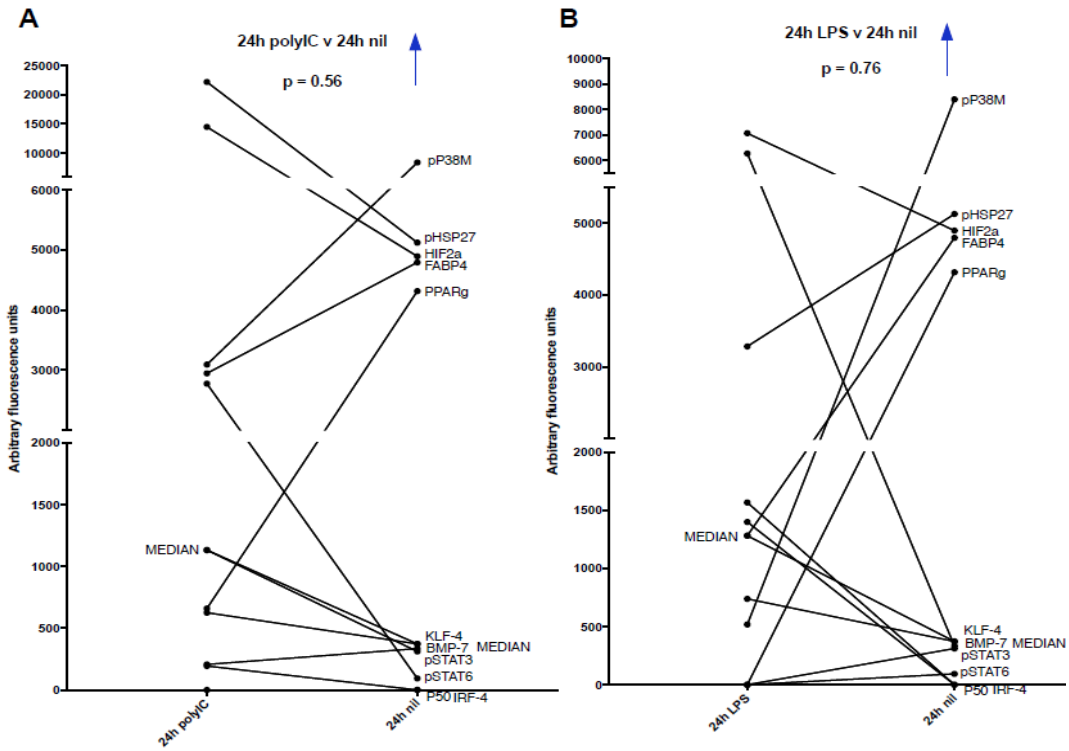
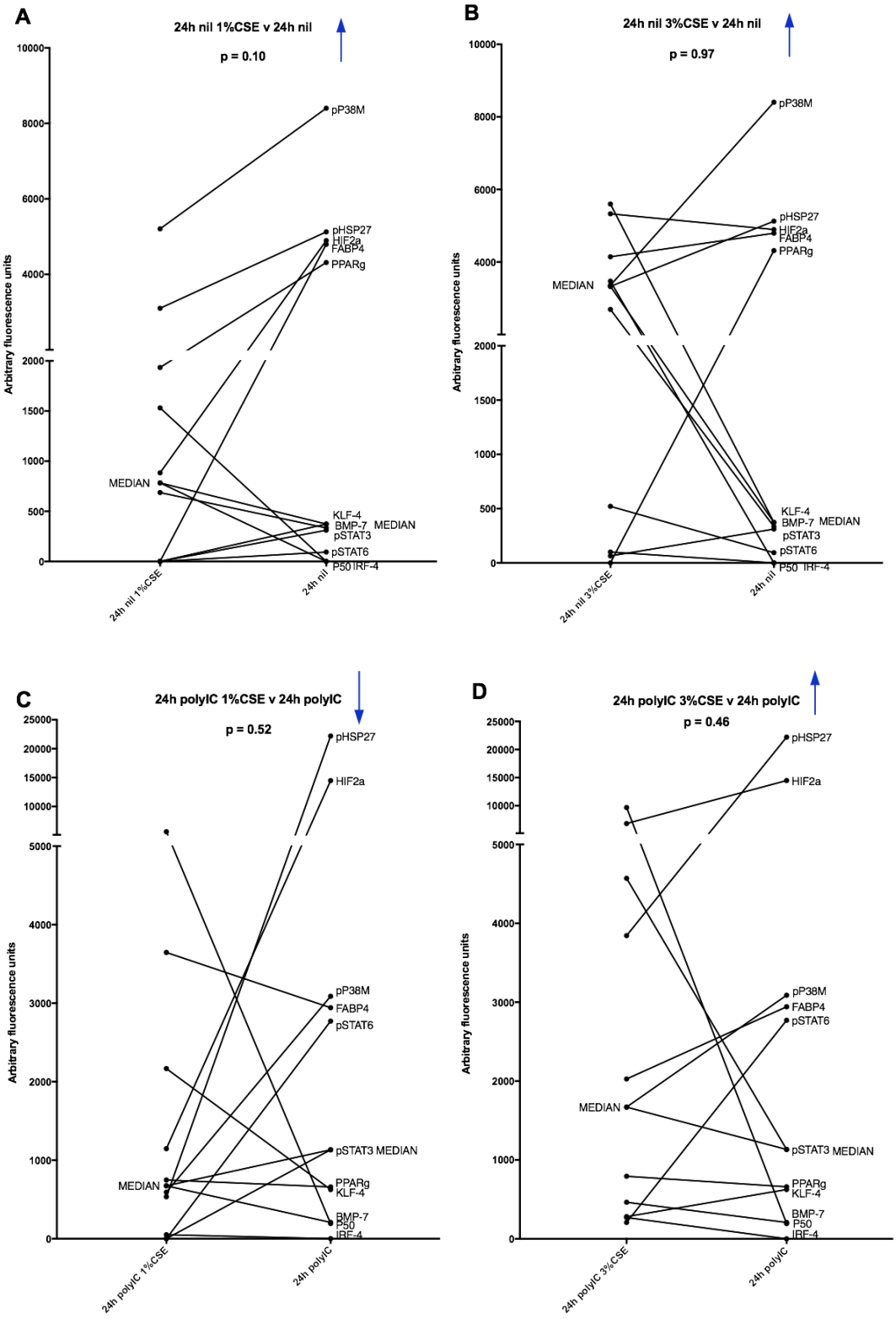


Figure 5.4: RPPA of median values for signalling molecule expression after 24 h incubation with only Poly-IC or LPS.

A) graph showing the change in the median value expression for all signalling molecules investigated after treatment with Poly-IC compared to untreated macrophages (nil). B) graph showing the change in the median value expression for all signalling molecules investigated after treatment with lipopolysaccharide (LPS) compared to untreated macrophages (nil). MEDIAN = the point for the median value for all the signalling molecules combined. Blue arrow at the top indicates whether the median of the values goes down or goes up with the treatment. Data were analysed for statistical significance using by Wilcoxon matched pairs test and values of $P < 0.05$ were considered significant: p values in red show significant differences, p values in green show 'borderline significant' (between 0.05 and 0.1), and p values in black are not near significant (> 0.1).

The results for the macrophages after treatment with CSE without or with Poly-IC or LPS show an increase in signalling molecule expression (except for 1 % CSE plus Poly-IC), but none of the changes were statistically significant.



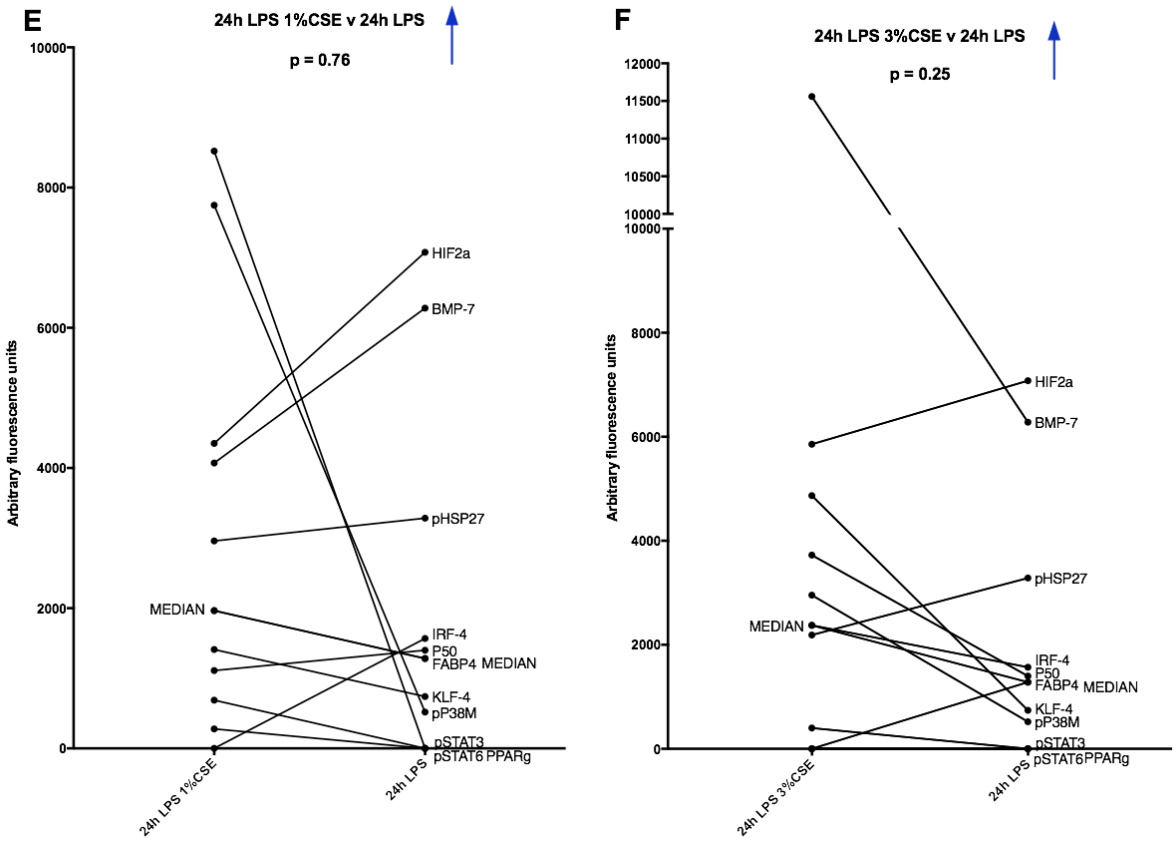
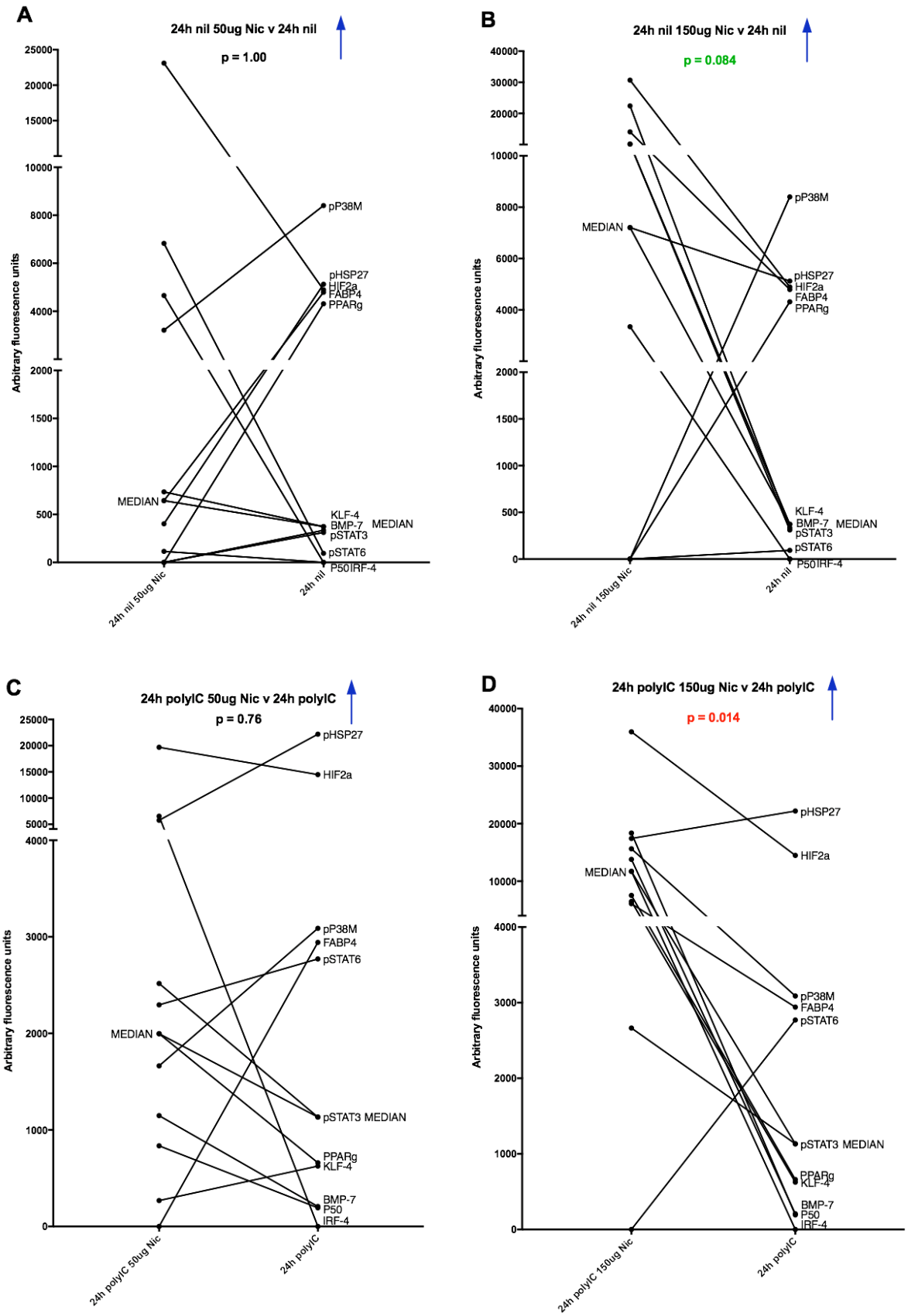


Figure 5.5: RPPA of median values for for signalling molecule expression after 24 h incubation with CSE +/- Poly-IC or LPS.

Graphs showing the change in the median value expression for the markers investigated after treatment with A) 1 % CSE, B) 3 % CSE, C) 1 % CSE + Poly-IC, D) 3% CSE + Poly-IC, E) 1 % CSE + lipopolysaccharide (LPS), and F) 3 % CSE + LPS compared to the relevant controls. MEDIAN = the point for the median value for all the signalling molecules combined. Blue arrow at the top indicates whether the median of the values goes down or goes up with the treatment. Data were analysed for statistical significance using by Wilcoxon matched pairs test with the Bonferroni correction, and values of $P < 0.05$ were considered significant: p values in red show significant differences, p values in green 'borderline significant' (between 0.05 and 0.1), and p values in black are not near significant (> 0.1).

The results for the macrophages after treatment with nicotine without or with Poly-IC or LPS showed an increase in signalling molecule expression (except for 150 µg/ml nicotine plus LPS). The only statistically significant increase was with 150 µg/ml nicotine plus poly-IC ($P = 0.014$), and the increase for 150 µg/ml alone was borderline significant ($P = 0.084$).


A
24h nil 50ug Nic v 24h nil
p = 1.00
Arbitrary fluorescence units
pP38M
pHSP27
HIF2a
FABP4
PPARg
MEDIAN
KLF-4
BMP-7
pSTAT3
MEDIAN
pSTAT6
P50IRF-4
24h nil 50ug Nic
24h nil
B
24h nil 150ug Nic v 24h nil
p = 0.084
Arbitrary fluorescence units
pP38M
MEDIAN
pHSP27
HIF2a
FABP4
PPARg
KLF-4
BMP-7
pSTAT3
MEDIAN
pSTAT6
P50IRF-4
24h nil 150ug Nic
24h nil
C
24h polyIC 50ug Nic v 24h polyIC
p = 0.76
Arbitrary fluorescence units
pHSP27
HIF2a
pP38M
FABP4
pSTAT6
MEDIAN
pSTAT3 MEDIAN
PPARg
KLF-4
BMP-7
P50
IRF-4
24h polyIC 50ug Nic
24h polyIC
D
24h polyIC 150ug Nic v 24h polyIC
p = 0.014
Arbitrary fluorescence units
pHSP27
HIF2a
MEDIAN
pP38M
FABP4
pSTAT6
pSTAT3 MEDIAN
PPARg
KLF-4
BMP-7
P50
IRF-4
24h polyIC 150ug Nic
24h polyIC

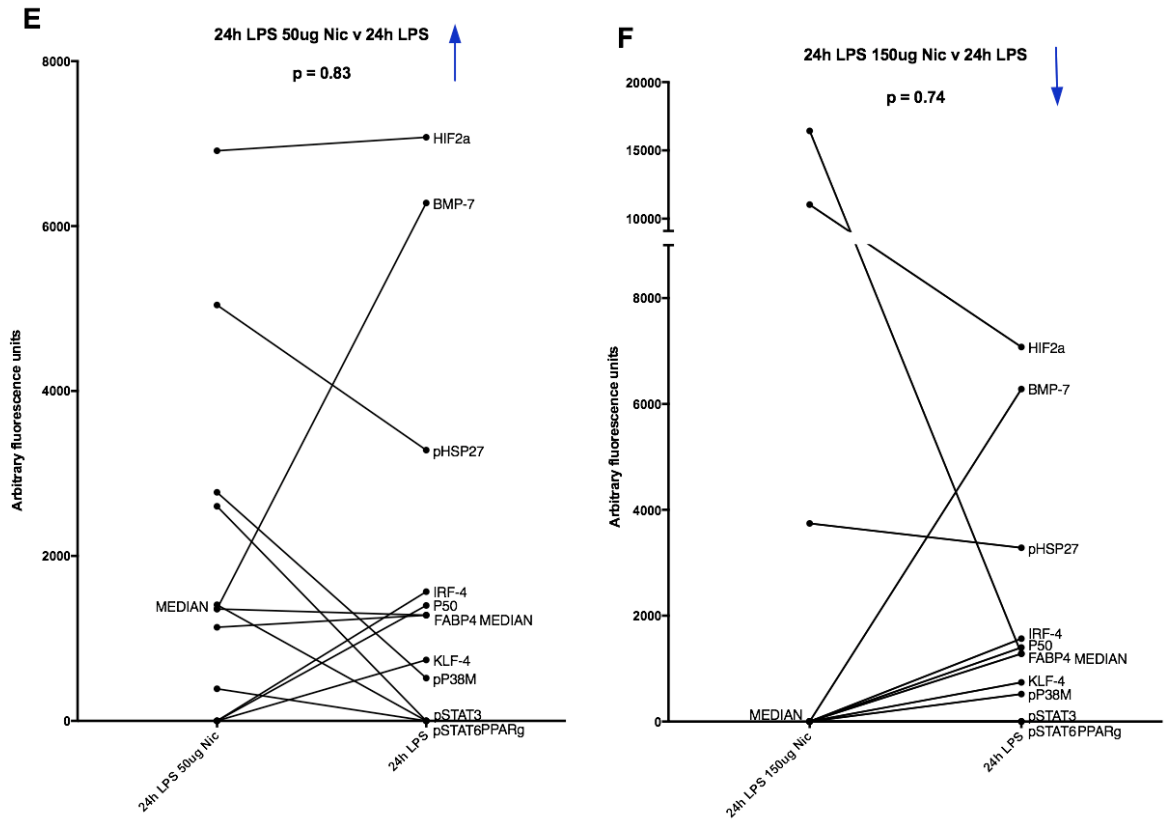


Figure 5.6: RPPA of median values for signalling molecule expression after 24 h incubation with nicotine +/- Poly-IC or LPS.

Graphs showing the change in the median value expression for the signalling molecules investigated after treatment with A) 50 µg/ml nicotine (nic), B) 150 µg/ml nic, C) 50 µg/ml nic + Poly-IC, D) 150 µg/ml nic + Poly-IC, E) 50 µg/ml nic + lipopolysaccharide (LPS), and F) 150 µg/ml nic + LPS compared to the relevant controls. MEDIAN = the point for the median value for all the signalling molecules combined. Blue arrow at the top indicates whether the median of the values goes down or goes up with the treatment. Data were analysed for statistical significance using by Wilcoxon matched pairs test and values of $P < 0.05$ were considered significant: p values in red show significant differences, p values in green show 'borderline significant' (between 0.05 and 0.1), and p values in black are not near significant (> 0.1).

5.2.3. Analysis of RPPA after 48 hours of incubation

After 48 hours of incubation with the different combinations of LPS, Poly-IC, CSE and nicotine, the final set of samples were taken for analysis of changes in protein expression by RPPA (and culture supernatants were again saved for analysis of secreted cytokines by ELISA). As with the 2 and 24 hour samples, the 48-hour results were initially analysed for statistical significance using a paired t-test with Bonferroni

correction to compare the treated and untreated samples, but due to the previously mentioned problems, the data were then re-analysed to assess the global changes across all signalling molecules, with CSE or nicotine treatments compared to no CSE or nicotine. Data were analysed for statistical significance by Wilcoxon matched pairs test and values of $P < 0.05$ were considered significant. The results for Poly-IC and LPS only after 48 h showed that Poly-IC generally produced a significant increase in expression ($P = 0.014$), whereas LPS mainly caused a suppression of the signalling molecule expression (borderline significant, $P = 0.078$).

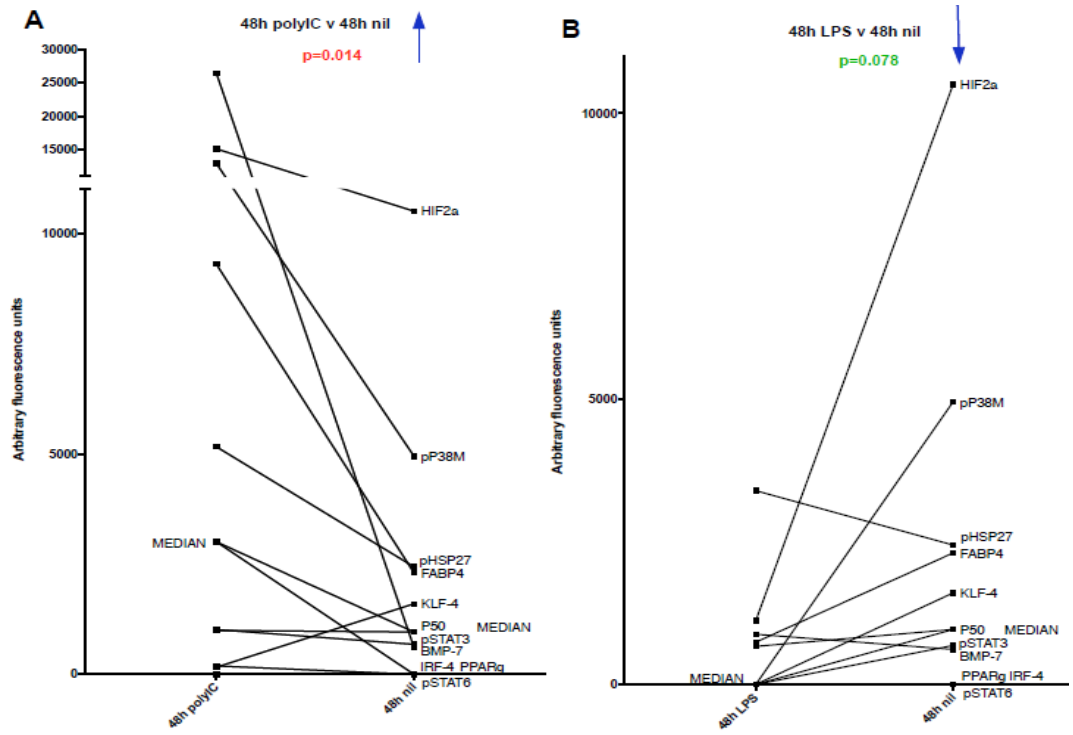
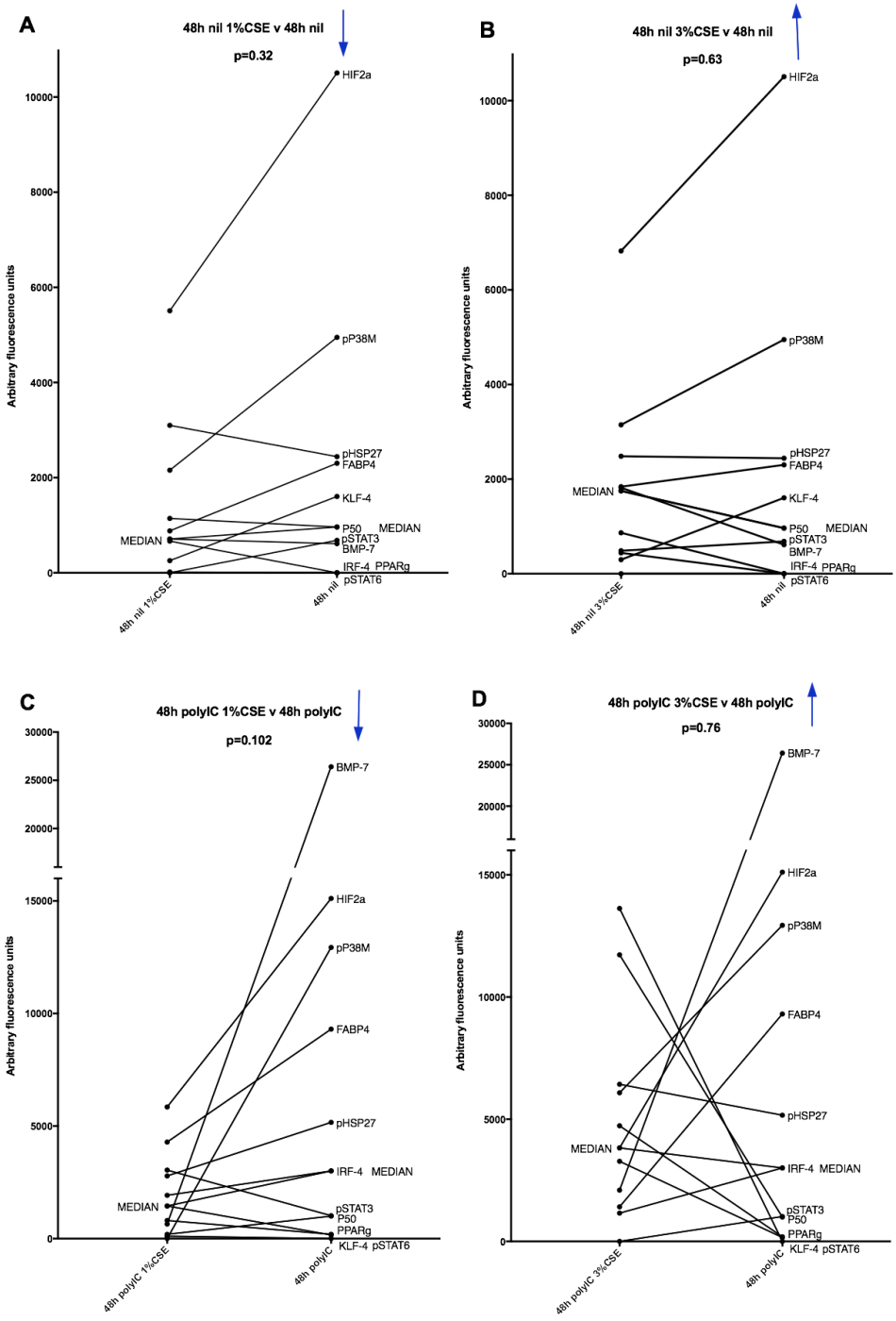


Figure 5.7: RPPA of median values for signalling molecule expression after 48 h incubation with only Poly-IC or LPS.

A) graph showing the change in the median value expression for all signalling molecules investigated after treatment with Poly-IC compared to untreated macrophages (nil). B) graph showing the change in the median value expression for all signalling molecules investigated after treatment with lipopolysaccharide (LPS) compared to untreated macrophages (nil). MEDIAN = the point for the median value for all the signalling molecules combined. Blue arrow at the top indicates whether the median of the values goes down or goes up with the treatment. Data were analysed for statistical significance using by Wilcoxon matched pairs test and values of $P < 0.05$ were considered significant: p values in red show significant differences, p values in green show 'borderline significant' (between 0.05 and 0.1), and p values in black are not near significant (> 0.1).

The results after 48 h treatment with CSE without and with Poly-IC or LPS showed that 1 % CSE with or without Poly-IC suppressed the expression of the markers (not significant), whereas 1 % CSE with LPS significantly increased signalling molecule expression (P = 0.039). 3 % CSE increased signalling molecules expression without or with poly-IC or LPS, but none of these changes were statistically significant.



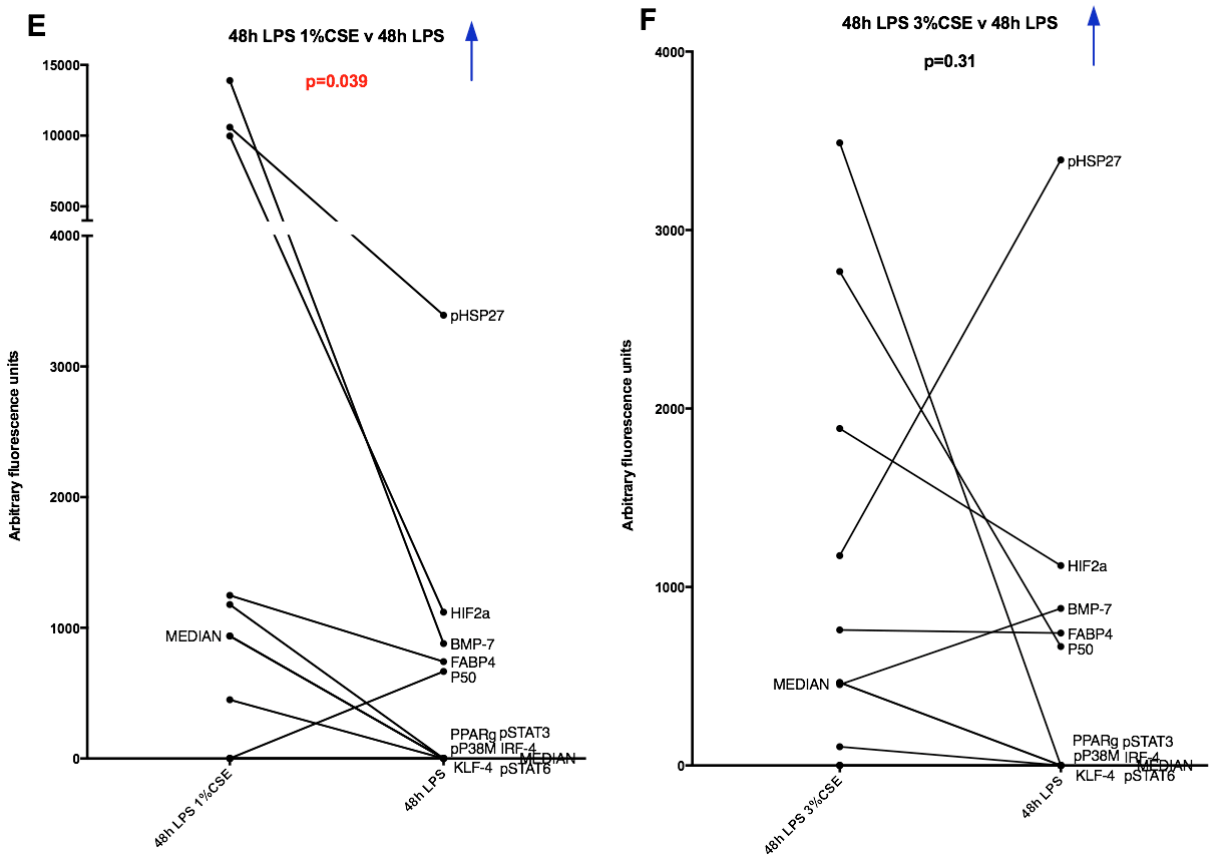
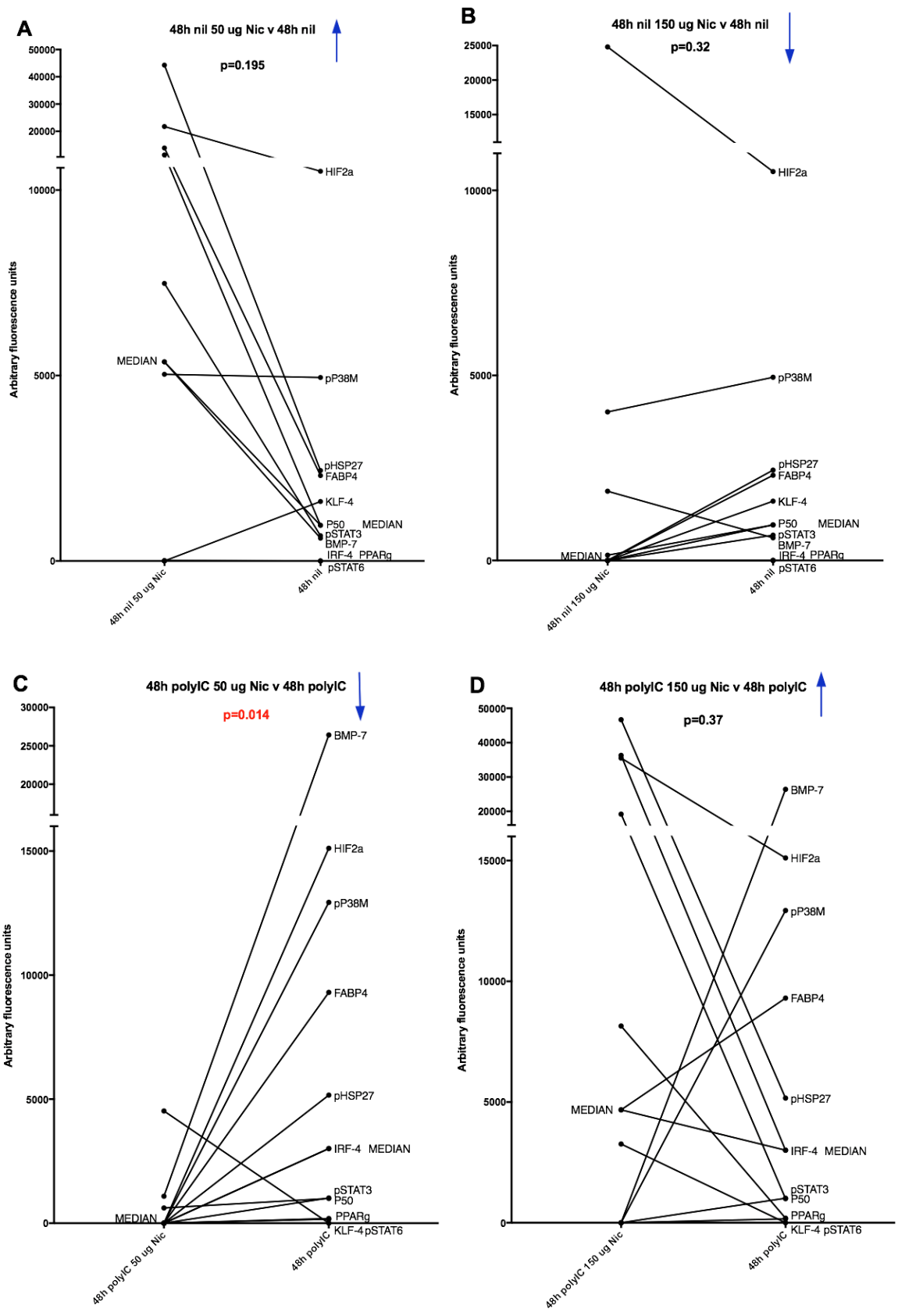


Figure 5.8: RPPA of median values for signalling molecule expression after 48 h incubation with CSE +/- Poly-IC or LPS.

Graphs showing the change in the median value expression for the signalling molecules investigated after treatment with A) 1 % CSE, B) 3 % CSE, C) 1 % CSE+ Poly-IC, D) 3% CSE+ Poly-IC, E) 1 % CSE + lipopolysaccharide (LPS), and F) 3 % CSE + LPS compared to the relevant controls. MEDIAN = the point for the median value for all the signalling molecules combined. Blue arrow at the top indicates whether the median of the values goes down or goes up with the treatment. Data were analysed for statistical significance using by Wilcoxon matched pairs test and values of $P < 0.05$ were considered significant: p values in red show significant differences, p values in green show 'borderline significant' (between 0.05 and 0.1), and p values in black are not near significant (> 0.1).

The results after treatment with nicotine without or with Poly-IC or LPS for 48 h (Figure 5.9) showed both up-regulation and down-regulation of the signalling molecules investigated. Most changes were not statistically significant, but 50 µg/ml nicotine with Poly-IC significantly down-regulated the signalling molecules ($P = 0.014$) and 150 µg/ml nicotine with LPS up-regulated the signalling molecules (borderline significance, $P = 0.055$).


A
48h nil 50 ug Nic v 48h nil
p=0.195
Arbitrary fluorescence units
HIF2a
MEDIAN
pP38M
pHSP27
FABP4
KLF-4
P50
MEDIAN
pSTAT3
BMP-7
IRF-4
PPARg
pSTAT6
48h nil 50 ug Nic
48h nil
B
48h nil 150 ug Nic v 48h nil
p=0.32
Arbitrary fluorescence units
HIF2a
pP38M
pHSP27
FABP4
KLF-4
P50
MEDIAN
pSTAT3
BMP-7
IRF-4
PPARg
MEDIAN
pSTAT6
48h nil 150 ug Nic
48h nil
C
48h polyIC 50 ug Nic v 48h polyIC
p=0.014
Arbitrary fluorescence units
BMP-7
HIF2a
pP38M
FABP4
pHSP27
IRF-4
MEDIAN
pSTAT3
P50
PPARg
MEDIAN
KLF-4
pSTAT6
48h polyIC 50 ug Nic
48h polyIC
D
48h polyIC 150 ug Nic v 48h polyIC
p=0.37
Arbitrary fluorescence units
BMP-7
HIF2a
pP38M
FABP4
pHSP27
MEDIAN
IRF-4
MEDIAN
pSTAT3
P50
PPARg
KLF-4
pSTAT6
48h polyIC 150 ug Nic
48h polyIC

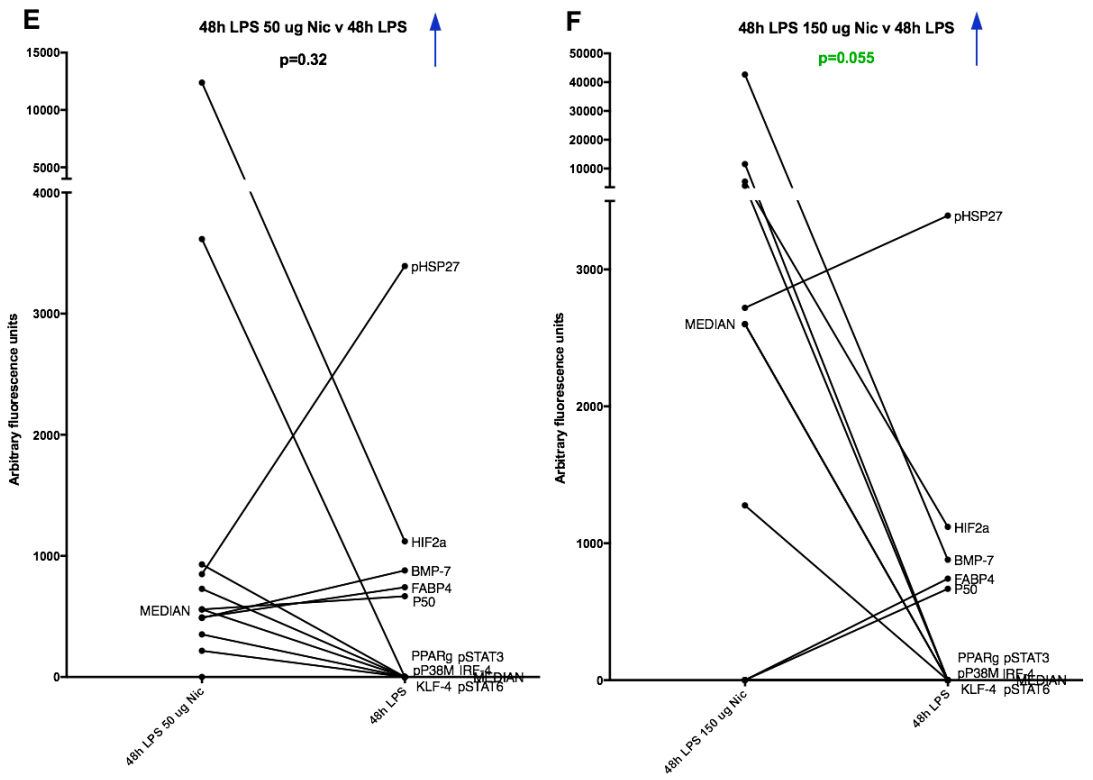


Figure 5.9: RPPA of median values for signalling molecule expression after 48 h incubation with CSE +/- Poly-IC or LPS.

Graphs showing the change in the median value expression for the signalling molecules investigated after treatment with A) 50 µg/ml nicotine (nic), B) 150 µg/ml nic, C) 50 µg/ml nic + Poly-IC, D) 150 µg/ml nic + Poly-IC, E) 50 µg/ml nic + lipopolysaccharide (LPS), and F) 150 µg/ml nic + LPS compared to the relevant controls. MEDIAN = the point for the median value for all the signalling molecules combined. Blue arrow at the top indicates whether the median of the values goes down or goes up with the treatment. Data were analysed for statistical significance using by Wilcoxon matched pairs test and values of $P < 0.05$ were considered significant: p values in red show significant differences, p values in green show 'borderline significant' (between 0.05 and 0.1), and p values in black are not near significant (> 0.1).

Overall, these results show that the different treatment combinations produce some significant differences for the cell surface markers investigated. The changes in the expression of markers associated with M2 macrophages can be related to changes in M2 function and changes in expression of markers associated with M1 macrophages can be related to changes in M1 function. By combining all the results for the different markers, the overall effects of the different treatments can be seen.

5.2.4. Summary of RPPA results

Tables 5.1 and 5.2 summarise the data presented in figures 5.1 to 5.9 for the effects of CSE or nicotine on the global expression of signalling molecule by the monocyte-derived macrophages, either unstimulated, or stimulated with poly-IC or LPS. Table 5.1 summarises whether stimulation with Poly-IC or LPS significantly up-regulated (up-arrow) or down-regulated (down-arrow) the signalling molecules overall compared to unstimulated macrophages. Table 5.2 summarises whether CSE or nicotine caused significant down-regulation (down-arrow) or up-regulation (up-arrow) of the signalling molecules as a whole compared to the relevant control macrophages cultured without CSE or nicotine (i.e. unstimulated macrophages, or macrophages stimulated with poly-IC or LPS alone). Red arrows indicate significant changes ($P < 0.05$) and green arrows indicate borderline significant changes ($0.05 < P < 0.1$); dashes (–) indicate no significant change.

Table 5.1 Effects of stimulation of monocyte-derived macrophages with Poly-IC or LPS on the overall expression of the signalling molecules investigated by RPPA, compared to unstimulated macrophages ('Nil')*.

Time point	Poly-IC versus Nil	LPS versus Nil
2 hours	↓	↓
24 hours	–	–
48 hours	↑	↓

*Red arrows indicate significant changes ($P < 0.05$) and green arrows indicate borderline significant changes ($0.05 < P < 0.1$); dashes (–) indicate no significant change.

Compared to unstimulated macrophages ('Nil'), stimulation for 2 hours with either Poly-IC or LPS induced an overall down-regulation of the signalling molecules (Table 5.1, Figure 5.1). At first sight, this seems unexpected; however, it should be borne in

mind that, as outlined in the Introduction to this chapter, the majority of the signalling molecules investigated are associated with M2 differentiation (i.e. STAT-6, PPAR-γ, KLF-4, IRF-4, BMP-7, HIF-2a, pSTAT-3), rather than M1 differentiation, and so this is consistent with both poly-IC and LPS initially favouring macrophage differentiation towards M1, as reported by others (reviewed by Martinez and Gordon, 2014). After 24 hours of stimulation, there were no overall significant differences in the signalling molecule levels between unstimulated and stimulated macrophages (Table 5.1, Figure 5.4) whereas, by 48 hours of stimulation, Poly-IC had significantly upregulated the overall signalling molecule expression, but LPS again showed a trend towards down-regulation of the signalling molecules (Table 5.1, Figure 5.7).

Table 5.2 Effects of CSE and nicotine on the overall expression of signalling molecules investigated by RPPA in monocyte-derived macrophages, either unstimulated or stimulated with Poly-IC or LPS*.

Time point	Nil				Poly-IC				LPS			
	1% CSE	3% CSE	50 Nic	150 Nic	1% CSE	3% CSE	50 Nic	150 Nic	1% CSE	3% CSE	50 Nic	150 Nic
2h	↓	↓	–	↓	↓	–	↑	–	↓	↓	–	–
24h	–	–	–	↑	–	–	–	↑	–	–	–	–
48h	–	–	–	–	–	–	↓	–	↑	–	–	↑

*Red arrows indicate significant changes ($P < 0.05$) and green arrows indicate borderline significant changes ($0.05 < P < 0.1$); dashes (–) indicate no significant change.

After just 2 hours of treatment, 1 % and 3 % CSE caused a significant down-regulation of the overall expression of the signalling molecules in both unstimulated and LPS-stimulated macrophages, with just a trend towards down-regulation by 1 % CSE in

Poly-IC stimulated macrophages (Table 5.2, Figure 5.2). By contrast, CSE had no significant effects on the overall signalling molecule expression, in either unstimulated or stimulated macrophages, after 24 hours and 48 hours of incubation (Table 5.2, Figures 5.5 and 5.8); the only exception was significant up-regulation of signalling molecules in LPS-stimulated macrophages treated with 1% CSE for 48 hours (Table 5.2, Figure 5.8). Thus, CSE appeared to induce acute down-regulation of the signalling molecules overall, but these effects ameliorated over a period of 24-48 hours of culture.

The effects of nicotine on the overall expression of the signalling molecules investigated was less clear-cut or consistent than the effects of CSE described above (Table 5.2). In four instances, nicotine induced significant, or borderline-significant, overall up-regulation of the signalling molecules and gave significant down-regulation in two instances; however, under most conditions, nicotine had no significant effects (Figure 5.2).

5.2.5. ELISA of IL-10 and TNF-α

To complement the investigation of the effects of CSE and nicotine on the expression of signalling molecules by RPPA, secreted cytokines produced in the same experiments were also assayed by ELISA. The cytokines chosen to be investigated were TNF-α and IL-10, as changes in their expression should help determine if the different conditions used produced more M1 or M2 type macrophages, respectively (Martinez and Gordon, 2014). The supernatants assayed were those from the monocyte-derived macrophages cultured for 24 or 48 hours without or with Poly-IC or LPS, and without or with CSE or nicotine. The supernatants from the 2 hour cultures were not assayed as very little, if any cytokine production and secretion is likely to have occurred at this early time point.

To investigate the limits of detection for the assay, cytokine standard curves for IL-10 and TNF-α were produced. The results (Figure 5.10) show that this assay can detect a sufficiently wide range of concentrations of these two cytokines, making it a suitable test for analysing the culture supernatants of the cell samples used for the RPPA experiments.

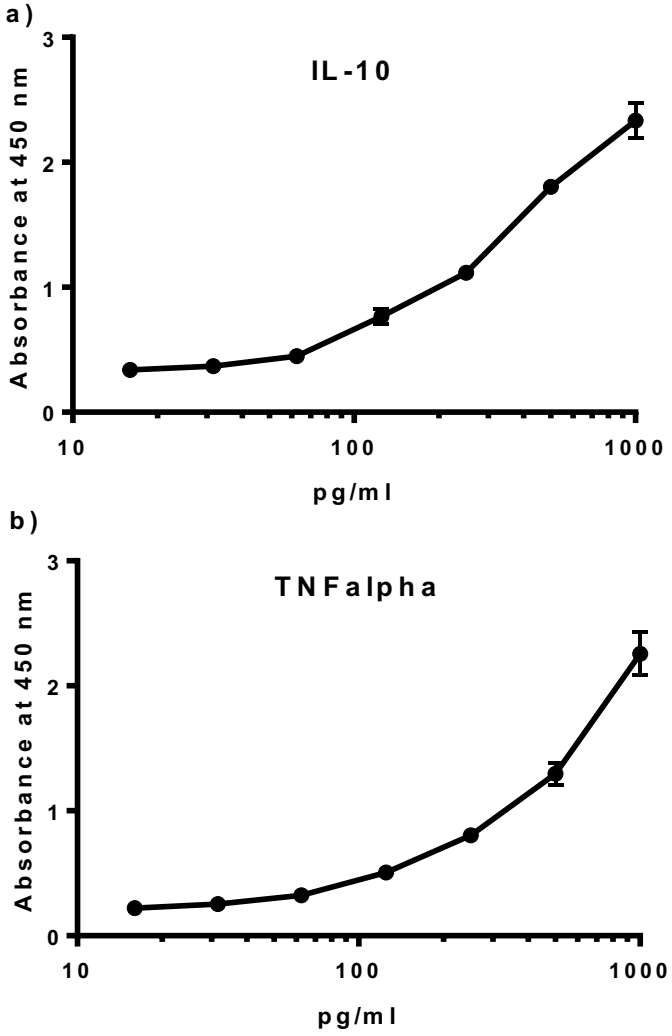


Figure 5.10: ELISA standard curves for a) IL-10 and b) TNF-α.

Data are mean and error bars are SD (n = 3).

5.2.6. Analysis of ELISA results after 24 h incubation

The results after 24 h of incubation with the different combinations of treatments showed that there were some differences between the expression of IL-10 and TNF-α. For the macrophages cultured without CSE or nicotine, the results for IL-10 (Figure 5.11a) showed that there was a significant increase in expression after stimulation with Poly-IC ($p = 0.0078$) or LPS ($p = 0.0156$). Similar results were obtained for TNF-α (Figure 5.11b), which also showed a significant increase in expression after stimulation with Poly-IC or LPS ($p = 0.0156$ for both).

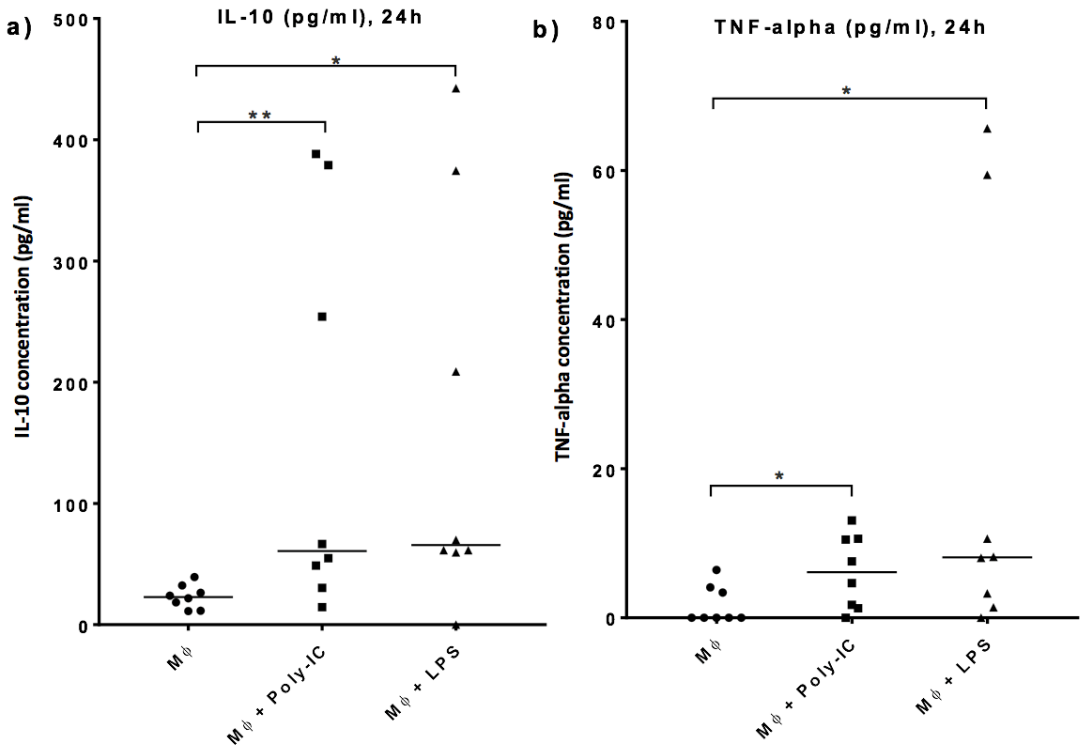


Figure 5.11: ELISA of cytokine expression after 24 h incubation of macrophages without or with Poly-IC or LPS.

The analysis is shown as scatter plots, each dot represents one experimental repeat, and the bar represents the median (n = 8) for a) expression of IL-10 and b) expression of TNF-α. Data were analysed for statistical significance by a Wilcoxon matched pairs test with Bonferroni correction, P values ≤ 0.025 were considered as significant, * = P < 0.025, ** = P < 0.01.

For the macrophage samples treated with or without Poly-IC or LPS and with or without CSE, the results for IL-10 (Figure 5.12) showed that there was a significant increase in expression only after treatment with 1 % CSE (p = 0.0078) with macrophage without Poly-IC or LPS. No other changes were statistically significant. This may be partly due to the wide variation in IL-10 levels produced by macrophages from different donors.

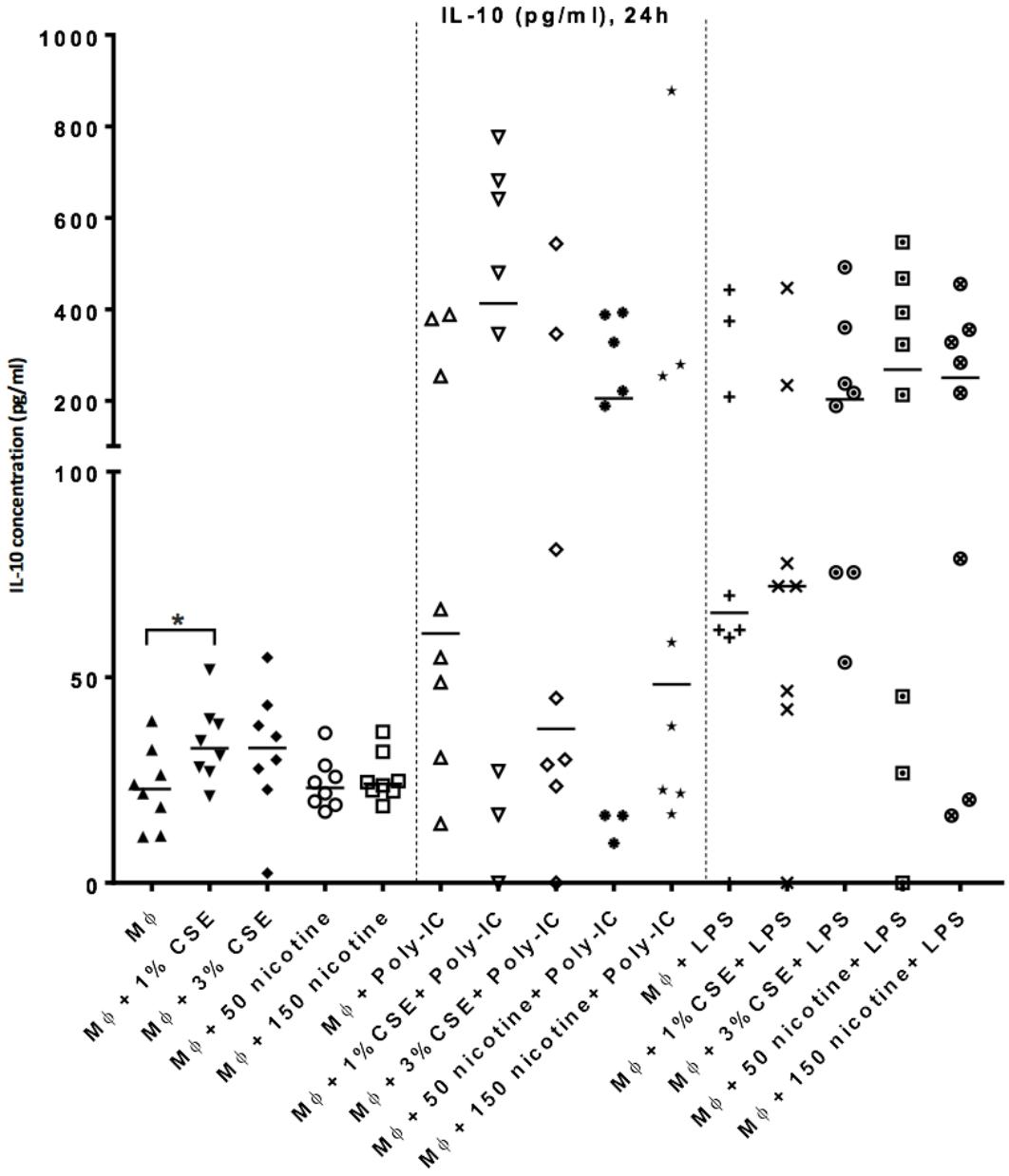


Figure 5.12: ELISA of IL-10 expression after 24 h incubation with all treatment conditions.
The analysis is shown as scatter plots, each dot represents one independent experimental repeat (donor sample), and the bar represents the median (n = 8). Data were analysed for statistical significance by a Wilcoxon matched pairs test with Bonferroni correction. P values ≤ 0.0125 were considered as significant for + 1 % CSE, + 3 % CSE, + 50 nic and + 150 nic, * = P < 0.0125.

For the macrophage samples treated with or without Poly-IC or LPS and with or without CSE, the results for TNF-α (Figure 5.13) showed that there was a significant increase in expression after treatment with 3 % CSE with Poly-IC (p = 0.0078), and 1 % CSE

with LPS (p = 0.0078). There was also an increase in expression for TNF-α after treatment with 1 % CSE (p = 0.0391), 3 % CSE (p = 0.0312), 1 % CSE with Poly-IC (0.0156), 3 % CSE with LPS (0.0156) and 50 µg/ml nicotine with LPS (p = 0.0312); these values were p < 0.05 but above the Bonferroni cut-off for significance, so they are considered to show a trend towards significance (borderline significant).

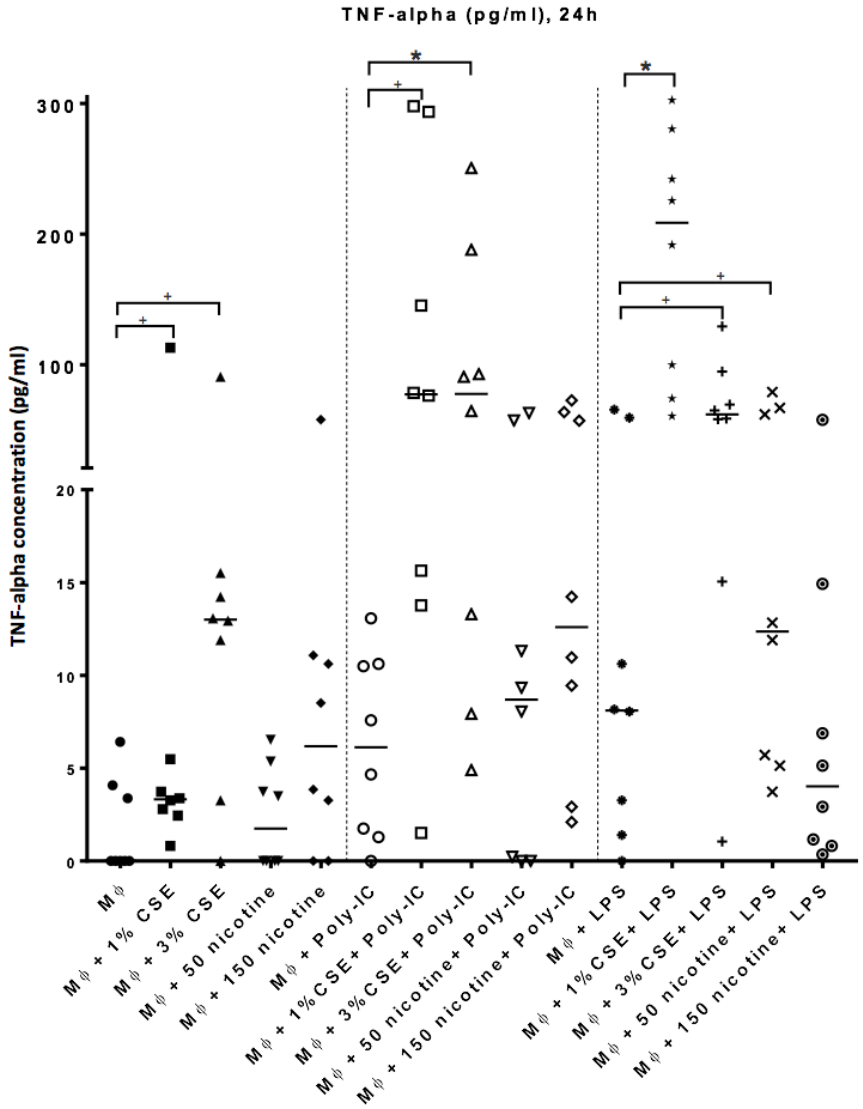


Figure 5.13: ELISA of TNF-α expression after 24 h incubation with all treatment conditions.
The analysis is shown as scatter plots, each dot represents independent experimental repeats (donors), and the bar represents the median (n = 8). Data were analysed for statistical significance by a Wilcoxon matched pairs test with Bonferroni correction. P values ≤ 0.0125 were considered as significant for + 1 % CSE, + 3 % CSE, + 50 nic and + 150 nic, * = P < 0.0125, + = are p < 0.05, but greater than the Bonferroni cut-off.

5.2.7. Analysis of ELISA results after 48 h incubation

The results after 48 h of incubation with the different combinations of treatment showed that there were some differences between the expression of IL-10 and TNF-α, with some similarities to the results after 24 h of treatment.

For the macrophage cultured without CSE or nicotine, the results for IL-10 (Figure 5.14a) are similar to those after 24h treatment, they showed that there was a significant increase in expression after treatment with Poly-IC ($p = 0.0156$) and LPS ($p = 0.0078$), and the results for TNF-α (Figure 5.14b) showed a similar pattern with a significant increase in expression after treatment with Poly-IC ($p = 0.0078$) and LPS ($p = 0.0234$).

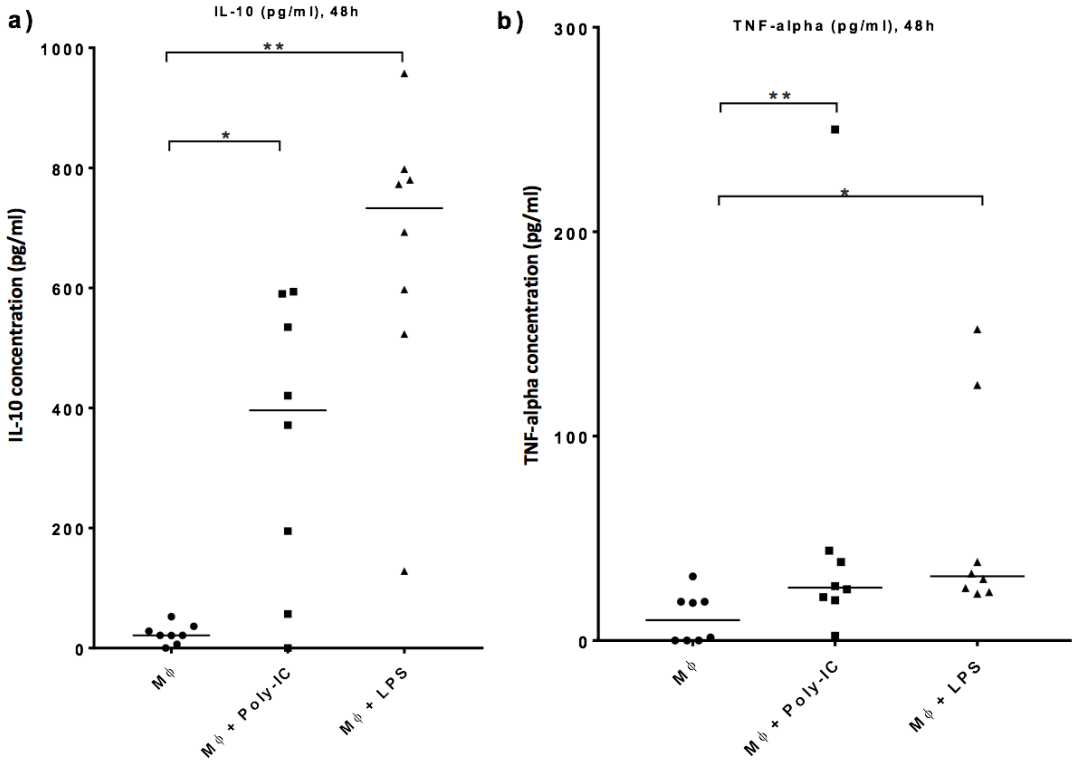


Figure 5.14: ELISA of cytokine expression after 48 h incubation of macrophages without or with Poly-IC or LPS.

The analysis is shown as scatter plots, each dot represents independent experimental repeats (donors), and the bar represents the median (n = 8) for a) expression of IL-10 and b) expression of TNF-α. Data were analysed for statistical significance by a Wilcoxon matched pairs test with Bonferroni correction, P values ≤ 0.025 were considered as significant, * = $P < 0.025$, ** = $P < 0.01$.

For the macrophage samples treated with or without Poly-IC or LPS with CSE or nicotine for 48 h, the results for IL-10 (Figure 5.15) showed that there was a significant decrease in expression after treatment with 50 or 150 μg/ml nicotine with LPS ($p = 0.0078$ for both), which is different to the results seen after 24 h. There was also a decrease in expression after 48 h treatment with 50 or 150 μg/ml nicotine and Poly-IC ($p = 0.0156$) and 50 μg/ml nicotine only ($p = 0.0391$), and an increase in IL-10 expression after 48 h treatment with 3 % CSE ($p = 0.0234$), but these values were $p < 0.05$ but above the Bonferroni cut-off for significance, so they show a trend towards significance (borderline significant).

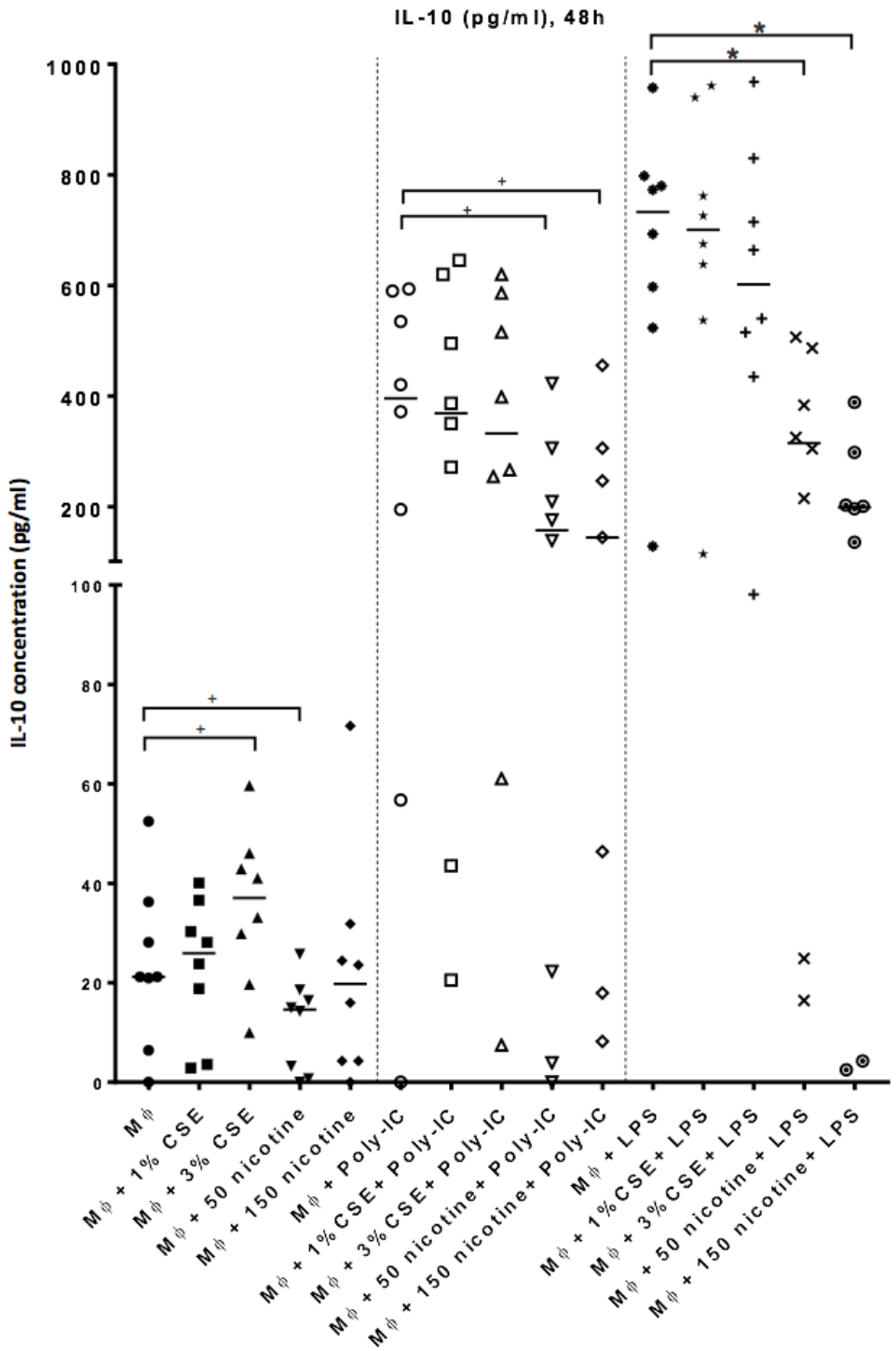


Figure 5.15: ELISA of IL-10 expression after 48 h incubation with all treatment conditions.

The analysis is shown as scatter plots, each dot represents independent experimental repeats (donors), and the bar represents the median (n = 8). Data were analysed for statistical significance by a Wilcoxon matched pairs test with Bonferroni correction, P values ≤ 0.0125 were considered as significant for + 1 % CSE, + 3 % CSE, + 50 nic and + 150 nic. * = P < 0.0125, + = are p < 0.05, but greater than the Bonferroni cut-off.

For the macrophage samples treated with or without Poly-IC or LPS and with or without CSE or nicotine for 48 h, the results for TNF-α (Figure 5.16) showed that, as with the 24 h results, there was a significant increase in expression for some of the treatments; 3 % CSE, 150 μg/ml nicotine, and 1 and 3 % CSE with LPS ($p = 0.0078$ for all). There was also an increase in expression after 48 h treatment with 1 % CSE and 50 μg/ml nicotine ($p = 0.0156$ for both), but as with some other results, these values were $p < 0.05$ but above the Bonferroni cut-off for significance, so they only show a trend towards significance (borderline significant).

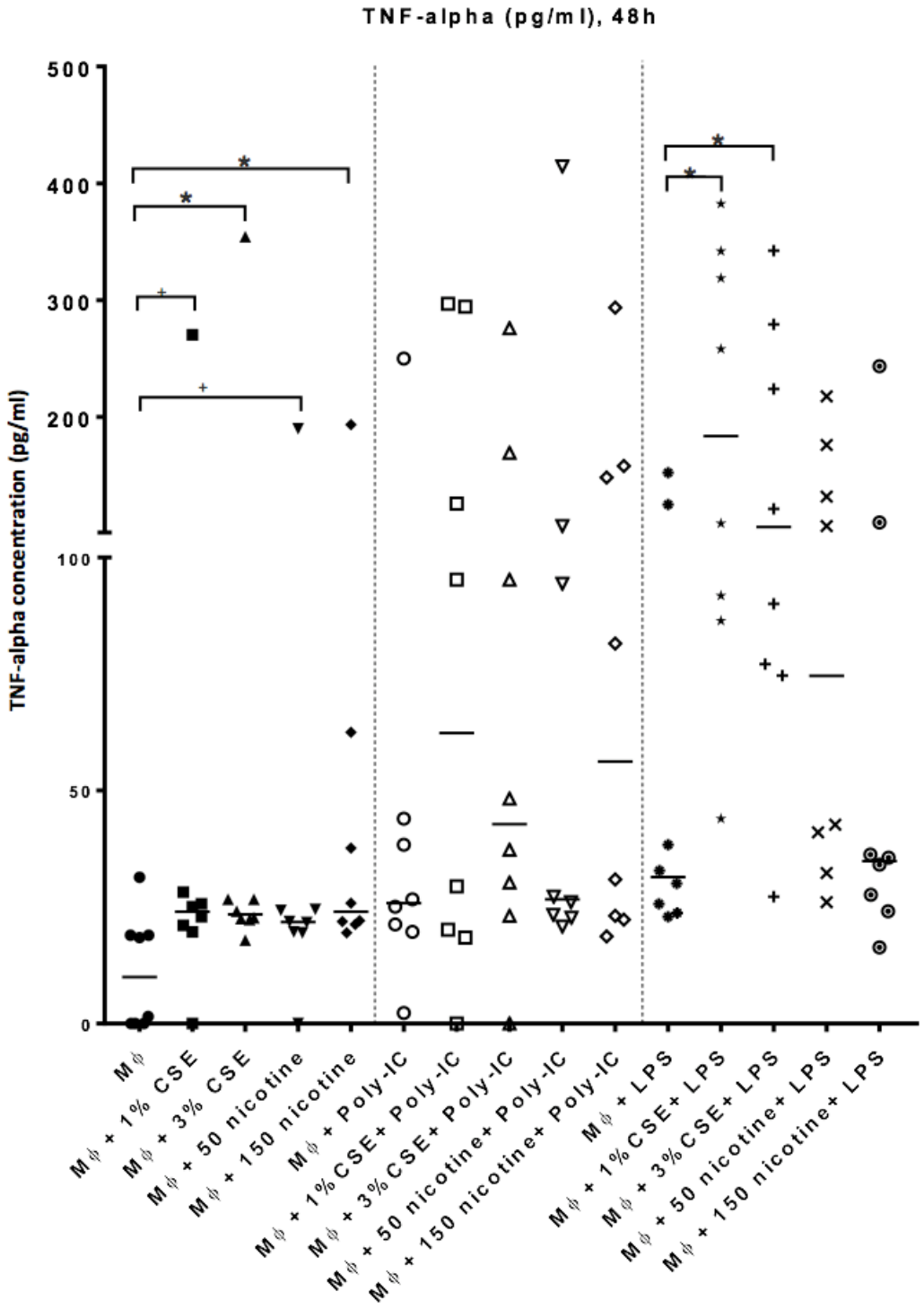


Figure 5.16: ELISA of TNF-α expression after 48 h incubation with all treatment conditions.

The analysis is shown as scatter plots, each dot represents independent experimental repeats (donors), and the bar represents the median (n = 8). Data were analysed for statistical significance by a Wilcoxon matched pairs test with Bonferroni correction, P values ≤ 0.0125 were considered as significant for + 1 % CSE, + 3 % CSE, + 50 nic and + 150 nic. * = P < 0.0125, + = are p < 0.05, but greater than the Bonferroni cut-off.

5.2.8. Summary of ELISA results

Tables 5.3 and 5.4 summarise the data presented in figures 5.11 to 5.16 for the effects of CSE or nicotine on the global expression of IL-10 and TNF-α by the monocyte-derived macrophages, either unstimulated, or stimulated with poly-IC or LPS. Table 5.3 summarises whether stimulation with Poly-IC or LPS significantly up-regulated (up-arrow) or down-regulated (down-arrow) the cytokines compared to unstimulated macrophages. Table 5.4 summarises whether CSE or nicotine caused significant down-regulation (down-arrow) or up-regulation (up-arrow) of the cytokines compared to the relevant control macrophages cultured without CSE or nicotine (i.e. unstimulated macrophages, or macrophages stimulated with poly-IC or LPS alone). Red arrows indicate significant changes ($P \leq 0.0125$) and green arrows indicate borderline significant changes ($0.0125 < P < 0.05$); dashes (–) indicate no significant change.

Table 5.3 Effects of stimulation of monocyte-derived macrophages with Poly-IC or LPS on the expression of the cytokines investigated by ELISA, compared to unstimulated macrophages ('Nil')*.

Cytokine/Time point	Poly-IC versus Nil	LPS versus Nil
IL-10/24 hours	↑	↑
IL-10/48 hours	↑	↑
TNF-α/24 hours	↑	↑
TNF-α/48 hours	↑	↑

*Red arrows indicate significant changes ($P \leq 0.0125$).

As shown in Table 5.3 (and Figures 5.11 and 5.14), both poly-IC and LPS significantly up-regulated production of secreted IL-10 and TNF-α at 24 hours and 48 hours of stimulation.

Table 5.4 Effects of CSE and nicotine on the expression of the cytokines investigated by ELISA in monocyte-derived macrophages, either unstimulated or stimulated with Poly-IC or LPS*.

Cyto-kine	Nil				Poly-IC				LPS			
Time point	1% CSE	3% CSE	50 Nic	150 Nic	1% CSE	3% CSE	50 Nic	150 Nic	1% CSE	3% CSE	50 Nic	150 Nic
IL-10 24h	↑	–	–	–	–	–	–	–	–	–	–	–
IL-10 48h	–	↑	↓	–	–	–	↓	↓	–	–	↓	↓
TNFa 24h	↑	↑	–	–	↑	↑	–	–	↑	↑	↑	–
TNFa 48h	↑	↑	↑	↑	–	–	–	–	↑	↑	–	–

*Red arrows indicate significant changes ($P \leq 0.0125$) and green arrows indicate borderline significant changes ($0.0125 < P < 0.05$); dashes (–) indicate no significant change.

Table 5.4 shows that, after 24 h of culture, IL-10 production was only increased by treatment of unstimulated macrophages with 1 % CSE (Figure 5.12). TNF-α production was increased by treatment with 1 % or 3 % CSE in all stimulation conditions (but some of the results only show a trend/borderline significance following Bonferroni correction), and there was also a high but borderline significant increase in expression after treatment with 50 µg/ml nicotine and LPS (Figure 5.13).

After 48 h of treatment, TNF-α expression was enhanced by 1 and 3 % CSE +/- LPS, and 50 and 150 µg/ml nicotine only (with some of the results only showing a trend/borderline significance) (Table 5.4, Figure 5.16). IL-10 expression was suppressed by nicotine under all conditions and only up-regulated by 3 % CSE alone (Table 5.4, Figure 5.15).

Overall, the summary of the cytokine results in Table 5.4 indicates that, in general, TNF-α production was up-regulated by both CSE and nicotine treatment of unstimulated and LPS-stimulated macrophages and, to a lesser extent in poly-IC-stimulated macrophages. In contrast, IL-10 production was generally down-regulated by treatment with nicotine for 48 hours of unstimulated and stimulated macrophages; up-regulation of IL-10 production was only observed in unstimulated macrophages treated with CSE.

5.3. Discussion

The results of the RPPA investigation imply that the treatment conditions used, and the markers selected were relevant and appropriate to detect differences produced as part of the investigation of how CSE and nicotine affect macrophages. However, it is important to bear in mind that studies have shown that, despite the many advantages of RPPA, there are some key limitations that need to be considered, including the level of sensitivity, specificity, spatial resolution and tissue sample preparation methods (Solier & Langen, 2014). The current limited availability of the required highly specific antibodies is a drawback of RPPA (Solier & Langen, 2014). Sensitivity is also an issue as the complexity of the biological mixture, such as blood, means there is a limit on the detection sensitivity of low-abundant proteins, meaning the RPPA assay may not be able to detect them accurately, which can lead to substantial variability between samples (Solier & Langen, 2014; Aziz, 2015). Sample preparation can also influence the accuracy of RPPA results, as the processes of blood collection, processing, handling and storage of samples can affect the levels of cytokines in the blood (Aziz, 2015).

The initial analyses of the RPPA data for each individual signalling molecule did not show any clearly significant changes associated with CSE or nicotine treatment, and some of the expression values for the signalling molecules were very similar to the blank controls (blocking buffer only), which suggests that there was either low expression of some signalling molecules in the macrophages derived from some domors, or there were technical issues with the RPPA. Therefore, the data was re-

analysed following subtraction of all the relevant blank values from the corresponding values for the signalling molecule expression.

A further consideration, based on previous experience and publications from our laboratory, is that the significance of many biological and pathological phenomena are not determined by changes in individual molecules, but cumulative or 'global' changes in multiple parameters. For example, investigations in our laboratory (using the same RPPA technology employed in this thesis) of elevated expression of pro-inflammatory signalling molecules in the PBMCs of patients with the autoinflammatory disease TRAPS (TNF Receptor Associated Periodic Syndrome) showed that the absolute changes in individual signalling molecule expression compared to healthy controls were not numerically large, but it was the changes across multiple signalling molecules in multiple signalling pathways that was most relevant (Negm *et al*., 2014). Similarly, and even more pertinently, the potential pathological importance of the suppression of pro-inflammatory signalling molecules in human monocyte-derived dendritic cells by CSE and nicotine was again based on the broad range of molecules affected rather than the level of the changes in individual molecules (Alkhattabi *et al*., 2018). Our group has also shown that, in COPD, the relevance to the disease of changes in cytokine levels is related to the co-elevation of multiple cytokines, rather than any individual cytokine (Selvarajah *et al*., 2016). On this basis, the RPPA data for the effects of CSE and nicotine on signalling molecule expression in macrophages was re-analysed: the median values were calculated for each signalling molecule for the 8 individual donors, then the changes across all signalling molecules were analysed, with CSE or nicotine treatments compared to no CSE or nicotine at the different time points and without or with stimulation of the cells with Poly-IC or LPS. The most consistent effect then observed was overall down-regulation of expression of the signalling molecules analysed after 2 hours treatment with CSE (Table 5.2). Since the majority of the signalling molecules assessed are associated with M2 macrophages, this would suggest that CSE promotes a bias away from M2 and towards M1 function.

Fewer significant effects were observed after 24 or 48 hours of culture and those that did occur were mainly associated with the presence of nicotine alone in the cultures,

rather than CSE. This suggests that the effects of CSE ameliorate after its initial acute effects and/or the macrophages are able to re-equilibrate their signalling networks.

To complement the RPPA data on the effects of CSE and nicotine on signalling molecules, analysis of the changes in IL-10 and TNF-α expression was carried out using ELISA to further investigate whether there was an increase in M1 or M2 macrophages after the different treatments. IL-10 is a major immunosuppressive cytokine, which has a major role in limiting the extent of the activation of both the innate and the adaptive immune cells to maintain a homeostatic state (Cao *et al.*, 2006). TNF-α has been shown to play a pivotal role in orchestrating the cytokine cascade in many inflammatory diseases as it is a "master-regulator" of inflammatory cytokine production, with macrophages being both the major producers of and highly responsive to TNF-α (Salari *et al*., 2013). IL-10 is principally produced by M2 macrophages, and TNF-α by M1 macrophages (Martinez and Gordon, 2014).

The results of the ELISA showed the different treatments used did produce some significant differences in the expression levels of IL-10 and TNF-α. Poly-IC and LPS both significantly enhanced TNF-α and IL-10 expression compared to unstimulated macrophages. TNF-α expression was generally increased by all CSE treatments and some nicotine treatments, whereas IL-10 expression was increased only after treatment with 1 % CSE for 24 h and 3% CSE for 48 h, but IL-10 expression was suppressed after 48 h with all nicotine treatments.

Overall, the most consistent findings from the RPPA data and the ELISA data are that CSE induced overall down-regulation of the signalling molecules at 2 hours, and that CSE up-regulated TNF-α production at 24 and 48 hours (Tables 5.2 and 5.4). Since the majority of the signalling molecules analysed were M2-related, and TNF-α is predominantly produced by M1 macrophages, these findings support the idea that CSE suppresses M2 function and favours M1 activation. Although the effects of CSE on the signalling molecules ameliorated by 24 hours of treatment, the early effects observed at 2 hours of treatment might trigger the intra-cellular processes resulting in TNF-α production at 24-48 hours.

The effects of nicotine on global expression of the signalling molecules was less consistent than that of CSE (Table 5.2). However, with respect to cytokine production, nicotine again up-regulated TNF-α production in certain conditions, and down-regulated IL-10 production (Table 5.4). As with CSE, this is also consistent with nicotine promoting a general shift towards M1 function.

When comparing these results to other studies that have investigated the effects of CSE and nicotine on different cell types, there are both similarities and differences. For example, others have reported that CSE promotes TNF-a production by the human monocytic U937 cell line (Demirjian *et al*., 2006). In a study conducted on human monocyte-derived DCs there was significant down-regulation by CSE and nicotine of the signalling molecules and other proteins investigated (Alkhattabi, 2018). However, the present results are different to those investigating the effects of nicotine on antimicrobial activity and cytokine responses of alveolar macrophages when exposed to *Legionella pneumophila*, which found reduced production of TNF-α linked to nicotine-induced impairment of antimicrobial activity (Matsunaga *et al*. 2001), which fits with other studies that have shown nicotine suppresses TNF-α expression in PBMC and THP-1 cells (Ouyang *et al*., 2000; Ween *et al*., 2017). Our results for IL-10 fit with previous studies that have shown nicotine can both increase IL-10 expression in naive mouse macrophages and activated monocytes and suppress expression in the U937 human monocytic cell line (Wolk *et al*., 2000; Bai *et al*., 2017; Rioux & Castonguay, 2001). The differences between these results and the results of this study may be due to the populations of macrophages in these studies being different, which would mean the reaction to the treatments used could vary.

It is clear that CSE and nicotine exert somewhat different effects. As mentioned previously, the other components in CSE can produce a different effect than nicotine alone; a study of nicotine and CSE reinforcing properties in rats used ligand binding autoradiography to investigate the affinity of CSE and nicotine for monoamine oxidase (MAO), an enzyme that breaks down serotonin, norepinephrine and dopamine, which is strongly inhibited in smokers (Costello *et al*., 2014). They found that CSE, but not nicotine, significantly reduced the activity of MAO-A and MAO-B (Costello *et al*., 2014). This could mean that the CSE could be affecting other markers that were not

investigated in this study, or the combined effects of the nicotine and the rest of the components in the CSE mean the overall changes seen are different to those of nicotine alone.

A recent study into the expression of M1 and M2 macrophages found that not all the macrophages in the body can be divided into M2 or M1 macrophages; for AM (obtained by BAL) in a steady state the majority of the population expressed a dual M1/M2 phenotype (Mitsi *et al*., 2018). This phenotype may be helpful to maintain a balance between protective immunity and immune tolerance in the alveolar space, as it can allow a quick and adaptive response to multiple elements in the local environment (Mitsi *et al*., 2018). Our predominant finding that CSE up-regulates M1 function raises the possibility that smoking promotes an inflammatory environment in the tissues of the lung. Consistent with this, others have recently shown that, compared to healthy non-smokers, smokers with normal lung function (and COPD patients) have increased numbers of M1 macrophages in the tissues of the small airways (Suji Eapen *et al*., 2017). However, the same study showed increased numbers of M2 macrophages in the bronco-alveolar lavage of smokers and COPD patients: this is consistent with some of the mixed results we observed for the changes in both signalling molecules and cytokine production.

5.3.1. Conclusions

This study indicates that CSE predominantly promotes M1 macrophage function. However, some of the results with CSE, and still more so with nicotine, also provide evidence for the promotion of M2 function in some conditions. These *in vitro* observations are consistent with *in vivo* findings that smoking differentially promotes M1 and M2 macrophage function in different lung compartments, or the generation of macrophages with a mixed M1/M2 phenotype.

6. General discussion

6.1. Discussion

COPD is a chronic and complex disease that affects many people all around the world (Price *et al*., 2011; Rab *et al*., 2013). The severity of COPD ranges from mild to severe, with different levels of bronchitis and emphysema observed in patients, and as the disease progresses there are increasing death rates. There are many risk factors that are involved in the development of COPD, however smoking cigarettes carries the highest associated risk for all forms of the disease (Kotz *et al*., 2014). Constituents of cigarette smoke are the main cause of COPD, and the progression of COPD is related to numerous changes in the immune system (Sharma *et al*., 2009). The overreaction of immune and epithelial responses can play a negative role associated with COPD development; so, ongoing research is trying to understand the role of the immune system in COPD and our interest is focused on the role of macrophages in this disease (Taylor, 2010; Sharma *et al*., 2009). As antigen presenting cells, macrophages play an important role not only in innate immunity, but also in the adaptive response of the immune system (Tetley, 2002; Pappas *et al.,* 2013). By understanding the relation of macrophages with other cells, this research might help to provide alternative and more effective plans for therapeutic approaches to limit the progressions of COPD or eliminate it.

Several studies have been carried out to evaluate the function of macrophages in COPD, but the results are not consistent (White *et al.,* 2003). This is mainly due to the fact that the importance of macrophages in COPD is not limited to their role in fighting the disease, but they also have a role in increasing the severity of the disease, as chronic accumulation of macrophages at sites of inflammation is consistent with the chronicity and the gradual development of COPD (Tetley, 2002). Macrophages have been shown to recruit inflammatory cells to the lungs and release proteases that cause some of the damage seen in COPD (for example alveolar destruction and mucus hypersecretion) (White *et al.,* 2003).

There is also evidence that macrophages are ultimately changed because of the continuous activation by chemokines and cytokines during the progression of the disease (Russell *et al.*, 2002). Hodge *et al*. (2003) showed that macrophages from COPD patients had a decreased phagocytic ability, therefore, they proposed that this response would be partly related to the pathogenesis of COPD due to the accumulation of apoptotic epithelial cells. Studies showed that the number of apoptotic epithelial cells is higher in COPD patients than in healthy smokers and non-smokers, which is consistent with their role in COPD development (Hodge *et al*., 2003). It has been suggested that it is through secreting a number of proteins that activate neutrophilic inflammation (TNF-α, IL-8, and leukotriene B4) that macrophages mediate their function in COPD (Barnes, 2004). These studies suggest more research is needed to identify what activates the macrophages, their possible role in the development of COPD, and the mechanism of their interaction with other immune cells in this disease.

Studies have shown that smoking cigarettes can significantly increase the risk of not only COPD, but other diseases as well, including Crohn's disease, tuberculosis, rheumatoid arthritis, pneumonia, cancer (mouth, lung, larynx, oesophagus and bladder), microbial infections (bacterial meningitis, periodontitis and respiratory infections) and heart disease (atherosclerosis, coronary heart disease, aneurysms and high blood pressure), and smoking has also been linked with delayed recovery from injuries (Sopori, 2002; Ji *et al*., 2017; Ween *et al*., 2017). After chronic use CS can exert its adverse effects by altering a large number of immunological functions, both in the adaptive and innate immune response (Sopori, 2002; Ween *et al*., 2017). For example, CS greatly increases the number of AMs; this can result in increased concentrations of elastase and lysosomal enzymes, which would damage lung parenchymal cells and connective tissue, and the AMs also produce increased levels of MMP activity and oxygen radicals which are important for killing intracellular pathogens (Sopori, 2002). Despite the increases in MMP activity and oxygen radicals, studies have shown AMs from smokers are functionally impaired with changes to their physiology, morphology, enzyme activity and biochemistry (Sopori, 2002). This can cause lower levels of proinflammatory cytokines to be secreted, which means they have cannot kill and/or phagocytose bacteria such as *Pseudomonas aeruginosa*, *Listeria monocytogenes* and

Staphylococcus aureus as easily, also there is inhibition of their early response to pathogens and up-regulation of local host defences, and a greater susceptibility to viruses such as sarcoma and influenza (Sopori, 2002; Ween *et al*., 2017).

There is further evidence of the role of CS in lung cancer, as studies have shown that some of the substrates of CYP2A13 in CS such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, pyrene, 1-nitropyrene, 1-acetylpyrene and 1-hydroxypyrene can be metabolically activated by CYP2A13 and are associated with an increased incidence of lung cancer, and 2-amino-3-methylimiidazo [4,5-f] quinolone has also been shown to be associated with lung cancer in mice (Ji *et al*., 2017).

Nicotine is considered to be a safer alternative to tobacco; it accounts for 95 % of the total alkaloid content in tobacco, and when it is a high alkaline pH it becomes non-ionised, meaning it is readily absorbed across the oral mucosa, skin, nose, and the epithelium of the lungs (Ji *et al*., 2017; Mishra *et al*., 2015). Nicotine is then metabolised by CYP2A13 and other related enzymes in the lung and liver to produce different metabolites which can affect different biological processes (Ji *et al*., 2017). Nicotine has been shown to affect all systems of the body with increased risk of respiratory, gastrointestinal and cardiovascular disorders, with a decreased immune response, and adverse effects on the kidneys and reproductive system (Mishra *et al*., 2015). Even though nicotine is not generally considered to be a carcinogen, there are an increasing number of studies that disagree (Mishra *et al*., 2015; Czyżykowski *et al*., 2016).

Nicotine has been shown to be involved in cancer and can act through suppression of apoptosis, promotion of angiogenesis, proliferation, migration and invasion of cancer cells (Czyżykowski *et al*., 2016; Mishra *et al*., 2015). Nicotine can act through membranous nicotinic acetylcholine receptors, leading to activation of intracellular pathways including RAS/RAF/MEK/ERK and JAK/STAT, with increased NF-κB activity, stimulation of pro-angiogenic factors, increased transcription of mitogenic promoters, and inhibition of the mitochondrial death pathway (Czyżykowski *et al*., 2016). Nicotine can also affect oxidative stress, cell proliferation, and DNA mutation (Mishra *et al*., 2015).

One of the approaches to understand the functions of macrophages is to use *in vitro* cell lines as model systems, as these allow researchers to manipulate the interactions that macrophages have with their environment in general and with other immune cells (Tsuchiya *et al.,* 1980). A widely-used cell line in the laboratory is THP-1, which is a human pro-myeloid cell line developed by Tsuchiya *et al*. (1980) that is responsive to external factors and sensitive to culture conditions. PBMCs are a convenient model of human primary monocyte–macrophages that can easily be manipulated and their interactions with the environment and other immune cells studied; this made them a suitable alternative to the THP-1 cells which produced inconsistent results (Slota *et al*., 2011).

The aims of the project were to investigate the effects of cigarette smoke on macrophages, initially, using THP-1 cells then later with PBMCs. The cells were initially cultured and differentiated with PMA, and stained for CD markers specific for monocytes and/or macrophages to confirm the identity of the cells by flow cytometry, before deciding whether to use them in further studies exposing them to CSE to investigate the changes to macrophages in COPD. The PBMC-derived macrophages where chosen for further study, so they were exposed to CSE and nicotine in combination with Poly-IC and LPS and any changes in CD markers were measured by flow cytometry. The PBMC-derived macrophages were then investigated for changes in different cell surface markers that are key regulators of macrophage function. The markers were selected based on their function and association with either the M1 or the M2 macrophage phenotype, as changes in expression of these markers could help to clarify how CSE and nicotine affect macrophage regulation and function.

The initial studies into the THP-1 cell line showed there were some similarities with previous studies, but there were also some differences in expression of the markers investigated when compared to the literature values; the results showed that some monocyte markers were still found among the differentiated macrophages, and some macrophage markers were expressed on THP-1 cells without stimulation (Park *et al*., 2007; Antal-Szalmas *et al*., 1997; Mittar, Paramban and McIntyre, 2011). Some studies have suggested that the THP-1 cell line can be used successfully for studying more general immune mechanisms *in-vitro* (Park *et al*., 2007), whereas others have found

variability in the THP-1 results making them un-reliable for further study (Vaddi and Newton, 1994; Markotic *et al.,* 2007). Overall, these results together with reports in the literature that THP-1 cells are of limited value as a model of primary human monocytes and macrophages, suggests that further study in this cell line would be of little use. Therefore, another type of cell, PBMCs, were investigated for their suitability for use in studying the effects of CSE (Chapter 4).

On day 0 both CD14 negative and CD14 positive PBMC cells were tested for macrophage cell surface markers and our results fit with previous studies suggested that on day 0 the CD14 positive cells were highly enriched and cells collected were monocytes, which was as expected (Khazen *et al.*, 2005). Subsequently, these monocytes were cultured for 7 days in the presence of M-CSF (Gleissner, 2012) and then treated with the relevant combination of LPS, Poly-IC, CSE and nicotine for a further 24 h. Cells were then harvested before staining for expression of CD14, CD16, CD206 and CD11b and analysed by flow cytometry.

The flow cytometry results for PBMC showed that there was statistically significant reduction in CD206 expression, which is consistent with these stimulators pushing the phenotype of the day 8 M0 macrophages towards a M1 phenotype, which would have reduced CD206 expression. Other studies of cell surface marker expression have shown similar results with a reduction in expression of CD206 on M1 macrophages compared to M0 or M2 macrophages and a reduced expression of CD206 in AM from COPD patients (Lathrop *et al.*, 2015; Jubrail, 2017; Yamasaki & van Eeden, 2018; Dewhurst *et al*., 2017). There was also a suppression of CD14 expression by nicotine with or without Poly-IC, and without LPS, which is consistent with other studies that show CD14 is down-regulated after M-CSF stimulation and in M2 type macrophages (Gleissner, 2012; Lathrop *et al.,* 2015). These studies also showed that CD16 followed the same pattern of expressions for the different macrophage types, high for M0 and M1 and low for M2, which means that the pattern of reduced CD16 expression seen in our results would fit with the different treatment conditions pushing the macrophages to an M2 phenotype, but as the results were not significant, no firm conclusions can be drawn (Lathrop *et al.*, 2015). Other studies on the effect of CS have shown an increase in the number of M2 AM in a mouse model (He *et al*., 2017). The results for CD206

were not consistent with the CD14 results; studies have shown that there are lower levels of CD206 expression in M0 and M1 macrophages and a higher level of expression in M2 macrophages, which could suggest that the treatments are producing a more complicated response than just an increase in macrophages with an M2 phenotype (Lathrop *et al.*, 2015).

This study also investigated the novel effect of using the different treatments of HDMS on the number of collected cells and maintenance of the phenotype of differentiated macrophages. The optimal concentration of HDMS was determined (1 % HMDS in 100 % Propanol), the flow cytometry results confirmed the presence of mature macrophages and the cell yield showed HDMS increased the number of recovered macrophages.

These results show that PBMC-derived macrophages are suitable for use to detect differences in cell surface markers after treatment with Poly-IC, LPS, CSE and nicotine, therefore they were used in the RPPA studies to further investigate the effect of CSE and nicotine on the pathways involved in macrophage signalling.

RPPA, which is essentially a high-throughput "dot blot", has been shown to be a much more sensitive technique for detecting changes in the expression level of proteins than other commonly used techniques such as ELISA (Lu *et al*., 2016). RPPA has other advantages such as its sample preparation is similar to Western blot meaning it is familiar for most labs, it is quantitative, it allows many samples to be analysed simultaneously as more than 1000 can be assayed on a single array; different samples types can be analysed including tissues, cell lines, plasma, or serum; it requires a small sample volume - only nano-litre quantities meaning samples from needle or core biopsies or laser captures micro-dissected tumours can be analysed; serial dilution across the slide allows proteins with different levels of expression to be detected, which means quantification can be accurate; the fast extraction and denaturing of proteins decreases degradation, and RPPA complements other techniques such as mass spectrometry for measuring total protein and phosphoprotein, as long as there are high quality antibodies available (Tibes *et al.*, 2006; Mueller, Liotta and Espina, 2010; Lu *et al*., 2016; Boellner & Becker, 2015).

After the initial analyses of the RPPA data for the individual signalling molecules, the results did not show any clear significant changes associated with CSE or nicotine treatment, therefore, the data was re-analysed following subtraction of all the relevant blank values from the corresponding values for the signalling molecules expression; the median values were calculated for each signalling molecule for the 8 individual donors, then the changes across all signalling molecules were calculated, with CSE or nicotine treatments compared to no CSE or nicotine, and LPS and Poly-IC treatments compared to no LPS or Poly-IC. The results were then combined to allow the global effects of these treatments on the selected signalling molecules to be analysed. The clearest effects of CSE or nicotine were seen after 2 h for all treatments, with either up regulation of expression for the markers after treatment with nicotine in combination with LPS or Poly-IC, or down regulation of the markers after treatment with nicotine only and for all the CSE treatments. After 24 and 48 h exposure, the results were less clear and significant, after 24 h there was generally some enhancement of the expression of the signalling molecules, but after 48 h of treatment there were no consistent effects produced. Overall, this suggests that the macrophages re-equilibrate during chronic CSE exposure to find the balance between M1 and M2 type macrophages that best deals with the effects of the CSE or nicotine exposure. However, the acute effects of CSE suggested that it promoted an M1 bias since the majority of the signalling molecules investigated were associated with M2 function, and were down-regulated by CSE after 2hours exposure.

The effect on markers was further analysed by investigating the change in expression of IL-10 and TNF-α expression using ELISA. The results showed Poly-IC and LPS both significantly enhance TNF-α and IL-10 expression in all conditions, TNF-α expression was generally increased after all CSE treatments and some nicotine treatments, whereas IL-10 expression was increased only after treatment with 1 % CSE for 24 h and 3% CSE for 48 h, but IL-10 expression was suppressed after 48 h treatment with all nicotine treatments. Overall, both the RPPA signalling molecule results and ELISA cytokine results are consistent with CSE and nicotine promoting an M1 phenotype over an M2 phenotype, although the latter may also be favoured in certain conditions.

Our results that show an increased in the M1 profile fit with other studies that have shown that smoking and CS can trigger inflammation and oxidative stress, which results in an increase in M1 macrophages (Bhowmik *et al*., 2005). CS exposure in COPD associated macrophages, including alveolar macrophages, results in increased secretion of inflammatory mediators, such as TNF-α, ROS, and proteolytic enzymes (MMPs), which agrees with the changes seen in our study (Colarusso *et al*., 2017).

Other studies into the effect of smoking and COPD on macrophages that have also found an increase in M2 transcription, due to deficiencies in the processes that switch off inflammation and restore lung homeostasis in patients with COPD who smoke, and there are increased levels of arginase-1 (expressed by M2 macrophages) in the airways of COPD patients, leading to exacerbations and worsened lung functions (Bhowmik *et al*., 2005; Pires *et al*., 2012; Vlahos and Bozinovski, 2014). Our results are also supported by other studies that have shown that CS led to an increased AMs in the M2 gene profile, with this occurring more in smokers with a normal lung function than in smokers with COPD (Shaykhiev *et al*., 2009). Overall, these studies suggest that an increase in M2 activity is detrimental to patients with COPD.

When comparing these results to other studies that have investigated the effects of CSE and nicotine on different cell lines, there are clear differences; studies have found significant inhibition of cell markers in monocyte-derived DCs, TNF-α was down-regulated in alveolar macrophages when exposed to *Legionella pneumophila*, and in PBMC and THP-1 cells (Alkhattabi, 2018; Matsunaga *et al*. 2001; Ouyang *et al*., 2000; Ween *et al*., 2017). Our results for IL-10 fit with some previous studies that have shown nicotine can both increase IL-10 expression in naive mouse macrophages and activated monocytes and suppress expression in the U937 human macrophage cell line (Wolk *et al.*, 2000; Bai *et al*., 2017; Rioux & Castonguay, 2001). Other studies have shown that LPS stimulation of PBMCs can alter cell surface markers, which was also seen in this study; LPS stimulation produced a reduction in IL-10 expression, which is similar to our results, but there was also a reduction in TNF-α expression, which was not seen in our study (Wolk *et al*., 2000).

The apparent contradiction between studies indicating either that CS favours either M1 or M2 macrophages may be partly answered by a study that has recently shown that, compared to healthy non-smokers, smokers with normal lung function (and COPD patients) have increased numbers of M1 macrophages in the tissues of the small airways, but increased numbers of M2 macrophages in the bronco-alveolar lavage (Suji Eapen *et al*., 2017). : This is also consistent with some of the mixed results we observed for the changes in both signalling molecules and cytokine production. This result is further supported by studies that have also shown that CS can induce both an increase in pro-inflammatory macrophages (M1) and also an increase in M2 macrophages, with no predominance of either type, which suggests smoking induced a complex immune response in the lung, with tissue remodelling (Nurwidya *et al*., 2016; Hiemstra, 2013).

A study of the effect on differentiated THP-1 macrophages of nicotine and other E-cigarette components on proinflammatory cytokine secretion, phagocytosis, and phagocytic recognition molecule expression found nicotine decreased phagocytosis by 15.26 %; there was reduced expression of the phagocytic recognition molecules TLR-2 and SR-A1; there was increased IL-8 expression, while IL-6, IL-1b, TNF-α, MCP-1MIP-1a, and MIP-1b expression was reduced, which is different from our results (Ween *et al*., 2017). These changes suggest nicotine caused dysfunction in macrophage phagocytosis, as well as altering cytokine secretion pathways and phagocytic recognition receptors (Ween *et al*., 2017). Nicotine has also been reported by some to reduce TNF-α and IL-1b expression in PBMCs, and reduce the expression of other cytokines including MCP-1 and IL-6 in BALF of mice (Ouyang *et al*. 2000; Lerner *et al.* 2015; Sussan *et al*. 2015).

Further evidence for the effect of nicotine on autophagy comes from a study which showed nicotine impaired macrophage related killing of *Mycobacterium tuberculosis*, by inhibiting autophagosome formation in infected primary murine alveolar macrophages and THP-1 cells, and increasing THP-1 cell migration, which reflects the increased numbers of macrophages seen in the lungs of smokers (Bai *et al*., 2017). In this study nicotine also increased expression of TGF-β from Tregs and increased IL-10 expression in naive mouse macrophages co-cultured with nicotine-exposed Tregs, which is similar to our results and suggested nicotine plays an important role in

subverting macrophage control during *Mycobacterium tuberculosis* infection (Bai *et al*., 2017).

The initial analyses of the RPPA data for the individual signalling molecules did not show any clearly significant changes and some of the expression values for the signalling molecules were very similar to the blank controls, which suggests that there was either low expression of these signalling molecules, or there were technical issues with the RPPA. This fits with other studies that have shown that there are some key challenges with RPPA, including the level of sensitivity, specificity, spatial resolution, tissue sample preparation methods, and quantification and quality control; the conversion of the serial dilutions into a single relative concentration for each sample can be challenging, but this can be overcome by determining the amount of assayable protein in each spot by calculating the median of a large number of antibodies using analysis software such as RPPanalyzer or SuperCurve (Lu *et al.*, 2016; Solier & Langen, 2014). To help with the problem of limited specific antibodies, an open access antibodypedia-website (http://www.antibodypedia.com) has been created were antibodies that detect proteins of the human proteome have been evaluated and listed for others to use, but there are still some key proteins that are missing highly specific antibodies (Boellner & Becker, 2015). Sensitivity is also an issue as the complexity of biological mixtures, means there is a limit to the detection sensitivity of low-abundant proteins, meaning the RPPA assay may not be able to detect them accurately, which can lead to substantial variability between samples (Solier & Langen, 2014; Aziz, 2015; Lu *et al*., 2016). Sample preparation and other pre-analysis challenges can also influence the accuracy of RPPA results, as the processing, handling and storage of samples can affect the levels of detection (Aziz, 2015; Lu *et al*., 2016; Boellner & Becker, 2015).

There are other assays that could have been used in this study to help elucidate the changes caused by CSE and nicotine. For example, a phagocytosis assay, such as the one described by Ween *et al*. (2017) to detect the effect of E-cigarette components (including nicotine) on THP-1 cells, as previous studies have suggested that nicotine and CSE can affect the phagocytotic ability of macrophages.

CSE and nicotine have also been suggested to affect apoptosis, and there are many different apoptosis assays that can be applied to different studies depending on the study

design and the assay timing; for example, using annexin V and propidium iodide to stain cells for detection of flipping of phosphatidylserine by flow cytometry in early apoptosis, detecting the activity of caspases by using fluorescent substrate probes, or specific antibodies, or by detecting DNA fragmentation caused by apoptosis using DNA ladder assays (Wan *et al*., 2009). Related to apoptosis, another marker that could have been investigated in this study is high mobility group box 1 protein (HMGB1), as its expression has been shown to inhibit macrophage phagocytosis of apoptotic cells, and increased expression is negatively correlated to patients' lung function (Colarusso *et al*., 2017).

Another aspect that could have been investigated in this study was the effect of CSE and nicotine on the ability of macrophages to act as APCs by studying the expression of MHC-bound antigenic peptides that APCs use to activate T cells, such as the costimulatory B7 molecules CD86, CD80 which engage T-cell CD28; the adhesion molecules CD58 and CD54 which are involved in direct co-stimulation of T cells (Wolk *et al*., 2000).

One way to mimic the action and activation of macrophages *in vivo* is to use stem cells, for example induced pluripotent stem cells (iPSCs) or hematopoietic stem cells (Takata *et al*., 2017). Tissue macrophages are formed from yolk-sac progenitors during embryogenesis to form primitive yolk-sac macrophages, and studies have shown that iPSCs can be driven to undergo a process similar to this *in vitro*, after which they can be stimulated to terminally differentiate into specialized macrophages including alveolar macrophages (Takata *et al*., 2017). Studies have shown the potential for CSE to affect stem cells, for example low concentrations of CSE impaired cell migration, and downregulated key interleukins in mesenchymal stem cells (Wahl *et al*., 2016).

6.1.1. Conclusions

Macrophages have been shown to be important in many diseases, including COPD, and despite their role generally being a protective one, studies have shown that macrophages have a deleterious effect in COPD (Tetley, 2002). Some studies have suggested this arises due to an increase in the number of AMs with an M2 profile in COPD patients,

which can result in increased tissue damage and fibrosis (Vlahos and Bozinovski, 2014). As the relative roles of M1 and M2 macrophages are not fully understood, macrophages may be involved in both fighting and inducing COPD and other diseases, such as Crohn's disease, tuberculosis, rheumatoid arthritis, pneumonia, and cancer (Vlahos and Bozinovski, 2014; Sopori, 2002; Ji *et al*., 2017; Ween *et al*., 2017). Studies have also highlighted the deleterious effect of CS and nicotine through altering a large number of immunological functions, both in the adaptive and innate immune response (Sopori, 2002; Ween *et al*., 2017).

This study aimed to establish an *in vitro* model to study the effect of CSE on macrophage function. The initial experiments in the THP-1 cell line showed that these cells still had monocyte markers present after differentiation into macrophages; conversely, some of the macrophage markers were present before stimulation, and there were also inconsistencies in marker expression compared to the literature. Overall, these results meant that the THP-1 cell line was not a suitable choice for investigating how macrophages are affected by CSE or nicotine, therefore, PBMCs were chosen as an alternative.

In this thesis, a procedure was established to generate macrophages from $CD14^+$ monocytes isolated by positive selection and stimulated to mature using M-CSF. Our results showed that the monocytes isolated from buffy coats expressed the appropriate cell surface markers of differentiated macrophages and coating tissue culture plates with HMDS achieved high numbers of cells recovered with no effect on the expression of cell surface markers. The investigation into the effects of different combinations of CSE, nicotine, Poly-IC and LPS suggested these treatments can influence the expression of CD cell surface markers, with suppression of CD14 and CD206 expression. This suggested there were changes in both M2 and M1 macrophages, which shows that the response to the treatments used may be multi-factorial, and this could also cause detrimental effects in the lungs.

To further investigate the effects of these treatments on macrophages, the expression of markers that are key regulators of macrophage function and associated with either the M2 (mainly) or the M1 macrophage phenotype were investigated in PBMCs by RPPA. As the initial analysis did not produce clearly significant differences for the different

treatments, the data were re-analysed (after subtraction of background readings) to examine the global effects on all signalling molecules after the different treatments. The re-analysed results showed significant differences after 2 h treatment, with up or down regulation of the expression of the signalling molecules depending on CSE or nicotine, with or without Poly-IC or LPS stimulation, and a lesser effect of CSE or nicotine after chronic exposure for 24 h or 48 h. To help clarify these results, ELISA was used with two markers to try and distinguish the M1 and M2 regulation. The results showed that both CSE and nicotine treatments up-regulated TNF-α (an M1-related cytokine) and that nicotine down-regulated IL-10 (an M2-related cytokine), but that CSE also up-regulated IL-10 production in some circumstances.

These results show some similarities to the results for the CD markers, which suggested both an increase in M1 and M2 macrophage phenotype, which fits with the literature that has shown CSE and smoking can increase both M1 and M2 macrophage phenotypes (Bhowmik *et al*., 2005; Pires *et al*., 2012; Vlahos and Bozinovski, 2014). Therefore, this study provides evidence that CSE and nicotine promote macrophage functions in general, up-regulating the pro-inflammatory M1 function and the anti-inflammatory (and potentially fibrosis-promoting) M2 function, or that the macrophages are expressing a dual M1/M2 phenotype.

6.1.2. Future work

The results of this thesis show that RPPA is a suitable technique to use to detect changes in markers in macrophages, but the results of the study need to be confirmed in a wider range of conditions to see how applicable they are in general to allow conclusions to be drawn about how these markers are involved in COPD progression. To do this healthy smokers and smokers who have developed COPD could be recruited to the study, and blood samples taken. The blood samples would be used to produce monocyte-derived macrophages using the techniques described in this thesis, and then treated with CSE or nicotine with co-treatments of Poly-IC and LPS in the same concentrations used here. Then the samples would be investigated for changes in the same set of markers by RPPA to see if there are any differences between healthy smokers and COPD suffers.

This would help to clarify the role of these markers and how the different macrophage phenotypes may contribute to COPD. By analysing the number, rate and functionality of the macrophages collected from these patients a better understanding of the changes in each pathway should be achieved.

Another key investigation that can be run alongside the RPPA analysis is an examination of the HLA-II typing of all COPD patients and healthy smokers, by using PCR. Many autoimmune diseases can be characterised by their association with particular HLA-II alleles, and it has been suggested that HLA class II allele DRB1*14 may contribute to the pathogenesis of severe COPD with emphysema (Faner *et al*., 2013). Therefore, this study will help to identify if there are specific genes associated with COPD progression in the population of patients examined, and help clarify if there is a link between HLA-II type and emphysema in these patients. The complementary information obtained from RPPA and HLA-typing studies may then help to identify genetic factors that pre-dispose to COPD in relation to the effects of CSE and nicotine on macrophages.

7. References

A Agusti K Donaldson, W. M. and M Cosio, H. (2003) 'Does COPD have an autoimmune component', *Thorax*, 58, p. 832–834.

Aarbiou, J., Rabe, K. F. and Hiemstra, P. S. (2002) 'Role of defensins in inflammatory lung disease', *Ann Med*. 2002/07/11, 34(2), pp. 96–101.

Abboud, R. T. and Vimalanathan, S. (2008) 'Pathogenesis of COPD. Part I. The role of protease-antiprotease imbalance in emphysema', *Int J Tuberc Lung Dis*. 2008/03/29, 12(4), pp. 361–367.

Adler, K. B. and Li, Y. (2001) 'Airway epithelium and mucus: intracellular signaling pathways for gene expression and secretion', *Am J Respir Cell Mol Biol*. 2001/11/06, 25(4), pp. 397–400. doi: 10.1165/ajrcmb.25.4.f214.

Aguilar-Mahecha, A., Cantin, C., O'Connor-McCourt, M., Nantel, A. and Basik, M. (2009) 'Development of reverse phase protein microarrays for the validation of clusterin, a mid-abundant blood biomarker', *Proteome science*. BioMed Central, 7(1), p. 15.

Akbani, R., Becker, K.-F., Carragher, N., Goldstein, T., de Koning, L., Korf, U., Liotta, L., Mills, G. B., Nishizuka, S. S. and Pawlak, M. (2014) 'Realizing the Promise of Reverse Phase Protein Arrays for Clinical, Translational, and Basic Research: A Workshop Report The RPPA (Reverse Phase Protein Array) Society', *Molecular & cellular proteomics*. ASBMB, 13(7), pp. 1625–1643.

Akella, A. and Deshpande, S. B. (2013) 'Pulmonary surfactants and their role in pathophysiology of lung disorders'. NISCAIR-CSIR, India.

Alkhattabi, N., Todd, I., Negm, O., Tighe, P.J. and Fairclough, L.C. (2018). Tobacco smoke and nicotine suppress expression of activating signaling molecules in human dendritic cells. *Toxicology Letters*. 299, 40-46.

Ambarus, C. A., Krausz, S., van Eijk, M., Hamann, J., Radstake, T. R., Reedquist, K. A., Tak, P. P. and Baeten, D. L. (2012) 'Systematic validation of specific phenotypic markers for *in vitro* polarized human macrophages', *J Immunol Methods*. 2011/11/15, 375(1–2), pp. 196–206. doi: 10.1016/j.jim.2011.10.013.

Ambrosi, P. and Becchetti, A. (2013) 'Targeting neuronal nicotinic receptors in cancer: new ligands and potential side-effects', *Recent patents on anti-cancer drug discovery*. Bentham Science Publishers, 8(1), pp. 38–52.

Anderson, A. E. and Foraker, A. G. (1973) 'Centrilobular emphysema and panlobular emphysema: two different diseases 1', *Thorax*, 28(5), pp. 547–550.

Antal-Szalmas, P., Strijp, J. A., Weersink, A. J., Verhoef, J. and Van Kessel, K. P. (1997) 'Quantitation of surface CD14 on human monocytes and neutrophils.', *Journal of leukocyte biology*. Soc Leukocyte Biology, 61(6), pp. 721–728.

Aoshiba, K., Tamaoki, J. and Nagai, A. (2001) Acute cigarette smoke exposure induces apoptosis of alveolar macrophages, American journal of physiology. Lung cellular and molecular physiology, 281, pp. L1392-1401.

Ardito, F., Giuliani, M., Perrone, D., Troiano, G. and Muzio, L. Lo (2017) 'The crucial role of protein phosphorylation in cell signaling and its use as targeted therapy (Review)', *International Journal of Molecular Medicine*. D.A. Spandidos, 40(2), pp. 271–280. doi: 10.3892/ijmm.2017.3036.

Arredouani, M., Yang, Z., Ning, Y., Qin, G., Soininen, R., Tryggvason, K. and Kobzik, L. (2004) 'The Scavenger Receptor MARCO Is Required for Lung Defense against Pneumococcal Pneumonia and Inhaled Particles', in *J Exp Med*, pp. 267–272. doi: 10.1084/jem.20040731.

Al Ashry, H. S. and Strange, C. (2017) 'COPD in individuals with the PiMZ alpha-1 antitrypsin genotype', *European Respiratory Review*. Eur Respiratory Soc, 26(146), p. 170068.

Aziz, N. (2015) 'Measurement of Circulating Cytokines and Immune-Activation Markers by Multiplex Technology in the Clinical Setting: What Are We Really Measuring?', *Forum on immunopathological diseases and therapeutics*, 6(1–2), pp. 19–22. doi: 10.1615/ForumImmunDisTher.2015014162.

Badham, C. (1814) *An essay on bronchitis : with a supplement, containing remarks on simple pulmonary abscess, etc. etc. / by Charles Badham. - Version details - Trove*. 2nd ed. London : printed for J. Callow, medical bookseller.

Bai, X., Stitzel, J. A., Bai, A., Zambrano, C. A., Phillips, M., Marrack, P. and Chan, E. D. (2017) 'Nicotine Impairs Macrophage Control of Mycobacterium tuberculosis', *American Journal of Respiratory Cell and Molecular Biology*. American Thoracic Society, 57(3), pp. 324–333. doi: 10.1165/rcmb.2016-0270OC.

Baraldo, S., Turato, G., Badin, C., Bazzan, E., Beghe, B., Zuin, R., Calabrese, F., Casoni, G., Maestrelli, P., Papi, A., Fabbri, L. M. and Saetta, M. (2004) 'Neutrophilic infiltration within the airway smooth muscle in patients with COPD', *Thorax*. 2004/03/30, 59(4), pp. 308–312.

Barnes, P. J. (2004) 'Mediators of chronic obstructive pulmonary disease', *Pharmacol Rev*. 2004/12/17, 56(4), pp. 515–548. doi: 10.1124/pr.56.4.2.

Barnes, P. J. (2008) 'Immunology of asthma and chronic obstructive pulmonary disease', *Nat Rev Immunol*. 2008/02/16, 8(3), pp. 183–192. doi: 10.1038/nri2254.

Barnes, P. J. (2008) 'The cytokine network in asthma and chronic obstructive pulmonary disease', *The Journal of Clinical Investigation*. 2008/11/05. American Society for Clinical Investigation, 118(11), pp. 3546–3556. doi: 10.1172/JCI36130.

Barnes, P. J. (2009) 'The cytokine network in chronic obstructive pulmonary disease', *Am J Respir Cell Mol Biol*. United States, 41(6), pp. 631–638. doi: 10.1165/rcmb.2009-0220TR.

Barnes, P. J., Shapiro, S. D. and Pauwels, R. A. (2003) 'Chronic obstructive pulmonary disease: molecular and cellular mechanisms', *Eur Respir J*. 2003/10/30, 22(4), pp. 672–688.

Barnes, P. J. and Stockley, R. A. (2005) 'COPD: current therapeutic interventions and future approaches', *Eur Respir J*. 2005/06/03, 25(6), pp. 1084–1106. doi: 10.1183/09031936.05.00139104.

Beck-Schimmer, B., Schwendener, R., Pasch, T., Reyes, L., Booy, C. and Schimmer, R. C. (2005) 'Alveolar macrophages regulate neutrophil recruitment in endotoxin-induced lung injury', *Respir Res*. 2005/06/24, 6, p. 61. doi: 10.1186/1465-9921-6-61.

Beckett, E. L., Stevens, R. L., Jarnicki, A. G., Kim, R. Y., Hanish, I., Hansbro, N. G., Deane, A., Keely, S., Horvat, J. C., Yang, M., Oliver, B. G., van Rooijen, N., Inman,

M. D., Adachi, R., Soberman, R. J., Hamadi, S., Wark, P. A., Foster, P. S. and Hansbro, P. M. (2013) 'A new short-term mouse model of chronic obstructive pulmonary disease identifies a role for mast cell tryptase in pathogenesis', *J Allergy Clin Immunol*. 2013/02/06, 131(3), pp. 752–762. doi: 10.1016/j.jaci.2012.11.053.

Benvenuti, F. (2016) 'The Dendritic Cell Synapse: A Life Dedicated to T Cell Activation', *Frontiers in Immunology*. Frontiers Media S.A., 7, p. 70. doi: 10.3389/fimmu.2016.00070.

Berenson, C. S., Kruzel, R. L., Wrona, C. T., Mammen, M. J. and Sethi, S. (2015) 'Impaired Innate COPD Alveolar Macrophage Responses and Toll-Like Receptor-9 Polymorphisms', *PLOS ONE*. Public Library of Science, 10(9), p. e0134209. doi: 10.1371/journal.pone.0134209.

Bernhard, D., Huck, C.W., Jakschitz, T., Pfister, G., Henderson, B., Bonn, G.K. and Wick, G. (2004) Development and evaluation of an *in vitro* model for the analysis of cigarette smoke effects on cultured cells and tissues, Journal of pharmacological and toxicological methods, 50, pp. 45-51.

Bernhard, W. (2016) 'Lung surfactant: Function and composition in the context of development and respiratory physiology', *Annals of Anatomy-Anatomischer Anzeiger*. Elsevier, 208, pp. 146–150.

Bhowmik, A., Seemungal, T. A., Donaldson, G. C. and Wedzicha, J. A. (2005) 'Effects of exacerbations and seasonality on exhaled nitric oxide in COPD', *Eur Respir J*. 2005/12/02, 26(6), pp. 1009–1015. doi: 10.1183/09031936.05.00047305.

Bianchi, M. E. (2007) 'DAMPs, PAMPs and alarmins: all we need to know about danger', *Journal of leukocyte biology*. Soc Leukocyte Biology, 81(1), pp. 1–5.

BioLegend (2011) *Macrophage Polarization: M1 vs M2 [Online]*. Available at: www.biolegend.com/NewsLegend/022311/index.htm (Accessed: 25 August 2015).

Blander, J. M. and Sander, L. E. (2012) 'Beyond pattern recognition: five immune checkpoints for scaling the microbial threat', *Nat Rev Immunol*. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved., 12(3), pp. 215–225.

Boellner, S. and Becker, K.-F. (2015) ‘Reverse Phase Protein Arrays—Quantitative Assessment of Multiple Biomarkers in Biopsies for Clinical Use’, *Microarrays*. Edited by G. Roth. MDPI, 4(2), pp. 98–114. doi: 10.3390/microarrays4020098.

Bonet, T. (1679) *Sepulchretum sive anatonia pructica ex Cadaveribus Morbo denatis* .

Boorsma Draijer, C., Melgert, B N., C. E. (2013) ‘Macrophage Heterogeneity in Respiratory Diseases’, *Mediators Inflammation* , 2013, p. 769214, 19 pages.

Brandsma, C.-A., Hylkema, M. N., Geerlings, M., van Geffen, W. H., Postma, D. S., Timens, W. and Kerstjens, H. A. M. (2009) ‘Increased levels of (class switched) memory B cells in peripheral blood of current smokers’, *Respiratory Research*, 10(1), p. 108. doi: info:pmid/19909533.

Brebner, J. A., Stockley, R. A., Laurell, Brantly, Fagerhol, Eriksson, Shin, Mahr, Hutchison and Blanco (2013) ‘Recent advances in α-1-antitrypsin deficiency-related lung disease’, *Expert review of respiratory medicine*. Taylor & Francis, 7(3), pp. 213–230.

Briscoe, W. A. and Nash, E. S. (1965) ‘THE SLOW SPACE IN CHRONIC OBSTRUCTIVE PULMONARY DISEASES’, *Ann N Y Acad Sci*. 1965/03/24, 121, pp. 706–722.

Bromberg, J. F., Wrzeszczynska, M. H., Devgan, G., Zhao, Y., Pestell, R. G., Albanese, C. and Darnell, J. E. (1999) ‘Stat3 as an oncogene’, *Cell*. Elsevier, 98(3), pp. 295–303.

Brusselle, G. G., Joos, G. F. and Bracke, K. R. (2011) ‘New insights into the immunology of chronic obstructive pulmonary disease’, *The Lancet*. Elsevier, 378(9795), pp. 1015–1026.

Butcher, E. C. (1990) ‘Warner-Lambert/Parke-Davis Award lecture. Cellular and molecular mechanisms that direct leukocyte traffic.’, *The American journal of pathology*. American Society for Investigative Pathology, 136(1), p. 3.

Calderon-Garciduenas, L., Valencia-Salazar, G., Rodriguez-Alcaraz, A., Gambling, T. M., Garcia, R., Osnaya, N., Villarreal-Calderon, A., Devlin, R. B. and Carson, J. L. (2001) ‘Ultrastructural nasal pathology in children chronically and sequentially exposed to air pollutants’, *Am J Respir Cell Mol Biol*. 2001/02/13, 24(2), pp. 132–138.

doi: 10.1165/ajrcmb.24.2.4157.

Caligiuri, M. A. (2008) 'Human natural killer cells', *Blood*. 2008/07/25, 112(3), pp. 461–469. doi: 10.1182/blood-2007-09-077438.

Cao, S., Zhang, X., Edwards, J. P. and Mosser, D. M. (2006) 'NF-κB1 (p50) homodimers differentially regulate pro-and anti-inflammatory cytokines in macrophages', *Journal of Biological Chemistry*. ASBMB, 281(36), pp. 26041–26050.

Cardinale, A., Nastrucci, C., Cesario, A. and Russo, P. (2012) 'Nicotine: specific role in angiogenesis, proliferation and apoptosis', *Critical Reviews in Toxicology*. Taylor & Francis, 42(1), pp. 68–89. doi: 10.3109/10408444.2011.623150.

Chalmers, G. W., Macleod, K. J., Little, S. A., Thomson, L. J., McSharry, C. P. and Thomson, N. C. (2002) 'Influence of cigarette smoking on inhaled corticosteroid treatment in mild asthma', *Thorax*. BMJ Publishing Group Ltd, 57(3), pp. 226–230.

Chanput, W., Mes, J.J. and Wichers H.J. (2014) THP-1 cell line: an *in vitro* cell model for immune modulation approach. *International Immunopharmacology.* 23, 37-45.

Chen, G. Y. and Nuñez, G. (2010) 'Sterile inflammation: sensing and reacting to damage', *Nature reviews. Immunology*. NIH Public Access, 10(12), p. 826.

Cheng, S.F., X.S. Qin, Z.L. Han, X.F. Sun, Y.N. Feng, F. Yang, W. Ge, L. Li, Y. Zhao, M. De Felici, S.H. Zou, Y. Zhou, W. Shen, Nicotine exposure impairs germ cell development in human fetal ovaries cultured *in vitro*, Aging, (2018).

Chrysofakis, G., Tzanakis, N., Kyriakoy, D., Tsoumakidou, M., Tsiligianni, I., Klimathianaki, M. and Siafakas, N. M. (2004) 'Perforin expression and cytotoxic activity of sputum CD8+ lymphocytes in patients with COPD', *Chest*. 2004/01/14, 125(1), pp. 71–76.

Chung, K. F. (2001) 'Cytokines in chronic obstructive pulmonary disease', *European Respiratory Journal*. 2002/10/24, 34(34 suppl), p. 50s LP-59s.

Cohen, N. A., Zhang, S., Sharp, D. B., Tamashiro, E., Chen, B., Sorscher, E. J. and Woodworth, B. A. (2009) 'Cigarette smoke condensate inhibits transepithelial chloride transport and ciliary beat frequency', *Laryngoscope*. 2009/05/07, 119(11), pp. 2269–2274. doi: 10.1002/lary.20223.

Colarusso, C., Terlizzi, M., Molino, A., Pinto, A. and Sorrentino, R. (2017) 'Role of the inflammasome in chronic obstructive pulmonary disease (COPD)', *Oncotarget*. Impact Journals LLC, 8(47), pp. 81813–81824. doi: 10.18632/oncotarget.17850.

Coleman, M. M., Ruane, D., Moran, B., Dunne, P. J., Keane, J. and Mills, K. H. (2013) 'Alveolar macrophages contribute to respiratory tolerance by inducing FoxP3 expression in naive T cells', *Am J Respir Cell Mol Biol*. 2013/03/16, 48(6), pp. 773–780. doi: 10.1165/rcmb.2012-0263OC.

Cosio, M. G. and Majo, J. (2002) 'Inflammation of the airways and lung parenchyma in COPD: role of T cells', *Chest*. 2002/05/16, 121(5 Suppl), p. 160s–165s.

Costa, C., Rufino, R., Traves, S. L., Lapa, E. S. J. R., Barnes, P. J. and Donnelly, L. E. (2008) 'CXCR3 and CCR5 chemokines in induced sputum from patients with COPD', *Chest*. 2007/10/11, 133(1), pp. 26–33. doi: 10.1378/chest.07-0393.

Costello, M. R., Reynaga, D. D., Mojica, C. Y., Zaveri, N. T., Belluzzi, J. D. and Leslie, F. M. (2014) 'Comparison of the Reinforcing Properties of Nicotine and Cigarette Smoke Extract in Rats', *Neuropsychopharmacology*. Nature Publishing Group, 39(8), pp. 1843–1851. doi: 10.1038/npp.2014.31.

Craig, J. M., Scott, A. L. and Mitzner, W. (2017) 'Immune-Mediated Inflammation in the Pathogenesis of Emphysema: Insights from Mouse Models', *Cell and tissue research*, 367(3), pp. 591–605. doi: 10.1007/s00441-016-2567-7.

Croasdell, A., Duffney, P. F., Kim, N., Lacy, S. H., Sime, P. J. and Phipps, R. P. (2015) 'PPARγ and the innate immune system mediate the resolution of inflammation', *PPAR research*. Hindawi Publishing Corporation, 2015.

Culley, F. J. (2009) 'Natural killer cells in infection and inflammation of the lung', *Immunology*. 2009/09/11, 128(2), pp. 151–163. doi: 10.1111/j.1365-2567.2009.03167.x.

Czyykowski, R., Poowinczak-Przybyek, J. and Potemski, P. (2015) 'Nicotine-induced resistance of non-small cell lung cancer to treatment; possible mechanisms', *Advances in Hygiene & Experimental Medicine/Postepy Higieny i Medycyny Doswiadczalnej*, pp. 186–193.

Daffa, N. I., Tighe, P. J., Corne, J. M., Fairclough, L. C. and Todd, I. (2015) 'Natural and disease-specific autoantibodies in chronic obstructive pulmonary disease', *Clinical and Experimental Immunology*. Oxford, UK: BlackWell Publishing Ltd, 180(1), pp. 155–163. doi: 10.1111/cei.12565.

Daffner, S.D., S. Waugh, T.L. Norman, N. Mukherjee, J.C. France, Nicotine Increases Osteoblast Activity of Induced Bone Marrow Stromal Cells in a Dose-Dependent Manner: An *in vitro* Cell Culture Experiment, Global Spine J, 2 (2012) 153-158.

Daigneault, M., Preston, J. A., Marriott, H. M., Whyte, M. K. and Dockrell, D. H. (2010) 'The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages', *PLoS One*. 2010/01/20, 5(1), p. e8668. doi: 10.1371/journal.pone.0008668.

Davies, L. C., Jenkins, S. J., Allen, J. E. and Taylor, P. R. (2013) 'Tissue-resident macrophages', *Nature immunology*, 14(10), pp. 986–995. doi: 10.1038/ni.2705.

Davis, M. J., Tsang, T. M., Qiu, Y., Dayrit, J. K., Freij, J. B., Huffnagle, G. B. and Olszewski, M. A. (2013) 'Macrophage M1/M2 Polarization Dynamically Adapts to Changes in Cytokine Microenvironments in Cryptococcus neoformans Infection'. doi: 10.1128/mBio.00264-13.

Dehn, S., DeBerge, M., Yeap, X.-Y., Yvan-Charvet, L., Fang, D., Eltzschig, H. K., Miller, S. D. and Thorp, E. B. (2016) 'HIF-2α in Resting Macrophages Tempers Mitochondrial Reactive Oxygen Species To Selectively Repress MARCO-Dependent Phagocytosis', *The Journal of Immunology*. Am Assoc Immnol, 197(9), pp. 3639–3649.

Desmedt, M., Rottiers, P., Dooms, H., Fiers, W. and Grooten, J. (1998) 'Macrophages Induce Cellular Immunity by Activating Th1 Cell Responses and Suppressing Th2 Cell Responses', *The Journal of Immunology*, 160(11), p. 5300 LP-5308.

Dewhurst, J. A., Lea, S., Hardaker, E., Dungwa, J. V, Ravi, A. K. and Singh, D. (2017) 'Characterisation of lung macrophage subpopulations in COPD patients and controls', *Scientific Reports*. London: Nature Publishing Group UK, 7, p. 7143. doi: 10.1038/s41598-017-07101-2.

Doyle, A. G., Herbein, G., Montaner, L. J., Minty, A. J., Caput, D., Ferrara, P. and

Gordon, S. (1994) ‘Interleukin-13 alters the activation state of murine macrophages *in vitro*: comparison with interleukin-4 and interferon-gamma’, *Eur J Immunol*. 1994/06/01, 24(6), pp. 1441–1445. doi: 10.1002/eji.1830240630.

Droemann, D., Goldmann, T., Tiedje, T., Zabel, P., Dalhoff, K. and Schaaf, B. (2005) ‘Toll-like receptor 2 expression is decreased on alveolar macrophages in cigarette smokers and COPD patients’, *Respiratory research*. BioMed Central, 6(1), p. 68.

Duluc, D., Delneste, Y., Tan, F., Moles, M. P., Grimaud, L., Lenoir, J., Preisser, L., Anegon, I., Catala, L., Ifrah, N., Descamps, P., Gamelin, E., Gascan, H., Hebbar, M. and Jeannin, P. (2007) ‘Tumor-associated leukemia inhibitory factor and IL-6 skew monocyte differentiation into tumor-associated macrophage-like cells’, *Blood*. 2007/09/13, 110(13), pp. 4319–4330. doi: 10.1182/blood-2007-02-072587.

Egleton, R. D., Brown, K. C. and Dasgupta, P. (2008) ‘Nicotinic acetylcholine receptors in cancer: multiple roles in proliferation and inhibition of apoptosis’, *Trends in pharmacological sciences*. Elsevier, 29(3), pp. 151–158.

Fadok, V. A., Bratton, D. L., Konowal, A., Freed, P. W., Westcott, J. Y. and Henson, P. M. (1998) ‘Macrophages that have ingested apoptotic cells *in vitro* inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF’, *J Clin Invest*. 1998/03/21, 101(4), pp. 890–898. doi: 10.1172/jci1112.

Fairclough, L., Urbanowicz, R. A., Corne, J. and Lamb, J. R. (2008) ‘Killer cells in chronic obstructive pulmonary disease’, *Clin Sci (Lond)*. 2008/03/14, 114(8), pp. 533–541. doi: 10.1042/cs20070356.

Faner, R., Cruz, T., Casserras, T., López-Giraldo, A., Noell, G., Coca, I., Tal-Singer, R., Miller, B., Rodriguez-Roisin, R., Spira, A., Kalko, S. G. and Agustí, A. (2016) ‘Network Analysis of Lung Transcriptomics Reveals a Distinct B-Cell Signature in Emphysema’, *American Journal of Respiratory and Critical Care Medicine*. American Thoracic Society - AJRCCM, 193(11), pp. 1242–1253. doi: 10.1164/rccm.201507-1311OC.

Feghali-Bostwick, C. A., Gadgil, A. S., Otterbein, L. E., Pilewski, J. M., Stoner, M. W., Csizmadia, E., Zhang, Y., Sciurba, F. C. and Duncan, S. R. (2008) ‘Autoantibodies in

Patients with Chronic Obstructive Pulmonary Disease', *American Journal of Respiratory and Critical Care Medicine*. American Thoracic Society - AJRCCM, 177(2), pp. 156–163. doi: 10.1164/rccm.200701-014OC.

Fishwick, D., Barber, C. M. and Darby, A. C. (2010) 'Chronic Obstructive Pulmonary Disease and the workplace', *Chron Respir Dis*. 2010/02/27, 7(2), pp. 113–122. doi: 10.1177/1479972309354690.

Floreani, A. A., Wyatt, T. A., Stoner, J., Sanderson, S. D., Thompson, E. G., Allen-Gipson, D. and Heires, A. J. (2003) 'Smoke and C5a induce airway epithelial intercellular adhesion molecule-1 and cell adhesion', *Am J Respir Cell Mol Biol*. 2003/04/26, 29(4), pp. 472–482. doi: 10.1165/rcmb.2002-0143OC.

Foey, A. (2012) 'Mucosal macrophages: phenotype and functionality in homeostasis and pathology', in *macrophages: life cycle, functions and diseases*. p. 121–146.

Forrester, M.A., Wassall, H.J., Hall, L.S., Cao, H., Wilson, H.M., Barker, R.N. and Vickers, M.A. (2018). Similarities and differences in surface receptor expression by THP-1 monocytes and differentiated macrophages polarized using seven different conditioning regimens. *Cellular Immunology*. 332, 58-76.

Franke-Ullmann, G., Pfortner, C., Walter, P., Steinmuller, C., Lohmann-Matthes, M. L. and Kobzik, L. (1996) 'Characterization of murine lung interstitial macrophages in comparison with alveolar macrophages *in vitro*', *J Immunol*. 1996/10/01, 157(7), pp. 3097–3104.

Franklin, W. A., Veve, R., Hirsch, F. R., Helfrich, B. A. and Bunn Jr., P. A. (2002) 'Epidermal growth factor receptor family in lung cancer and premalignancy', *Semin Oncol*. 2002/03/15, 29(1 Suppl 4), pp. 3–14.

Fukuchi, Y. (2009) 'The aging lung and chronic obstructive pulmonary disease: similarity and difference', *Proc Am Thorac Soc*. 2009/11/26, 6(7), pp. 570–572. doi: 10.1513/pats.200909-099RM.

Gadgil, A. and Duncan, S. R. (2008) 'Role of T-lymphocytes and pro-inflammatory mediators in the pathogenesis of chronic obstructive pulmonary disease', *Int J Chron Obstruct Pulmon Dis*, 3(4), pp. 531–541.

Gerhart, C. L. G. M. M. D. S. R. E. R. M. N. R. J. M. (2008) 'CIGARETTE SMOKE VAPOR-PHASE EFFECTS IN THE RAT UPPER RESPIRATORY TRACT', *http://dx.doi.org/10.1080/089583798197420*. doi: 13n6cb5wcwucj0kr.

Ghorani, V., Boskabady, M. H., Khazdair, M. R. and Kianmeher, M. (2017) 'Experimental animal models for COPD: a methodological review', *Tobacco Induced Diseases*, 15(1), p. 25. doi: 10.1186/s12971-017-0130-2.

Givi, M. E., Folkerts, G., Wagenaar, G. T. M., Redegeld, F. A. and Mortaz, E. (2015) 'Cigarette smoke differentially modulates dendritic cell maturation and function in time', *Respiratory Research*. London: BioMed Central, 16, p. 131. doi: 10.1186/s12931-015-0291-6.

Gleissner, C. A. (2012) 'Macrophage phenotype modulation by CXCL4 in atherosclerosis', *Frontiers in physiology*. Frontiers Media SA, 3.

Global Initiative for Chronic Obstructive Lung Disease, G. (2004) *The GOLD global strategy for the management and prevention of COPD [Online]*. Available at: http://goldcopd.org (Accessed: 31 August 2015).

Goerdt, S. and Orfanos, C. E. (1999) 'Other Functions, Other Genes: Alternative Activation of Antigen-Presenting Cells', *Immunity*, 10(2), pp. 137–142. doi: https://doi.org/10.1016/S1074-7613(00)80014-X.

Gordon, S. (2008) 'Elie Metchnikoff: father of natural immunity', *Eur J Immunol*. 2008/11/29, 38(12), pp. 3257–3264. doi: 10.1002/eji.200838855.

Gordon, S. and Martinez, F. O. (2010) 'Alternative activation of macrophages: mechanism and functions', *Immunity*. Elsevier, 32(5), pp. 593–604.

Gordon, S., Plüddemann, A. and Martinez Estrada, F. (2014) 'Macrophage heterogeneity in tissues: phenotypic diversity and functions', *Immunological Reviews*. Wiley/Blackwell (10.1111), 262(1), pp. 36–55. doi: 10.1111/imr.12223.

Gordon, S. and Taylor, P. R. (2005) 'Monocyte and macrophage heterogeneity', *Nat Rev Immunol*. 2005/12/03, 5(12), pp. 953–964. doi: 10.1038/nri1733.

Van Gorp, V., Danschutter, D., Huyghens, L., Hachimi-Idrissi, S., Sarkozy, A., Chierchia, G. B., Henkens, S. and Brugada, P. (2010) 'Monitoring the safety of

antiepileptic medication in a child with Brugada syndrome', *International journal of cardiology*. Elsevier, 145(2), pp. e64–e67.

Gosens, R., Zaagsma, J., Meurs, H. and Halayko, A. J. (2006) 'Muscarinic receptor signaling in the pathophysiology of asthma and COPD', *Respiratory Research*, 7(1), p. 73. doi: info:pmid/16684353.

Gosman, M. M. E., Willemse, B. W. M., Jansen, D. F., Lapperre, T. S., van Schadewijk, A., Hiemstra, P. S., Postma, D. S., Timens, W. and Kerstjens, H. A. M. (2006) 'Increased number of B-cells in bronchial biopsies in COPD', *Eur Respir J*. 2006/01/03, 27(1), pp. 60–64. doi: 10.1183/09031936.06.00007005.

Gray, E. E. and Cyster, J. G. (2012) 'Lymph node macrophages', *Journal of innate immunity*. Karger Publishers, 4(5–6), pp. 424–436.

Guan, Y., Ranoa, D. R. E., Jiang, S., Mutha, S. K., Li, X., Baudry, J. and Tapping, R. I. (2010) 'Human TLRs 10 and 1 Share Common Mechanisms of Innate Immune Sensing but Not Signaling', *The Journal of Immunology*, 184(9), pp. 5094–5103. doi: 10.4049/jimmunol.0901888.

Guilliams, M., De Kleer, I., Henri, S., Post, S., Vanhoutte, L., De Prijck, S., Deswarte, K., Malissen, B., Hammad, H. and Lambrecht, B. N. (2013) 'Alveolar macrophages develop from fetal monocytes that differentiate into long-lived cells in the first week of life via GM-CSF', in *J Exp Med*, pp. 1977–1992. doi: 10.1084/jem.20131199.

Günthner, R. and Anders, H.-J. (2013) 'Interferon-regulatory factors determine macrophage phenotype polarization', *Mediators of inflammation*. Hindawi Publishing Corporation, 2013.

Hamm, J. T., Yee, S., Rajendran, N., Morrissey, R. L., Richter, S. J. and Misra, M. (2007) 'Histological alterations in male A/J mice following nose-only exposure to tobacco smoke', *Inhal Toxicol*. 2007/03/17, 19(5), pp. 405–418. doi: 10.1080/08958370601174875.

Hao, N. B., Lu, M. H., Fan, Y. H., Cao, Y. L., Zhang, Z. R. and Yang, S. M. (2012) 'Macrophages in tumor microenvironments and the progression of tumors', *Clin Dev Immunol*. 2012/07/11, 2012, p. 948098. doi: 10.1155/2012/948098.

Hardison, S. E. and Brown, G. D. (2012) ‘C-type lectin receptors orchestrate antifungal immunity’, *Nat Immunol*. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved., 13(9), pp. 817–822.

Hardy, H., Harris, J., Lyon, E., Beal, J. and Foey, A. D. (2013) ‘Probiotics, Prebiotics and Immunomodulation of Gut Mucosal Defences: Homeostasis and Immunopathology’, in *Nutrients*, pp. 1869–1912. doi: 10.3390/nu5061869.

Hashimoto, D., Chow, A., Noizat, C., Teo, P., Beasley, M. B., Leboeuf, M., Becker, C. D., See, P., Price, J., Lucas, D., Greter, M., Mortha, A., Boyer, S. W., Forsberg, E. C., Tanaka, M., van Rooijen, N., Garcia-Sastre, A., Stanley, E. R., Ginhoux, F., Frenette, P. S. and Merad, M. (2013) ‘Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes’, *Immunity*. 2013/04/23, 38(4), pp. 792–804. doi: 10.1016/j.immuni.2013.04.004.

He, S., Xie, L., Lu, J. and Sun, S. (2017) ‘Characteristics and potential role of M2 macrophages in COPD’, *International Journal of Chronic Obstructive Pulmonary Disease*. Dove Medical Press, 12, pp. 3029–3039. doi: 10.2147/COPD.S147144.

Heijink, I. H., Brandenburg, S. M., Noordhoek, J. A., Slebos, D.-J., Postma, D. S. and van Oosterhout, A. J. (2011) ‘Role of aberrant metalloproteinase activity in the pro-inflammatory phenotype of bronchial epithelium in COPD’, *Respiratory research*. BioMed Central, 12(1), p. 110.

Hersh, C. P., DeMeo, D. L. and Silverman, E. K. (2008) ‘National Emphysema Treatment Trial state of the art: genetics of emphysema’, *Proceedings of the American Thoracic Society*. American Thoracic Society, 5(4), pp. 486–493.

Hickman-Davis, J. M., O’Reilly, P., Davis, I. C., Peti-Peterdi, J., Davis, G., Young, K. R., Devlin, R. B. and Matalon, S. (2002) ‘Killing of Klebsiella pneumoniae by human alveolar macrophages’, *Am J Physiol Lung Cell Mol Physiol*. 2002/04/12, 282(5), pp. L944-56. doi: 10.1152/ajplung.00216.2001.

Hiemstra, P. S. (2013) ‘Altered Macrophage Function in Chronic Obstructive Pulmonary Disease’, *Annals of the American Thoracic Society*. American Thoracic Society - AJRCCM, 10(Supplement), pp. S180–S185. doi: 10.1513/AnnalsATS.201305-123AW.

Hodge, S., Hodge, G., Ahern, J., Jersmann, H., Holmes, M. and Reynolds, P. N. (2007) 'Smoking alters alveolar macrophage recognition and phagocytic ability: implications in chronic obstructive pulmonary disease', *Am J Respir Cell Mol Biol*. 2007/07/17, 37(6), pp. 748–755. doi: 10.1165/rcmb.2007-0025OC.

Hodge, S., Hodge, G., Scicchitano, R., Reynolds, P. N. and Holmes, M. (2003) 'Alveolar macrophages from subjects with chronic obstructive pulmonary disease are deficient in their ability to phagocytose apoptotic airway epithelial cells', *Immunol Cell Biol*. 2003/07/10, 81(4), pp. 289–296. doi: 10.1046/j.1440-1711.2003.t01-1-01170.x.

Hodge, S., Matthews, G., Mukaro, V., Ahern, J., Shivam, A., Hodge, G., Holmes, M., Jersmann, H. and Reynolds, P. N. (2011) 'Cigarette Smoke-Induced Changes to Alveolar Macrophage Phenotype and Function Are Improved by Treatment with Procysteine', *American Journal of Respiratory Cell and Molecular Biology. American Thoracic Society - AJRCMB*, 44(5), pp. 673–681.

Hogg, J. C. (2008) 'Lung structure and function in COPD', *Int J Tuberc Lung Dis*. 2008/04/19, 12(5), pp. 467–479.

Hogg, J. C. and Senior, R. M. (2002) 'Chronic obstructive pulmonary disease - part 2: pathology and biochemistry of emphysema', *Thorax*. 2002/08/30, 57(9), pp. 830–834.

Holt, P. G. and Stumbles, P. A. (2000) 'Regulation of immunologic homeostasis in peripheral tissues by dendritic cells: The respiratory tract as a paradigm', *Journal of Allergy and Clinical Immunology*, 105(3), pp. 421–429. doi: 10.1067/mai.2000.105010.

Hong, S. C. and Lee, S. H. (2010) 'Role of Th17 Cell and Autoimmunity in Chronic Obstructive Pulmonary Disease', *Immune Netw*, 10(4), pp. 109–114. doi: 10.4110/in.2010.10.4.109.

Hume, D. A. (2008) 'Macrophages as APC and the Dendritic Cell Myth', *The Journal of Immunology*, 181(9), p. 5829 LP-5835.

Hume, D. A. (2012) *The Biology of Macrophages [Online]*, *The Biology of Macrophages [Online]*.

Hutchinson, J. (1846) 'On the capacity of the lungs, and on the respiratory functions, with a view of establishing a precise and easy method of detecting disease by the

spirometer', *Med Chir Trans*, 29, pp. 137–252.

Improgo, M. R., Tapper, A. R. and Gardner, P. D. (2011) 'Nicotinic acetylcholine receptor-mediated mechanisms in lung cancer', *Biochemical pharmacology*. Elsevier, 82(8), pp. 1015–1021.

Jaguin, M., Houlbert, N., Fardel, O. and Lecureur, V. (2013) 'Polarization profiles of human M-CSF-generated macrophages and comparison of M1-markers in classically activated macrophages from GM-CSF and M-CSF origin', *Cell Immunol*. 2013/03/05, 281(1), pp. 51–61. doi: 10.1016/j.cellimm.2013.01.010.

Janeway, C. A. (1992) 'The immune system evolved to discriminate infectious nonself from noninfectious self', *Immunology today*. Elsevier, 13(1), pp. 11–16.

Jarvis, M. J., Boreham, R., Primatesta, P., Feyerabend, C. and Bryant, A. (2001) 'Nicotine Yield From Machine-Smoked Cigarettes and Nicotine Intakes in Smokers: Evidence From a Representative Population Survey', *JNCI: Journal of the National Cancer Institute*, 93(2), pp. 134–138.

Ji, M., Zhang, Y., Li, N., Wang, C., Xia, R., Zhang, Z. and Wang, S.-L. (2017) 'Nicotine Component of Cigarette Smoke Extract (CSE) Decreases the Cytotoxicity of CSE in BEAS-2B Cells Stably Expressing Human Cytochrome P450 2A13', *International Journal of Environmental Research and Public Health*. MDPI, 14(10), p. 1221. doi: 10.3390/ijerph14101221.

Jia Wang Patrick J. Tighe, Ian Todd, Jonathan M. Corne, Lucy C. Fairclough, R. A. U. (2013) 'Differential Activation of Killer Cells in the Circulation and the Lung: A Study of Current Smoking Status and Chronic Obstructive Pulmonary Disease (COPD)'. doi: 10.1371/journal.pone.0058556.

Jones, R. and Ostrem, A. (2011) 'Optimising pharmacological maintenance treatment for COPD in primary care', *Prim Care Respir J*. 2010/11/26, 20(1), pp. 33–45. doi: 10.4104/pcrj.2010.00069.

Joos, L., Pare, P. D. and Sandford, A. J. (2002) 'Genetic risk factors of chronic obstructive pulmonary disease', *Swiss Med Wkly*. 2002/04/16, 132(3–4), pp. 27–37. doi: 2002/03/smw-09752.

Jubrail, J. (2017) ‘Defective Alveolar Macrophage Function in COPD and Novel Treatments to Restore the Defects’, *EC Pulmonology and Respiratory Medicine*, SI.01, pp. 11–19.

Kang, M.-J., Lee, C. G., Lee, J.-Y., Cruz, C. S. Dela, Chen, Z. J., Enelow, R. and Elias, J. A. (2008) ‘Cigarette smoke selectively enhances viral PAMP–and virus-induced pulmonary innate immune and remodeling responses in mice’, *The Journal of clinical investigation*. Am Soc Clin Investig, 118(8), pp. 2771–2784.

Kapellos, T. S., Bassler, K., Aschenbrenner, A. C., Fujii, W. and Schultze, J. L. (2018) ‘Dysregulated Functions of Lung Macrophage Populations in COPD’, *Journal of Immunology Research*. Hindawi, 2018, p. 2349045. doi: 10.1155/2018/2349045.

Kapoor, N., Niu, J., Saad, Y., Kumar, S., Sirakova, T., Becerra, E., Li, X. and Kolattukudy, P. E. (2015) ‘Transcription factors STAT6 and KLF4 implement macrophage polarization via the dual catalytic powers of MCPIP’, *The Journal of Immunology*. Am Assoc Immnol, 194(12), pp. 6011–6023.

Karayama, M., Inui, N., Suda, T., Nakamura, Y., Nakamura, H. and Chida, K. (2010) ‘ANtiendothelial cell antibodies in patients with copd’, *Chest*, 138(6), pp. 1303–1308.

Kasahara, Y., Tuder, R. M., Taraseviciene-Stewart, L., Le Cras, T. D., Abman, S., Hirth, P. K., Waltenberger, J. and Voelkel, N. F. (2000) ‘Inhibition of VEGF receptors causes lung cell apoptosis and emphysema’, *J Clin Invest*. 2000/12/06, 106(11), pp. 1311–1319. doi: 10.1172/jci10259.

Kawai, T. and Akira, S. (2005) ‘Pathogen recognition with Toll-like receptors’, *Current opinion in immunology*. Elsevier, 17(4), pp. 338–344.

Kawai, T. and Akira, S. (2010) ‘The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors’, *Nat Immunol*. Nature Publishing Group, 11(5), pp. 373–384.

Khazen, W., M’Bika J, P., Tomkiewicz, C., Benelli, C., Chany, C., Achour, A. and Forest, C. (2005) ‘Expression of macrophage-selective markers in human and rodent adipocytes’, *FEBS Lett*. 2005/10/11, 579(25), pp. 5631–5634. doi: 10.1016/j.febslet.2005.09.032.

Kim, H. R., Appel, S., Vetterkind, S., Gangopadhyay, S. S. and Morgan, K. G. (2008) 'Smooth muscle signalling pathways in health and disease', *Journal of Cellular and Molecular Medicine*. Chichester, UK: John Wiley & Sons, Ltd, 12(6a), pp. 2165–2180. doi: 10.1111/j.1582-4934.2008.00552.x.

Kirkham, P. A., Caramori, G., Casolari, P., Papi, A. A., Edwards, M., Shamji, B., Triantaphyllopoulos, K., Hussain, F., Pinart, M., Khan, Y., Heinemann, L., Stevens, L., Yeadon, M., Barnes, P. J., Chung, K. F. and Adcock, I. M. (2011) 'Oxidative Stress–induced Antibodies to Carbonyl-modified Protein Correlate with Severity of Chronic Obstructive Pulmonary Disease', *American Journal of Respiratory and Critical Care Medicine*. American Thoracic Society, 184(7), pp. 796–802. doi: 10.1164/rccm.201010-1605OC.

Klagas, I., Goulet, S., Karakiulakis, G., Zhong, J., Baraket, M., Black, J. L., Papakonstantinou, E. and Roth, M. (2009) 'Decreased hyaluronan in airway smooth muscle cells from patients with asthma and COPD', *Eur Respir J*. 2009/03/14, 34(3), pp. 616–628. doi: 10.1183/09031936.00070808.

Kode, A., Yang, S.-R. and Rahman, I. (2006) 'Differential effects of cigarette smoke on oxidative stress and proinflammatory cytokine release in primary human airway epithelial cells and in a variety of transformed alveolar epithelial cells', *Respiratory research*. BioMed Central, 7(1), p. 132.

Kojima, J., Araya, J., Hara, H., Ito, S., Takasaka, N., Kobayashi, K., Fujii, S., Tsurushige, C., Numata, T., Ishikawa, T., Shimizu, K., Kawaishi, M., Saito, K., Kamiya, N., Hirano, J., Odaka, M., Morikawa, T., Hano, H., Arai, S., Miyazaki, T., Kaneko, Y., Nakayama, K. and Kuwano, K. (2013) 'Apoptosis inhibitor of macrophage (AIM) expression in alveolar macrophages in COPD', *Respir Res*. 2013/03/19, 14, p. 30. doi: 10.1186/1465-9921-14-30.

Kotz, D., Simpson, C. R., Viechtbauer, W., van Schayck, O. C. and Sheikh, A. (2014) 'Development and validation of a model to predict the 10-year risk of general practitioner-recorded COPD', *NPJ Prim Care Respir Med*. 2014/05/21, 24, p. 14011. doi: 10.1038/npjpcrm.2014.11.

Kroening, P. R., Barnes, T. W., Pease, L., Limper, A., Kita, H. and Vassallo, R. (2008)

‘Cigarette Smoke-Induced Oxidative Stress Suppresses Generation of Dendritic Cell IL-12 and IL-23 through ERK-Dependent Pathways’, *The Journal of Immunology*, 181(2), p. 1536 LP-1547.

Krombach, F., Gerlach, J. T., Padovan, C., Burges, A., Behr, J., Beinert, T. and Vogelmeier, C. (1996) ‘Characterization and quantification of alveolar monocyte-like cells in human chronic inflammatory lung disease’, *European Respiratory Journal*, 9(5), p. 984 LP-991.

Krombach, F., Münzing, S., Allmeling, A. M., Gerlach, J. T., Behr, J. and Dörger, M. (1997) ‘Cell size of alveolar macrophages: an interspecies comparison’, *Environ Health Perspect*, 105(Suppl 5), pp. 1261–1263.

Kropski, J. A., Lawson, W. E., Young, L. R. and Blackwell, T. S. (2013) ‘Genetic studies provide clues on the pathogenesis of idiopathic pulmonary fibrosis’, *Disease models & mechanisms*. The Company of Biologists Ltd, 6(1), pp. 9–17.

Kuo, Y.-B., Chang, C. A., Wu, Y.-K., Hsieh, M.-J., Tsai, C.-H., Chen, K.-T., Chen, C.-Y. and Chan, E.-C. (2010) ‘Identification and clinical association of anti-cytokeratin 18 autoantibody in COPD’, *Immunology Letters*, 128(2), pp. 131–136. doi: http://dx.doi.org/10.1016/j.imlet.2009.12.017.

Laennec, R. T. H. (1821) *A treatise on the diseases of the chest (English translation from the French)*. London: Forbes J ed.: T and G Underwood.

Lan, M. Y., Ho, C. Y., Lee, T. C. and Yang, A. H. (2007) ‘Cigarette smoke extract induces cytotoxicity on human nasal epithelial cells’, *Am J Rhinol*. 2007/04/12, 21(2), pp. 218–223.

Lane, N., Robins, R. A., Corne, J. and Fairclough, L. (2010) ‘Regulation in chronic obstructive pulmonary disease: the role of regulatory T-cells and Th17 cells’, *Clinical Science*, 119(2), pp. 75–86. doi: 10.1042/cs20100033.

Lathrop, S. K., Binder, K. A., Starr, T., Cooper, K. G., Chong, A., Carmody, A. B. and Steele-Mortimer, O. (2015) ‘Replication of Salmonella enterica serovar Typhimurium in human monocyte-derived macrophages’, *Infection and immunity*. Am Soc Microbiol, 83(7), pp. 2661–2671.

Leberl, M., Kratzer, A. and Taraseviciene-Stewart, L. (2013) 'Tobacco smoke induced COPD/emphysema in the animal model—are we all on the same page?', *Frontiers in Physiology*. Frontiers Media S.A., 4, p. 91. doi: 10.3389/fphys.2013.00091.

Lee, J. J., Liu, D., Lee, J. S., Kurie, J. M., Khuri, F. R., Ibarguen, H., Morice, R. C., Walsh, G., Ro, J. Y. and Broxson, A. (2001) 'Long-term impact of smoking on lung epithelial proliferation in current and former smokers', *Journal of the National Cancer Institute*. Oxford University Press, 93(14), pp. 1081–1088.

Lee, M. S. and Kim, Y.-J. (2007) 'Signaling pathways downstream of pattern-recognition receptors and their cross talk', *Annu. Rev. Biochem.* Annual Reviews, 76, pp. 447–480.

Lee, S.-H., Goswami, S., Grudo, A., Song, L., Bandi, V., Goodnight-White, S., Green, L., Hacken-Bitar, J., Huh, J., Bakaeen, F., Coxson, H. O., Cogswell, S., Storness-Bliss, C., Corry, D. B. and Kheradmand, F. (2007) 'Antielastin autoimmunity in tobacco smoking-induced emphysema', *Nat Med*. Nature Publishing Group, 13(5), pp. 567–569.

Leidinger, P., Keller, A., Heisel, S., Ludwig, N., Rheinheimer, S., Klein, V., Andres, C., Hamacher, J., Huwer, H., Stephan, B., Stehle, I., Lenhof, H.-P. and Meese, E. (2009) 'Novel autoantigens immunogenic in COPD patients', *Respiratory Research*. BioMed Central, 10(1), p. 20. doi: 10.1186/1465-9921-10-20.

Liao, X., Sharma, N., Kapadia, F., Zhou, G., Lu, Y., Hong, H., Paruchuri, K., Mahabeleshwar, G. H., Dalmas, E. and Venteclef, N. (2011) 'Krüppel-like factor 4 regulates macrophage polarization', *The Journal of clinical investigation*. American Society for Clinical Investigation, 121(7), p. 2736.

Liu, T., Gao, Y.-J. and Ji, R.-R. (2012) 'Emerging role of Toll-like receptors in the control of pain and itch', *Neuroscience Bulletin*, 28(2), pp. 131–144. doi: 10.1007/s12264-012-1219-5.

Liu, Y., Lee, K., Perez-Padilla, R., Hudson, N. L. and Mannino, D. M. (2008) 'Outdoor and indoor air pollution and COPD-related diseases in high-and low-income countries [State of the Art Series. Chronic obstructive pulmonary disease in high-and low-income countries. Edited by G. Marks and M. Chan-Yeung. Number 2 in the series]', *The international journal of tuberculosis and lung disease*. International Union Against

Tuberculosis and Lung Disease, 12(2), pp. 115–127.

Liu, X., Conner, H., Kobayashi, T., Kim, H., Wen, F., Abe, S., Fang, Q., Wang, X., Hashimoto, M., Bitterman, P. and Rennard, S.I. (2005) Cigarette smoke extract induces DNA damage but not apoptosis in human bronchial epithelial cells, *American journal of respiratory cell and molecular biology*, 33, pp. 121-129.

Löfdahl, J. M., Wahlström, J. and Sköld, C. M. (2006), Different inflammatory cell pattern and macrophage phenotype in chronic obstructive pulmonary disease patients, smokers and non- smokers. *Clinical & Experimental Immunology*, 145: 428-437.

Lohmann-Matthes, M. L., Steinmuller, C. and Franke-Ullmann, G. (1994) 'Pulmonary macrophages', *Eur Respir J*. 1994/09/01, 7(9), pp. 1678–1689.

Lu, Y., Ling, S., Hegde, A. M., Byers, L. A., Coombes, K., Mills, G. B. and Akbani, R. (2016) 'Using Reverse-Phase Protein Arrays (RPPAs) as Pharmacodynamic Assays for Functional Proteomics, Biomarker Discovery, and Drug Development in Cancer', *Seminars in oncology*, 43(4), pp. 476–483. doi: 10.1053/j.seminoncol.2016.06.005.

Luppi, F., Aarbiou, J., van Wetering, S., Rahman, I., de Boer, W. I., Rabe, K. F. and Hiemstra, P. S. (2005) 'Effects of cigarette smoke condensate on proliferation and wound closure of bronchial epithelial cells *in vitro*: role of glutathione', *Respiratory Research*, 6(1), p. 140. doi: info:pmid/16309548.

MacNee, W. (2005) 'Pathogenesis of chronic obstructive pulmonary disease', *Proc Am Thorac Soc*. 2005/11/04, 2(4), pp. 251–258. doi: 10.1513/pats.200504-045SR.

Mahadeva, R. and Shapiro, S. (2002) 'Chronic obstructive pulmonary disease • 3: Experimental animal models of pulmonary emphysema', *Thorax*. BMJ Group, 57(10), pp. 908–914. doi: 10.1136/thorax.57.10.908.

Mandel, W. and Cockrill (2013) 'Principles of Pulmonary Medicine E-Book, 6th Edition | Steven Weinberger, Barbara Cockrill, Jess Mandel | ISBN 9781455725342'. s.

Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A. and Locati, M. (2004) 'The chemokine system in diverse forms of macrophage activation and polarization', *Trends Immunol*. 2004/11/09, 25(12), pp. 677–686. doi: 10.1016/j.it.2004.09.015.

Markotić, A., Hensley, L., Daddario, K., Spik, K., Anderson, K. and Schmaljohn, C. (2007) 'Pathogenic hantaviruses elicit different immunoreactions in THP-1 cells and primary monocytes and induce differentiation of human monocytes to dendritic-like cells', *Collegium antropologicum*. Hrvatsko antropološko društvo, 31(4), pp. 1159–1167.

Marti-Lliteras, P., Regueiro, V., Morey, P., Hood, D. W., Saus, C., Sauleda, J., Agusti, A. G., Bengoechea, J. A. and Garmendia, J. (2009) 'Nontypeable Haemophilus influenzae clearance by alveolar macrophages is impaired by exposure to cigarette smoke', *Infect Immun*. 2009/07/22, 77(10), pp. 4232–4242. doi: 10.1128/iai.00305-09.

Martinez, F.O. and Gordon, S. (2014). The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Reports.* 6, 13.

Martinez, F. O., Helming, L., Milde, R., Varin, A., Melgert, B. N., Draijer, C., Thomas, B., Fabbri, M., Crawshaw, A., Ho, L. P., Ten Hacken, N. H., Cobos Jimenez, V., Kootstra, N. A., Hamann, J., Greaves, D. R., Locati, M., Mantovani, A. and Gordon, S. (2013) 'Genetic programs expressed in resting and IL-4 alternatively activated mouse and human macrophages: similarities and differences', in *Blood*. United States, pp. e57-69. doi: 10.1182/blood-2012-06-436212.

Martinon, F., Mayor, A. and Tschopp, J. (2009) 'The inflammasomes: guardians of the body', *Annual review of immunology*. Annual Reviews, 27, pp. 229–265.

Mason, R. J. (2006) 'Biology of alveolar type II cells', *Respirology*. Wiley Online Library, 11, pp. S12–S15.

Massaro, D. and Massaro, G. D. (2004) 'Estrogen regulates pulmonary alveolar formation, loss, and regeneration in mice', *Am J Physiol Lung Cell Mol Physiol*. 2004/08/10, 287(6), pp. L1154-9. doi: 10.1152/ajplung.00228.2004.

Maton, A. (1993) *Human Biology and Health*. 3rd edn. Pearson Prentice Hall, 1997.

Matsunaga, K., Klein, T. W., Friedman, H. and Yamamoto, Y. (2001) 'Involvement of Nicotinic Acetylcholine Receptors in Suppression of Antimicrobial Activity and Cytokine Responses of Alveolar Macrophages to Legionella pneumophila Infection by Nicotine', *The Journal of Immunology*, 167(11),

p. 6518 LP-6524.

Maus, U. A., Koay, M. A., Delbeck, T., Mack, M., Ermert, M., Ermert, L., Blackwell, T. S., Christman, J. W., Schlondorff, D., Seeger, W. and Lohmeyer, J. (2002) 'Role of resident alveolar macrophages in leukocyte traffic into the alveolar air space of intact mice', *Am J Physiol Lung Cell Mol Physiol*. 2002/05/11, 282(6), pp. L1245-52. doi: 10.1152/ajplung.00453.2001.

McKenzie, E. J., Su, Y.-P., Martinez-Pomares, L. and 隈本洋介 (2002) 'The mannose receptor, a bi-functional lectin with roles in homeostasis and immunity', *Trends in Glycoscience and Glycotechnology*. FCCA (Forum: Carbohydrates Coming of Age), 14(79), pp. 273–283.

Medzhitov, R. (2007) 'Recognition of microorganisms and activation of the immune response', *Nature*. Nature Publishing Group, 449(7164), p. 819.

Mian, M.F., Lauzon, N.M., Stampfli, M.R., Mossman, K.L. and Ashkar, A.A. (2008) Impairment of human NK cell cytotoxic activity and cytokine release by cigarette smoke, Journal of leukocyte biology, 83, pp. 774-784.

(2009) Exposure to cigarette smoke suppresses IL-15 generation and its regulatory NK cell functions in poly I:C-augmented human PBMCs, Molecular immunology, 46, pp. 3108-3116.

Michaeloudes, C., Kuo, C.-H., Haji, G., Finch, D. K., Halayko, A. J., Kirkham, P., Chung, K. F. and Adcock, I. M. (2017) 'Metabolic re-patterning in COPD airway smooth muscle cells', *The European Respiratory Journal*. European Respiratory Society, 50(5), p. 1700202. doi: 10.1183/13993003.00202-2017.

Mills, C. D., Kincaid, K., Alt, J. M., Heilman, M. J. and Hill, A. M. (2000) 'M-1/M-2 macrophages and the Th1/Th2 paradigm', *J Immunol*. 2000/06/08, 164(12), pp. 6166–6173.

Minematsu, N., Blumental-Perry, A. and Shapiro, S. D. (2011) 'Cigarette Smoke Inhibits Engulfment of Apoptotic Cells by Macrophages through Inhibition of Actin Rearrangement', *American Journal of Respiratory Cell and Molecular Biology*, 44(4), pp. 474–482. doi: 10.1165/rcmb.2009-0463OC.

Mishra, A., Chaturvedi, P., Datta, S., Sinukumar, S., Joshi, P. and Garg, A. (2015) 'Harmful effects of nicotine', *Indian Journal of Medical and Paediatric Oncology : Official Journal of Indian Society of Medical & Paediatric Oncology*. India: Medknow Publications & Media Pvt Ltd, 36(1), pp. 24–31. doi: 10.4103/0971-5851.151771.

Mitsi, E., Kamng'ona, R., Rylance, J., Solórzano, C., Jesus Reiné, J., Mwandumba, H. C., Ferreira, D. M. and Jambo, K. C. (2018) 'Human alveolar macrophages predominately express combined classical M1 and M2 surface markers in steady state', *Respiratory Research*. BioMed Central, 19, p. 66. doi: 10.1186/s12931-018-0777-0.

Mittar, D., Paramban, R. and McIntyre, C. (2011) 'Flow Cytometry and High-Content Imaging to Identify Markers of Monocyte-Macrophage Differentiation', *BD Biosciences, August*.

Mohamadzadeh, M. and Luftig, R. (2004) 'Dendritic cells: In the forefront of immunopathogenesis and vaccine development - A review', *J Immune Based Ther Vaccines*, 2, p. 1. doi: 10.1186/1476-8518-2-1.

Morgagni, G. and Johnson, P. (1769) *The seats and causes of disease. In: Alexander B, Miller A, Caldwell T, translators. Investigated by anatomy; in five books, containing a great variety of dissections, with remarks.* London.

Morris, D. G., Huang, X., Kaminski, N., Wang, Y., Shapiro, S. D., Dolganov, G., Glick, A. and Sheppard, D. (2003) 'Loss of integrin alpha(v)beta6-mediated TGF-beta activation causes Mmp12-dependent emphysema', *Nature*. 2003/03/14, 422(6928), pp. 169–173. doi: 10.1038/nature01413.

Mortaz, E., Kraneveld, A. D., Smit, J. J., Kool, M., Lambrecht, B. N., Kunkel, S. L., Lukacs, N. W., Nijkamp, F. P. and Folkerts, G. (2009) 'Effect of Cigarette Smoke Extract on Dendritic Cells and Their Impact on T-Cell Proliferation', *PLoS ONE*. Edited by O. Neyrolles. San Francisco, USA: Public Library of Science, 4(3), p. e4946. doi: 10.1371/journal.pone.0004946.

Mueller, C., Liotta, L. A. and Espina, V. (2010) 'Reverse phase protein microarrays advance to use in clinical trials', *Molecular oncology*. Wiley Online Library, 4(6), pp. 461–481.

Murarescu, E. D., Mitrofan, E. C. and Mihailovici, M. S. (2007) 'Chronic obstructive pulmonary disease in a new concept', *Rom J Morphol Embryol*. 2007/10/05, 48(3), pp. 207–214.

Murray, A. B. and Morrison, B. J. (1986) 'The effect of cigarette smoke from the mother on bronchial responsiveness and severity of symptoms in children with asthma', *Journal of allergy and clinical immunology*. Elsevier, 77(4), pp. 575–581.

Murray, L. A., Dunmore, R., Camelo, A., Da Silva, C. A., Gustavsson, M. J., Habiel, D. M., Hackett, T. L., Hogaboam, C. M., Sleeman, M. A. and Knight, D. A. (2017) 'Acute cigarette smoke exposure activates apoptotic and inflammatory programs but a second stimulus is required to induce epithelial to mesenchymal transition in COPD epithelium', *Respiratory research*. BioMed Central, 18(1), p. 82.

Nathan, C. F. and Hibbs Jr., J. B. (1991) 'Role of nitric oxide synthesis in macrophage antimicrobial activity', *Curr Opin Immunol*. 1991/02/11, 3(1), pp. 65–70.

Navarrette, C. R., Sisson, J. H., Nance, E., Allen-Gipson, D., Hanes, J. and Wyatt, T. A. (2012) 'Particulate Matter in Cigarette Smoke Increases Ciliary Axoneme Beating Through Mechanical Stimulation', *Journal of Aerosol Medicine and Pulmonary Drug Delivery*. 140 Huguenot Street, 3rd FloorNew Rochelle, NY 10801USA: Mary Ann Liebert, Inc., 25(3), pp. 159–168. doi: 10.1089/jamp.2011.0890.

Negm, O.H., Mannsperger, H.A., McDermott, E.M., Drewe, E., Powell, R.J., Todd, I., Fairclough, L.C. and Tighe, P.J. (2014). *A pro-inflammatory signalome is constitutively activated by C33Y mutant TNF receptor 1in TNF receptor associated periodic syndrome (TRAPS).* European Journal of Immunology. 44, 2096-2110.

Núñez, B., Sauleda, J., Antó, J. M., Julià, M. R., Orozco, M., Monsó, E., Noguera, A., Gómez, F. P., Garcia-Aymerich, J. and Agustí, A. (2011) 'Anti-Tissue Antibodies Are Related to Lung Function in Chronic Obstructive Pulmonary Disease', *American Journal of Respiratory and Critical Care Medicine*, 183(8), pp. 1025–1031. doi: 10.1164/rccm.201001-0029OC.

Nurwidya, F., Damayanti, T. and Yunus, F. (2016) 'The Role of Innate and Adaptive Immune Cells in the Immunopathogenesis of Chronic Obstructive Pulmonary Disease', *Tuberculosis and Respiratory Diseases*. The Korean Academy of Tuberculosis and

Respiratory Diseases, 79(1), pp. 5–13. doi: 10.4046/trd.2016.79.1.5.

O'Donnell, R., Breen, D., Wilson, S. and Djukanovic, R. (2006) 'Inflammatory cells in the airways in COPD', *Thorax*, 61(5), pp. 448–454. doi: 10.1136/thx.2004.024463.

O'Leary, L., Sevinç, K., Papazoglou, I. M., Tildy, B., Detillieux, K., Halayko, A. J., Chung, K. F. and Perry, M. M. (2016) 'Airway smooth muscle inflammation is regulated by microRNA- 145 in COPD ', *Febs Letters*. Hoboken: John Wiley and Sons Inc., 590(9), pp. 1324–1334. doi: 10.1002/1873-3468.12168.

Oldenburg, M., Krüger, A., Ferstl, R., Kaufmann, A., Nees, G., Sigmund, A., Bathke, B., Lauterbach, H., Suter, M., Dreher, S., Koedel, U., Akira, S., Kawai, T., Buer, J., Wagner, H., Bauer, S., Hochrein, H. and Kirschning, C. J. (2012) 'TLR13 Recognizes Bacterial 23S rRNA Devoid of Erythromycin Resistance–Forming Modification', *Science*, 337(6098), pp. 1111–1115. doi: 10.1126/science.1220363.

Osthoff, R. C., General Electric Research Laboratory N.Y., S., Kantor, S. W. and General Electric Research Laboratory N.Y., S. (1957) *Organosilazane Compounds*. John Wiley & Sons, Inc. doi: 10.1002/9780470132364.ch16.

Ouyang, Y., Virasch, N., Hao, P., Aubrey, M. T., Mukerjee, N., Bierer, B. E. and Freed, B. M. (2000) 'Suppression of human IL-1β, IL-2, IFN-γ, and TNF-α production by cigarette smoke extracts', *Journal of Allergy and Clinical Immunology*. Elsevier, 106(2), pp. 280–287. doi: 10.1067/mai.2000.107751.

Ovchinnikov, D. A. (2008) 'Macrophages in the embryo and beyond: much more than just giant phagocytes', *Genesis*. 2008/09/11, 46(9), pp. 447–462. doi: 10.1002/dvg.20417.

Packard, T. A., Li, Q. Z., Cosgrove, G. P., Bowler, R. P. and Cambier, J. C. (2013) 'COPD is associated with production of autoantibodies to a broad spectrum of self-antigens, correlative with disease phenotype', *Immunologic research*, 55(0), pp. 48–57. doi: 10.1007/s12026-012-8347-x.

Pappas, K., Papaioannou, A. I., Kostikas, K. and Tzanakis, N. (2013) 'The role of macrophages in obstructive airways disease: chronic obstructive pulmonary disease and asthma', *Cytokine*. 2013/10/03, 64(3), pp. 613–625. doi: 10.1016/j.cyto.2013.09.010.

Park, E. K., Jung, H. S., Yang, H. I., Yoo, M. C., Kim, C. and Kim, K. S. (2007) 'Optimized THP-1 differentiation is required for the detection of responses to weak stimuli', *Inflammation research*. Springer, 56(1), pp. 45–50.

Parkes, G., Greenhalgh, T., Griffin, M. and Dent, R. (2008) 'Effect on smoking quit rate of telling patients their lung age: the Step2quit randomised controlled trial', *BMJ*, 336(7644), p. 598 LP-600.

Pesci, A., Balbi, B., Majori, M., Cacciani, G., Bertacco, S., Alciato, P. and Donner, C. F. (1998) 'Inflammatory cells and mediators in bronchial lavage of patients with chronic obstructive pulmonary disease', *European Respiratory Journal*. Eur Respiratory Soc, 12(2), pp. 380–386.

Pifer, R., Benson, A., Sturge, C. R. and Yarovinsky, F. (2011) 'UNC93B1 Is Essential for TLR11 Activation and IL-12-dependent Host Resistance to Toxoplasma gondii', *Journal of Biological Chemistry*, 286(5), pp. 3307–3314. doi: 10.1074/jbc.M110.171025.

Pilette, C., Ouadrhiri, Y., Godding, V., Vaerman, J. P. and Sibille, Y. (2001) 'Lung mucosal immunity: immunoglobulin-A revisited', *Eur Respir J*. 2001/10/09, 18(3), pp. 571–588.

Pires, K. M., Lanzetti, M., Rueff-Barroso, C. R., Castro, P., Abrahao, A., Koatz, V. L., Valenca, S. S. and Porto, L. C. (2012) 'Oxidative damage in alveolar macrophages exposed to cigarette smoke extract and participation of nitric oxide in redox balance', *Toxicol In Vitro*. 2012/06/06, 26(6), pp. 791–798. doi: 10.1016/j.tiv.2012.05.011.

Pons, A. R., Noguera, A., Blanquer, D., Sauleda, J., Pons, J. and Agustí, A. G. N. (2005) 'Phenotypic characterisation of alveolar macrophages and peripheral blood monocytes in COPD', *European Respiratory Journal*, 25(4), p. 647 LP-652.

Price, D., Freeman, D., Cleland, J., Kaplan, A. and Cerasoli, F. (2011) 'Earlier diagnosis and earlier treatment of COPD in primary care', *Prim Care Respir J*. 2010/09/28, 20(1), pp. 15–22. doi: 10.4104/pcrj.2010.00060.

Rab, A., Rowe, S. M., Raju, S. V, Bebok, Z., Matalon, S. and Collawn, J. F. (2013) 'Cigarette smoke and CFTR: implications in the pathogenesis of COPD', *Am J Physiol*

Lung Cell Mol Physiol. 2013/08/13, 305(8), pp. L530-41. doi: 10.1152/ajplung.00039.2013.

Reynolds, H. Y. (2011) 'Bronchoalveolar lavage and other methods to define the human respiratory tract milieu in health and disease', *Lung*. 2011/02/26, 189(2), pp. 87–99. doi: 10.1007/s00408-011-9284-5.

Richens, T. R., Linderman, D. J., Horstmann, S. A., Lambert, C., Xiao, Y.-Q., Keith, R. L., Boé, D. M., Morimoto, K., Bowler, R. P., Day, B. J., Janssen, W. J., Henson, P. M. and Vandivier, R. W. (2009) 'Cigarette Smoke Impairs Clearance of Apoptotic Cells through Oxidant-dependent Activation of RhoA', *American Journal of Respiratory and Critical Care Medicine*, 179(11), pp. 1011–1021. doi: 10.1164/rccm.200807-1148OC.

Rioux, N. and Castonguay, A. (2001) '4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone modulation of cytokine release in U937 human macrophages', *Cancer Immunology, Immunotherapy*, 49(12), pp. 663–670. doi: 10.1007/s002620000157.

Robinson, A. B., Johnson, K. D., Bennion, B. G. and Reynolds, P. R. (2012) 'RAGE signaling by alveolar macrophages influences tobacco smoke-induced inflammation', *Am J Physiol Lung Cell Mol Physiol*. 2012/04/17, 302(11), pp. L1192-9. doi: 10.1152/ajplung.00099.2012.

Robinson, A. B., Stogsdill, J. A., Lewis, J. B., Wood, T. T. and Reynolds, P. R. (2012) 'RAGE and tobacco smoke: insights into modeling chronic obstructive pulmonary disease', *Frontiers in Physiology*. Frontiers Media S.A., 3, p. 301. doi: 10.3389/fphys.2012.00301.

Roth, M. (2008) 'Pathogenesis of COPD. Part III. Inflammation in COPD [State of the Art Series. Chronic obstructive pulmonary disease in high- and low-income countries. Edited by G. Marks and M. Chan-Yeung. Number 3 in the series]', *The International Journal of Tuberculosis and Lung Disease*, 12(4), pp. 375–380.

Le Rouzic, O., Koné, B., Kluza, J., Marchetti, P., Hennegrave, F., Olivier, C., Kervoaze, G., Vilain, E., Mordacq, C., Just, N., Perez, T., Bautin, N., Pichavant, M. and Gosset, P. (2016) 'Cigarette smoke alters the ability of human dendritic cells to promote anti-Streptococcus pneumoniae Th17 response', *Respiratory Research*. London: BioMed Central, 17, p. 94. doi: 10.1186/s12931-016-0408-6.

Russell, R. E., Culpitt, S. V, DeMatos, C., Donnelly, L., Smith, M., Wiggins, J. and Barnes, P. J. (2002) 'Release and activity of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 by alveolar macrophages from patients with chronic obstructive pulmonary disease', *Am J Respir Cell Mol Biol*. 2002/04/24, 26(5), pp. 602–609. doi: 10.1165/ajrcmb.26.5.4685.

Salari, S., Seibert, T., Chen, Y.-X., Hu, T., Shi, C., Zhao, X., Cuerrier, C. M., Raizman, J. E. and O'Brien, E. R. (2013) 'Extracellular HSP27 acts as a signaling molecule to activate NF-κB in macrophages', *Cell Stress and Chaperones*. Springer, 18(1), pp. 53–63.

Schaal, C. and Chellappan, S. P. (2014) 'Nicotine-mediated cell proliferation and tumor progression in smoking-related cancers', *Molecular cancer research : MCR*, 12(1), pp. 14–23. doi: 10.1158/1541-7786.MCR-13-0541.

Schembri, F., Sridhar, S., Perdomo, C., Gustafson, A. M., Zhang, X., Ergun, A., Lu, J., Liu, G., Bowers, J., Vaziri, C., Ott, K., Sensinger, K., Collins, J. J., Brody, J. S., Getts, R., Lenburg, M. E. and Spira, A. (2009) 'MicroRNAs as modulators of smoking-induced gene expression changes in human airway epithelium', in *Proc Natl Acad Sci U S A*, pp. 2319–2324. doi: 10.1073/pnas.0806383106.

Schreier, S. (2009) 'Influence of oxidatively modified LDL on the transcription factor HIF-1α and the scavenger receptor SR-BI in THP-1 macrophages'. uniwien.

Schwende, H., Fitzke, E., Ambs, P. and Dieter, P. (1996) 'Differences in the state of differentiation of THP-1 cells induced by phorbol ester and 1,25-dihydroxyvitamin D3', *J Leukoc Biol*. 1996/04/01, 59(4), pp. 555–561.

Selvarajah, S., Todd, I., Tighe, P.J., John, M., Bolton, C.E., Harrison, T. and Faorclough, L.C. (2016). Multiple circulating cytokines are co-elevated in chronic obstructive pulmonary disease. Mediators of Inflammation. Article ID 3604842. http://dx.doi.org/10.1155/2016/3604842

Shahab, L., Jarvis, M. J., Britton, J. and West, R. (2006) 'Prevalence, diagnosis and relation to tobacco dependence of chronic obstructive pulmonary disease in a nationally representative population sample', *Thorax*. 2006/10/17, 61(12), pp. 1043–1047. doi: 10.1136/thx.2006.064410.

Sharma, A., Fix, B. V, Delnevo, C., Cummings, K. M. and O'Connor, R. J. (2016) 'Trends in market share of leading cigarette brands in the USA: national survey on drug use and health 2002–2013', *BMJ Open*. BMA House, Tavistock Square, London, WC1H 9JR: BMJ Publishing Group, 6(1), p. e008813. doi: 10.1136/bmjopen-2015-008813.

Sharma, G., Hanania, N. A. and Shim, Y. M. (2009) 'The aging immune system and its relationship to the development of chronic obstructive pulmonary disease', *Proc Am Thorac Soc*. 2009/11/26, 6(7), pp. 573–580. doi: 10.1513/pats.200904-022RM.

Shaykhiev, R., Krause, A., Salit, J., Strulovici-Barel, Y., Harvey, B. G., O'Connor, T. P. and Crystal, R. G. (2009) 'Smoking-dependent reprogramming of alveolar macrophage polarization: implication for pathogenesis of chronic obstructive pulmonary disease', *J Immunol*. 2009/07/29, 183(4), pp. 2867–2883. doi: 10.4049/jimmunol.0900473.

Sheehan, K. M., Calvert, V. S., Kay, E. W., Lu, Y., Fishman, D., Espina, V., Aquino, J., Speer, R., Araujo, R. and Mills, G. B. (2005) 'Use of reverse phase protein microarrays and reference standard development for molecular network analysis of metastatic ovarian carcinoma', *Molecular & Cellular Proteomics*. ASBMB, 4(4), pp. 346–355.

Shi, Z., Cai, Z., Sanchez, A., Zhang, T., Wen, S., Wang, J., Yang, J., Fu, S. and Zhang, D. (2011) 'A Novel Toll-like Receptor That Recognizes Vesicular Stomatitis Virus', *Journal of Biological Chemistry*, 286(6), pp. 4517–4524. doi: 10.1074/jbc.M110.159590.

Sica, A. and Mantovani, A. (2012) 'Macrophage plasticity and polarization: in vivo veritas', *The Journal of Clinical Investigation*. American Society for Clinical Investigation, 122(3), pp. 787–795. doi: 10.1172/JCI59643.

Singla, D. K., Singla, R. and Wang, J. (2016) 'BMP-7 treatment increases M2 macrophage differentiation and reduces inflammation and plaque formation in apo E-/-mice', *PloS one*. Public Library of Science, 11(1), p. e0147897.

Slota, M., Lim, J. B., Dang, Y. and Disis, M. L. (2011) 'ELISpot for measuring human immune responses to vaccines', *Expert Rev Vaccines*, 10(3), pp. 299–306. doi:

10.1586/erv.10.169.

Smith, L. A., Paszkiewicz, G. M., Hutson, A. D. and Pauly, J. L. (2010) 'Inflammatory response of lung macrophages and epithelial cells to tobacco smoke: a literature review of ex vivo investigations', *Immunol Res*. 2010/01/23, 46(1–3), pp. 94–126. doi: 10.1007/s12026-009-8133-6.

Snider, G. L. (1989) 'Chronic Obstructive Pulmonary Disease: Risk Factors, Pathophysiology and Pathogenesis', *Annual Review of Medicine*. Annual Reviews, 40(1), pp. 411–429. doi: 10.1146/annurev.me.40.020189.002211.

Solier, C. and Langen, H. (2014) 'Antibody-based proteomics and biomarker research—Current status and limitations', *PROTEOMICS*. Wiley-Blackwell, 14(6), pp. 774–783. doi: 10.1002/pmic.201300334.

Somborac-Bačura, A., Popović-Grle, S., Zovko, V. and Žanić-Grubišić, T. (2018) 'Cigarette Smoke Induces Activation of Polymorphonuclear Leukocytes', *Lung*, 196(1), pp. 27–31. doi: 10.1007/s00408-017-0077-3.

Sopori, M. (2002) 'Effects of cigarette smoke on the immune system', *Nature Reviews Immunology*. Nature publishing group, 2(5), p. 372.

Springer, T. A. (1990) 'Adhesion receptors of the immune system', *Nature*. Springer, 346(6283), pp. 425–434.

Stahl, P. D. (1992) 'The mannose receptor and other macrophage lectins', *Curr Opin Immunol*. 1992/02/01, 4(1), pp. 49–52.

Stampfli, M. R. and Anderson, G. P. (2009) 'How cigarette smoke skews immune responses to promote infection, lung disease and cancer', *Nat Rev Immunol*. Nature Publishing Group, 9(5), pp. 377–384.

Stefanska, A. M. and Walsh, P. T. (2009) 'Chronic Obstructive Pulmonary Disease: Evidence for an Autoimmune Component', *Cell Mol Immunol*. 2009/05/01. Nature Publishing Group, 6(2), pp. 81–86. doi: 10.1038/cmi.2009.11.

Stein, M., Keshav, S., Harris, N. and Gordon, S. (1992) 'Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation', *J Exp Med*. 1992/07/01, 176(1), pp. 287–292.

Stevenson, C. S. and Birrell, M. A. (2011) 'Moving towards a new generation of animal models for asthma and COPD with improved clinical relevance', *Pharmacology & Therapeutics*, 130(2), pp. 93–105. doi: https://doi.org/10.1016/j.pharmthera.2010.10.008.

Stockley, R. A. (2002) 'Neutrophils and the pathogenesis of COPD', *Chest*. 2002/05/16, 121(5 Suppl), p. 151s–155s.

Stringer, K.A., M. Tobias, H.C. O'Neill, C.C. Franklin, Cigarette smoke extract-induced suppression of caspase-3-like activity impairs human neutrophil phagocytosis, American journal of physiology. Lung cellular and molecular physiology, 292 (2007) L1572-1579.

Su, Y., W. Cao, Z. Han, E.R. Block, Cigarette smoke extract inhibits angiogenesis of pulmonary artery endothelial cells: the role of calpain, American journal of physiology. Lung cellular and molecular physiology, 287 (2004) L794-800.

Suji Eapen, M., Hansbro, P.M., McAlinden, K., Kim, R.Y., Ward, C., Hackett, T-L., Walters, E.H. and Sohal, S.S. (2017). Abnormal M1/M2 macrophage phenotype profiles in the small airway walland lumen in smokers and chronic pbstructive pulmonary disease. *Scientific Reports.* 7, 13392.

Sutherland, E. R. and Cherniack, R. M. (2004) 'Management of chronic obstructive pulmonary disease', *N Engl J Med*. 2004/06/25, 350(26), pp. 2689–2697. doi: 10.1056/NEJMra030415.

Sutherland, E. R. and Martin, R. J. (2003) 'Airway inflammation in chronic obstructive pulmonary disease: comparisons with asthma', *J Allergy Clin Immunol*. 2003/11/12, 112(5), p. 819–27; quiz 828. doi: 10.1016/s0091.

Symposium., C. G. (1959) 'Terminology, definitions, and classification of chronic pulmonary emphysema and related conditions. '. Thorax.

Takabayshi, K., Corr, M., Hayashi, T., Redecke, V., Beck, L., Guiney, D., Sheppard, D. and Raz, E. (2006) 'Induction of a homeostatic circuit in lung tissue by microbial compounds', *Immunity*. 2006/04/19, 24(4), pp. 475–487. doi: 10.1016/j.immuni.2006.02.008.

Takata, K., Kozaki, T., Lee, C. Z. W., Thion, M. S., Otsuka, M., Lim, S., Utami, K. H., Fidan, K., Park, D. S., Malleret, B., Chakarov, S., See, P., Low, D., Low, G., Garcia-Miralles, M., Zeng, R., Zhang, J., Goh, C. C., Gul, A., Hubert, S., Lee, B., Chen, J., Low, I., Shadan, N. B., Lum, J., Wei, T. S., Mok, E., Kawanishi, S., Kitamura, Y., Larbi, A., Poidinger, M., Renia, L., Ng, L. G., Wolf, Y., Jung, S., Önder, T., Newell, E., Huber, T., Ashihara, E., Garel, S., Pouladi, M. A. and Ginhoux, F. (2017) 'Induced-Pluripotent-Stem-Cell-Derived Primitive Macrophages Provide a Platform for Modeling Tissue-Resident Macrophage Differentiation and Function', *Immunity*. Elsevier, 47(1), p. 183–198.e6. doi: 10.1016/j.immuni.2017.06.017.

Takizawa, H., Tanaka, M., Takami, K., Ohtoshi, T., Ito, K., Satoh, M., Okada, Y., Yamasawa, F., Nakahara, K. and Umeda, A. (2001) 'Increased expression of transforming growth factor-beta1 in small airway epithelium from tobacco smokers and patients with chronic obstructive pulmonary disease (COPD)', *Am J Respir Crit Care Med*. 2001/05/24, 163(6), pp. 1476–1483. doi: 10.1164/ajrccm.163.6.9908135.

Talhout, R., Schulz, T., Florek, E., Van Benthem, J., Wester, P. and Opperhuizen, A. (2011) 'Hazardous compounds in tobacco smoke', *International journal of environmental research and public health*. Molecular Diversity Preservation International, 8(2), pp. 613–628.

Tamashiro, E., Cohen, N. A., Palmer, J. N. and Lima, W. T. (2009) 'Effects of cigarette smoking on the respiratory epithelium and its role in the pathogenesis of chronic rhinosinusitis', *Braz J Otorhinolaryngol*. 2010/03/09, 75(6), pp. 903–907.

Tamashiro, E., Xiong, G., Anselmo-Lima, W. T., Kreindler, J. L., Palmer, J. N. and Cohen, N. A. (2009) 'Cigarette smoke exposure impairs respiratory epithelial ciliogenesis', *Am J Rhinol Allergy*. 2009/04/30, 23(2), pp. 117–122. doi: 10.2500/ajra.2009.23.3280.

Tamimi, A., Serdarevic, D. and Hanania, N. A. (2012) 'The effects of cigarette smoke on airway inflammation in asthma and COPD: therapeutic implications', *Respir Med*. 2011/12/27, 106(3), pp. 319–328. doi: 10.1016/j.rmed.2011.11.003.

Taylor, J. D. (2010) 'COPD and the response of the lung to tobacco smoke exposure', *Pulmonary Pharmacology & Therapeutics*. Academic Press, 23(5), pp. 376–383. doi:

10.1016/J.PUPT.2010.04.003.

Taylor, M. E. (2001) ‘Structure and function of the macrophage mannose receptor’, *Results Probl Cell Differ*. 2001/02/24, 33, pp. 105–121.

Taylor, P. R., Gordon, S. and Martinez-Pomares, L. (2005) ‘The mannose receptor: linking homeostasis and immunity through sugar recognition’, *Trends Immunol*. 2005/01/26, 26(2), pp. 104–110. doi: 10.1016/j.it.2004.12.001.

Tedesco, S., De Majo, F., Kim, J., Trenti, A., Trevisi, L., Fadini, G.P., Bolego, C., Zandstra, P.W., Cignarella, A. and Vitiello, L. (2018). Convenience versus biological significance: are PMA-differentiaited THP-1 cells a reliable substitute for blood-derived macrophages when studying *in vitro* polarization? *Frontiers in Pharmacology.* 9, article 71.

Teramoto, S. (2016) ‘Recent progress in genetic background of chronic obstructive pulmonary disease (COPD)’, *Nihon rinsho. Japanese journal of clinical medicine*, 74(5), pp. 733–742.

Tetley, T. D. (2002) ‘Macrophages and the pathogenesis of COPD’, *CHEST Journal*. American College of Chest Physicians, 121(5_suppl), p. 156S–159S.

Thomson, N. C., Chaudhuri, R. and Livingston, E. (2004) ‘Asthma and cigarette smoking’, *European respiratory journal*. Eur Respiratory Soc, 24(5), pp. 822–833.

Tibes, R., Qiu, Y., Lu, Y., Hennessy, B., Andreeff, M., Mills, G. B. and Kornblau, S. M. (2006) ‘Reverse phase protein array: validation of a novel proteomic technology and utility for analysis of primary leukemia specimens and hematopoietic stem cells’, *Molecular cancer therapeutics*. AACR, 5(10), pp. 2512–2521.

Tiffeneau R, P. A. (1947) ‘Air circulant et air captif dans l’exploration de la function ventilatrice pulmonaire. ’, 37.

Tinti, F., M. Soory, Mechanisms for redox actions of nicotine and glutathione in cell culture, relevant to periodontitis, Scientific reports, 2 (2012) 566.

De Torres, J. P., Casanova, C., Hernandez, C., Abreu, J., Aguirre-Jaime, A. and Celli, B. R. (2005) ‘Gender and COPD in patients attending a pulmonary clinic’, *Chest*. 2005/10/21, 128(4), pp. 2012–2016. doi: 10.1378/chest.128.4.2012.

De Torres, J. P., Casanova, C., Hernandez, C., Abreu, J., Montejo de Garcini, A., Aguirre-Jaime, A. and Celli, B. R. (2006) 'Gender associated differences in determinants of quality of life in patients with COPD: a case series study', *Health Qual Life Outcomes*, 4, p. 72. doi: 10.1186/1477-7525-4-72.

Tschernig, T., Veith, N. T., Diler, E., Bischoff, M., Meier, C. and Schicht, M. (2016) 'The importance of surfactant proteins—New aspects on macrophage phagocytosis', *Annals of Anatomy-Anatomischer Anzeiger*. Elsevier, 208, pp. 142–145.

Tsuchiya, S., Kobayashi, Y., Goto, Y., Okumura, H., Nakae, S., Konno, T. and Tada, K. (1982) 'Induction of maturation in cultured human monocytic leukemia cells by a phorbol diester', *Cancer Res*. 1982/04/01, 42(4), pp. 1530–1536.

Tsuchiya, S., Yamabe, M., Yamaguchi, Y., Kobayashi, Y., Konno, T. and Tada, K. (1980) 'Establishment and characterization of a human acute monocytic leukemia cell line (THP-1)', *Int J Cancer*. 1980/08/01, 26(2), pp. 171–176.

Tuncman, G., Erbay, E., Hom, X., De Vivo, I., Campos, H., Rimm, E. B. and Hotamisligil, G. S. (2006) 'A genetic variant at the fatty acid-binding protein aP2 locus reduces the risk for hypertriglyceridemia, type 2 diabetes, and cardiovascular disease', *Proceedings of the National Academy of Sciences*. National Acad Sciences, 103(18), pp. 6970–6975.

Ueno, H., Palucka, A. K. and Banchereau, J. (2010) 'The expanding family of dendritic cell subsets', *Nature Biotechnology*, 28, pp. 813–815. doi: doi:10.1038/nbt0810-813.

Unanue, E. R. (1984) 'Antigen-Presenting Function of the Macrophage', *Annual Review of Immunology*. Annual Reviews, 2(1), pp. 395–428. doi: 10.1146/annurev.iy.02.040184.002143.

Urbanowicz, R. A., Lamb, J. R., Todd, I., Corne, J. M. and Fairclough, L. C. (2009) 'Altered effector function of peripheral cytotoxic cells in COPD', *Respir Res*, 10(1), p. 53. doi: 10.1186/1465-9921-10-53.

Urbanowicz, R. A., Lamb, J. R., Todd, I., Corne, J. M. and Fairclough, L. C. (2010) 'Enhanced effector function of cytotoxic cells in the induced sputum of COPD patients', *Respir Res*, 11(1), p. 76. doi: 10.1186/1465-9921-11-76.

Vaddi, K. and Newton, R. C. (1994) ‘Comparison of biological responses of human monocytes and THP-1 cells to chemokines of the intercrine-beta family.’, *Journal of leukocyte biology*. Soc Leukocyte Biology, 55(6), pp. 756–762.

Varol, C., Mildner, A. and Jung, S. (2015) ‘Macrophages: Development and Tissue Specialization’, *Annual Review of Immunology*. Annual Reviews, 33(1), pp. 643–675. doi: 10.1146/annurev-immunol-032414-112220.

Vassallo, R., Tamada, K., Lau, J. S., Kroening, P. R. and Chen, L. (2005) ‘Cigarette Smoke Extract Suppresses Human Dendritic Cell Function Leading to Preferential Induction of Th-2 Priming’, *The Journal of Immunology*, 175(4), p. 2684 LP-2691.

van de Velde, N. C., Mottram, P. L. and Hogarth, P. M. (2006) ‘FcγRII and multi-system autoimmune disease’, in *Springer seminars in immunopathology*. Springer, pp. 329–338.

Vissers, M., Habets, M. N., Ahout, I. M. L., Jans, J., de Jonge, M. I., Diavatopoulos, D. A. and Ferwerda, G. (2013) ‘An *in vitro* model to study immune responses of human peripheral blood mononuclear cells to human respiratory syncytial virus infection’, *Journal of visualized experiments: JoVE*. MyJoVE Corporation, (82).

Vlahos, R. and Bozinovski, S. (2014) ‘Role of alveolar macrophages in chronic obstructive pulmonary disease’, *Front Immunol*. 2014/10/14, 5, p. 435. doi: 10.3389/fimmu.2014.00435.

Wahl, E. A., Schenck, T. L., Machens, H.-G. and Egaña, J. T. (2016) ‘Acute stimulation of mesenchymal stem cells with cigarette smoke extract affects their migration, differentiation, and paracrine potential’, *Scientific Reports*. Nature Publishing Group, 6, p. 22957. doi: 10.1038/srep22957.

Wan, J., Johnson, M., Schilz, J., Djordjevic, M. V, Rice, J. R. and Shields, P. G. (2009) ‘Evaluation of *In Vitro* Assays For Assessing the Toxicity of Cigarette Smoke and Smokeless Tobacco’, *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology*, 18(12), pp. 3263–3304. doi: 10.1158/1055-9965.EPI-09-0965.

Wanner, A. (2009) ‘COPD: new lessons from alpha1-antitrypsin deficiency?’, *Chest*. 2009/05/08, 135(5), pp. 1342–1344. doi: 10.1378/chest.08-2341.

Wanner, A., Salathe, M. and O’Riordan, T. G. (1996) ‘Mucociliary clearance in the airways’, *Am J Respir Crit Care Med*. 1996/12/01, 154(6 Pt 1), pp. 1868–1902. doi: 10.1164/ajrccm.154.6.8970383.

Ween, M. P., Whittall, J. J., Hamon, R., Reynolds, P. N. and Hodge, S. J. (2017) ‘Phagocytosis and Inflammation: Exploring the effects of the components of E-cigarette vapor on macrophages’, *Physiological Reports*. Hoboken: John Wiley and Sons Inc., 5(16), p. e13370. doi: 10.14814/phy2.13370.

Wen, L., Jiang, K., Yuan, W., Cui, W. and Li, M. D. (2016) ‘Contribution of variants in CHRNA5/A3/B4 gene cluster on chromosome 15 to tobacco smoking: from genetic association to mechanism’, *Molecular neurobiology*. Springer, 53(1), pp. 472–484.

Wongtrakool, C., K. Grooms, K.M. Bijli, K. Crothers, A.M. Fitzpatrick, C.M. Hart, Nicotine stimulates nerve growth factor in lung fibroblasts through an NFkappaB-dependent mechanism, PLoS One, 9 (2014) e109602.

White, E. S., Flaherty, K. R., Carskadon, S., Brant, A., Iannettoni, M. D., Yee, J., Orringer, M. B. and Arenberg, D. A. (2003) ‘Macrophage Migration Inhibitory Factor and CXC Chemokine Expression in Non-Small Cell Lung Cancer: Role in Angiogenesis and Prognosis’, *Clinical Cancer Research*, 9(2), pp. 853–860.

Whitsett, J. A., Wert, S. E. and Weaver, T. E. (2015) ‘Diseases of Pulmonary Surfactant Homeostasis’, *Annual review of pathology*, 10, pp. 371–393. doi: 10.1146/annurev-pathol-012513-104644.

WHO, 310 Fact sheet (2014) ‘WHO | The top 10 causes of death’, *WHO*. doi: /entity/mediacentre/factsheets/fs310/en/index.html.

Van Winkle, L. S., Evans, M. J., Brown, C. D., Willits, N. H., Pinkerton, K. E. and Plopper, C. G. (2001) ‘Prior exposure to aged and diluted sidestream cigarette smoke impairs bronchiolar injury and repair’, *Toxicol Sci*. 2001/02/27, 60(1), pp. 152–164.

Wolk, K., Döcke, W.-D., von Baehr, V., Volk, H.-D. and Sabat, R. (2000) ‘Impaired antigen presentation by human monocytes during endotoxin tolerance’, *Blood*, 96(1),

p. 218 LP-223.

Wright, J. L., Lawson, L. M., Pare, P. D., Kennedy, S., Wiggs, B. and Hogg, J. C. (1984) 'The detection of small airways disease', *Am Rev Respir Dis*. 1984/06/01, 129(6), pp. 989–994.

Wright, W. R., Parzych, K., Crawford, D., Mein, C., Mitchell, J. A. and Paul-Clark, M. J. (2012) 'Inflammatory transcriptome profiling of human monocytes exposed acutely to cigarette smoke', *PLoS One*. 2012/03/01, 7(2), p. e30120. doi: 10.1371/journal.pone.0030120.

Yamasaki, K. and van Eeden, S. F. (2018) 'Lung Macrophage Phenotypes and Functional Responses: Role in the Pathogenesis of COPD', *International Journal of Molecular Sciences*. MDPI, 19(2), p. 582. doi: 10.3390/ijms19020582.

Yang, Y., Kim, S. C., Yu, T., Yi, Y.-S., Rhee, M. H., Sung, G.-H., Yoo, B. C. and Cho, J. Y. (2014) 'Functional roles of p38 mitogen-activated protein kinase in macrophage-mediated inflammatory responses', *Mediators of Inflammation*. Hindawi Publishing Corporation, 2014.

Yarovinsky, F., Zhang, D., Andersen, J. F., Bannenberg, G. L., Serhan, C. N., Hayden, M. S., Hieny, S., Sutterwala, F. S., Flavell, R. A., Ghosh, S. and Sher, A. (2005) 'TLR11 Activation of Dendritic Cells by a Protozoan Profilin-Like Protein', *Science*, 308(5728), pp. 1626–1629. doi: 10.1126/science.1109893.

Yin, Y., Hou, G., Li, E., Wang, Q. and Kang, J. (2014) 'PPARgamma agonists regulate tobacco smoke-induced Toll like receptor 4 expression in alveolar macrophages', *Respir Res*. 2014/03/13, 15, p. 28. doi: 10.1186/1465-9921-15-28.

YOSHIDA, M. and WHITSETT, J. A. (2006) 'Alveolar macrophages and emphysema in surfactant protein-D-deficient mice', *Respirology*. Wiley/Blackwell (10.1111), 11(s1), pp. S37–S40. doi: 10.1111/j.1440-1843.2006.00806.x.

Zeimpekoglou, K. (2008) 'The role of B-lymphocytes in lung disease', *Pneumon*, 21(1), pp. 196–199.

Zhang, G., Zhang, H., Liu, Y., He, Y., Wang, W., Du, Y., Yang, C and Gao F. (2014). CD44 clustering is involved in monocyte differentiation. *Acta Biochim Biophys Sin* 46,

540-547.

Zhou, L., Shen, L-H, Hu, L-H., Ge, H., Pu, J., Chai, D-J., Shao, Q., Wang, L., Weng, J-Z. and He, B. (2010). Retinoid X receptor agonistsinhibit phorbol-12-myristate-13-acetate (PMA)-induced differentiationof monocytic THP-1 cells into macrophages. *Molecular and cellular biochemistry.* 335, 283-289.

Zlotnik, A. and Yoshie, O. (2000) 'Chemokines: A New Classification System and Their Role in Immunity', *Immunity*, 12(2), pp. 121–127. doi: https://doi.org/10.1016/S1074-7613(00)80165-X.

Zuniga, E. I., McGavern, D. B., Pruneda-Paz, J. L., Teng, C. and Oldstone, M. B. (2004) 'Bone marrow plasmacytoid dendritic cells can differentiate into myeloid dendritic cells upon virus infection', *Nat Immunol*. 2004/11/09, 5(12), pp. 1227–1234. doi: 10.1038/ni1136.

Zuo, H., Han, B., Poppinga, W. J., Ringnalda, L., Kistemaker, L. E. M., Halayko, A. J., Gosens, R., Nikolaev, V. O. and Schmidt, M. (2018) 'Cigarette smoke up- regulates PDE3 and PDE4 to decrease cAMP in airway cells', *British journal of pharmacology*. Wiley Online Library.