THE ROLE OF TRANSIENT RECEPTOR POTENTIAL (TRP) CHANNELS IN THE PATHOGENESIS OF COPD

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Abstract

COPD is currently the 4th most prevalent cause of death worldwide. Despite the global impact, there are no currently available treatments which impede disease progression. This lack of effective therapies is largely due to an inadequate understanding of the mechanisms which drive disease progression.

Cigarette smoke (CS), the most important risk factor for COPD, is thought to initiate an inflammatory response in the lungs which becomes self-propagating and dysregulated. Chronically, this inflammatory response drives structural and functional changes. The mechanisms by which CS elicits this inflammatory response, however, remain unclear. Certain CS constituents are known to activate Transient Receptor Potential (TRP) ion channels. A number of TRP channels are actively expressed in the lung tissue or inflammatory cells, and a further few are also implicated in the generation of inflammation. Owing to these features, it was hypothesised that TRP channels A1, C6, M2, M8, V1 and V4 have a role in CS-induced airway inflammation and, consequently, the pathogenesis of COPD.

To test this hypothesis, three murine models of induced airway inflammation were characterised: acute CS, sub-chronic CS and endotoxin (LPS). Lung-tissue TRP channel expression levels were measured in these models alongside human lung-parenchyma samples from non-smokers, smokers and emphysema patients.

Mice deficient for specific TRP channels were profiled in the CS-model and the LPS-model to establish the role of TRP channels in the initiation of inflammation in disease and non-disease settings. TRPV1-/-, TRPV4-/- and TRPM8-/- mice exhibited significantly reduced levels of airway inflammation compared to wild-types after acute CS, but normal responses to the innate (LPS) challenge.

This data suggests that modulation of TRP channels could represent a novel anti-inflammatory approach for combating smoke induced diseases like COPD without impacting on the normal, essential innate defence mechanisms.

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Statement

The work in this thesis is my own unless otherwise referenced. The initial characterisation of the *in vivo* acute cigarette smoke model and the *in vivo* acute LPS models was performed by the Respiratory Pharmacology group. The initial characterisation experiments for the sub-chronic cigarette smoke exposure model were designed by Suffwan Eltom and Joe Rastrick.

Copyright Declaration

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List of Abbreviations

ATP	Adenosine Triphosphate
AMP	Adenosine Monophosphate
BAL	Bronchoalveolar Lavage
BALF	Bronchoalveolar Lavage Fluid
CCL	Chemokine C-C Motif Ligand
cDNA	Complimentary Deoxyribonucleic Acid
CGRP	Calcitonin Gene-Related Peptide
CNS	Central Nervous System
COPD	Chronic Obstructive Pulmonary Disease
COX	Cyclooxygenase
CS	Cigarette Smoke
CSE	Cigarette Smoke Extract
CSM	Cigarette Smoke Conditioned Medium
Ct	Critical Threshold
CXCL	Complimentary C-X-C Motif Ligand
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleoside Triphosphate
DRG	Dorsal root ganglia
EDTA	Ethylenediamineteteraacetic Acid
EET	Epoxyeicosatrienoic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
ERK	Extracellular Signal-Related Kinase
FBS	Foetal Bovine Serum
FEV_1	Forced Expiratory Volume in One Second
FVC	Forced Vital Capacity
G-CSF	Granulocyte-Colony Stimulating Factor
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
GOLD	Global Initiative for Chronic Obstructive Lung Disease
GRO	Growth Related Oncogene
HDAC-2	Histone Deacetylase 2
HDM	House Dust Mite
HRP	Horse Radish Peroxidase
IFN	Interferon
IL	Interleukin
IP3	Inositol 1,4,5- Trisphosphate
KC	Keratinocyte-Derived Chemokine
KO	Knock-Out
LABA	Long-Acting β_2-Agonist
LMN	Lymphomononuclear Cells

LPS	Lipopolysaccharide
MCP	Monocyte Chemotactic Protein
MIP	Macrophage Inflammatory Protein
MMP	Matrix Metalloproteinase
mRNA	Messenger Ribonucleic Acid
MUC5AC	Mucin 5AC
MyD88	Myeloid Differentiation Primary Response Gene 88
NAD	Nicotinamide Adenine Dinucleotide
NF-κB	Nuclear Factor- κB
OVA	Ovalbumin
P2X	Purinoceptor P2X
P2Y	Purinoceptor P2Y
PAR	Protease-Activated Receptor
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDE	Phosphodiesterase
PG	Prostaglandin
pH	Hydrogen Potential
PI3k	Phospho-Inositol -3 Kinase
PIP_2	Phosphatidylinositol 4,5-Bisphosphate
PP_i	Inorganic Pyrophosphatase
PK	Protein Kinase
PLC	Phospholipase C
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
RT-PCR	Real Time – Polymerase Chain Reaction
SEM	Standard Error of the Mean
siRNA	Small interfering RNA
SNP	Single Nucleotide Polymorphism
Th1	T-Helper Cell-1
Th2	T-Helper Cell-2
TIMP	Tissue Inhibitor of Metalloproteinases
TLR	Toll-Like Receptor
TNF	Tumour Necrosis Factor
TRPA	Transient Receptor Potential Ankyrin
TRPC	Transient Receptor Potential Canonical
TRPM	Transient Receptor Potential Melastatin
TRPML	Transient Receptor Potential Mucolipin
TRPP	Transient Receptor Potential Polycystin
TRPV	Transient Receptor Potential Vanilloid
TSP	Total Smoke Particulate
UV	Ultra Violet
WBC	White Blood Cell
WHO	World Health Organisation
WT	Wild-type

Chapter 1

Introduction

1.1 COPD

1.1.1 Background

Chronic Obstructive Pulmonary Disease (COPD) is a term given to a group of airway pathologies which result in progressively worsening airflow obstruction, leading to increasing levels of disability and ultimately death. COPD is currently ranked as the fourth most common cause of death in the world by the World Health Organisation, after heart disease, stroke and lower-respiratory infections. COPD is therefore a more common cause of death worldwide than HIV/AIDS, diabetes or any form of cancer, and yet is comparatively less recognised as a global health issue (WHO, Factsheet no. 310).

It is of considerable note that COPD is the only 'top 10' global cause of death that is increasing in prevalence (Lopez and Mathers 2006). This is due to a combination of increasing numbers of smokers and the fact that there are no existing therapies which halt the relentless progression of this disease or which reduce the mortality rates. For these reasons COPD represents a large social and economic burden and, therefore, an important avenue for research in order to develop therapies suitable for this hitherto un-met medical need.

The current dogma is that dysregulated inflammation in the lungs becomes self-propagating and leads to damage, destruction and consequently functional changes. These processes lead to progressively worsening obstruction of airflow in and out of the lungs, manifesting as dyspnoea. Along with dyspnoea, COPD patients frequently exhibit chronic cough and chronic sputum production.

1.1.1.1 Definition and Diagnosis

The most frequently quoted definition for COPD is that of the Global Initiative for Chronic Obstructive Lung Disease (GOLD): *'a disease state characterised by airflow limitation that is not fully reversible. The airflow limitation is usually progressive and associated with abnormal inflammatory response of the lung to noxious particles or gases.'* (Mannino and Buist 2007)

Key indicators for diagnosing COPD include chronic cough, chronic sputum production, dyspnoea, family history of COPD and a history of exposure to cigarette smoke or other risk factors. Patients presenting with one or more of these indicators are normally only considered for COPD diagnosis if over 40 years of age (Rabe et al. 2007). Ultimately spirometry is required to make a clinical diagnosis. FEV_1 (Forced Expiratory Volume in 1 second) and FVC (Forced Vital Capacity) are the two spirometry parameters usually assessed for diagnosis of COPD. A post-bronchodilator FEV_1/FVC ratio of less than 0.7 is deemed to indicate persistent airflow limitation and therefore COPD (Vestbo et al. 2012).

COPD is normally categorised into progressive stages, in line with GOLD classification (Vestbo et al. 2012). These four stages are defined by severity of airflow limitation, measured by post-bronchodilator FEV_1 (see table 1.1).

GOLD classification	**Disease Severity**	**Post-bronchodilator FEV_1 (% predicted)**
Stage I	Mild	> 80%
Stage II	Moderate	80% - 50%
Stage III	Severe	50% - 30%
Stage IV	Very Severe	< 30%

Table 1.1 GOLD classification of disease severity *(adapted from GOLD Outpatient Management Reference Guide, 2012)*

Apart from severity of airflow limitation, other factors are separately assessed including symptoms, risk of exacerbations and comorbidities. Comorbidities frequently include cardiovascular disease, osteoporosis, depression-anxiety, skeletal muscle dysfunction and lung cancer. Once all the COPD disease factors of an individual patient have been assessed, a management plan is devised with the aims to reduce risk factors, relieve symptoms where possible, manage exacerbations and overall reduce mortality (Vestbo et al. 2012).

1.1.2 Burden of Disease

It is difficult to ascertain the true burden of COPD on the world in terms of prevalence, mortality and economics, especially due to under-recognition in developing countries where the disease is thought to be propagating the fastest. However, it is recognised that the social and economic burden of COPD has been steadily increasing for a number of years, and is projected to increase in the coming decades.

A meta-analysis of 62 studies reporting on populations from 28 different countries concluded that for COPD there was an overall pooled prevalence estimate of 7.6% (Halbert et al. 2006). Objective definitions, i.e. spirometric data, consistently predicted higher prevalence than patient-reported predictions however, so this figure is likely to be an under-estimate. The same study showed that COPD prevalence was significantly higher in those older than 40 years, as well as in smokers and ex-smokers compared to non-smokers (Halbert et al. 2006). Despite these observations, analysis of the Burden of Obstructive Lung Disease (BOLD) study revealed that never-smokers comprised 23.3% of COPD patients classified stage II and above (Lamprecht et al. 2011).

COPD was ranked as the sixth highest cause of death in 1990, is currently estimated as fourth, and is predicted to be the third by 2020 (Lopez et al. 2006). The increasing rate of mortality is thought to be driven by the increasing popularity of smoking (particularly in developing countries), as well as higher global life expectancies, due to advances in the treatment of other life threatening diseases. There are no current treatments which have been shown to reduce COPD associated mortality.

The majority of the economic burden of COPD is derived from critical care during exacerbations, the frequency of which increases with disease progression. In the USA in 2002, COPD healthcare burden totalled $18 billion in direct costs and $14.1 billion indirectly according to the National Heart, Lung and Blood Institute. The estimated costs for COPD patients is $1,000 to $8,000 per annum (Mannino & Buist 2007), and in the European Union it is estimated that COPD directly accounts for >3% of the total healthcare budget (Rabe et al. 2007).

1.1.3 Pathologies

COPD is a general term describing a group of respiratory diseases, frequently divided into chronic bronchitis, small airways disease and emphysema. Patients may present with one, two or all of these pathologies and in varying degrees of relative severity. The pathologies are all thought to be driven by inflammation and are characterised by increased levels of inflammatory cells and mediators in the relevant lung tissues (Di Stefano et al. 1998). The individual pathologies are all accompanied by typical functional changes resulting from repeated injury and repair.

1.1.3.1 Chronic bronchitis

Chronic Bronchitis describes persistent inflammation of the epithelium of the bronchi, which are the two main tubes supplying air to the lungs, formed by the bifurcation of the trachea. It is normally diagnosed by persistent sputum producing cough for at least three months per year, and for two consecutive years. The main characteristics of chronic bronchitis include increased production of mucus, defective mucociliary clearance and epithelial barrier disruption. Goblet cell hypertrophy (increased size) and hyperplasia (increased numerically) together lead to increased mucus production and contribute to disease severity, particularly in the latter stages of disease (Hogg, 2004). Increased numbers of inflammatory cells including neutrophils and macrophages are also a hallmark in chronic bronchitis, and are thought to play a key role in disease development (Tetley, 2005). The 'Reid Index' is used to diagnose disease severity using the ratio of gland to wall thickness, named after the scientist who first noticed that bronchial mucus glands were enlarged in chronic bronchitis (Reid, 1960).

1.1.3.2 Small airways disease

Small airways disease, alternatively known as bronchiolitis, refers to inflammation and remodelling of the conducting airways less than 2mm in diameter. It is these airways, between the fourth and fourteenth generations of airway branching, which are the major

site of airflow obstruction characterising COPD (Hogg 2004). Deposition of connective tissue in the adventitial compartment of the airway wall reduces the airway lumen size dramatically, thus increasing airway resistance (Saetta et al. 1994). Mucus-plugging is a common feature of small airways disease due to increased and chronic production of inflammatory exudate, although there is also evidence mucus plugs may be produced in the glands of larger airways and subsequently aspirated into smaller airways (Hogg et al. 2004).

1.1.3.3 Emphysema

Emphysema describes the destruction of structural lung tissues beyond the terminal bronchioles and was first described by Laennec in 1834 (Laennec 1834). Emphysema reduces maximum expiratory airflow due to decreased elastic recoil force (Mead et al. 1967). The destruction of alveoli leads to reduced lung tissue surface area, impairing gas exchange. To compensate for this, patients frequently exhibit thoracic cage expansion (barrel-chest), and a flattened diaphragm, leading to the common “pink puffer” description of severe emphysema sufferers.

Emphysema is commonly divided into two main types, centriacinar and panacinar. Centriacinar emphysema is most commonly found in the upper lobes of the lung, and may be characterised by large cavities (Kim et al., 1991; Thurlbeck, 1963). This form is normally associated with long-term cigarette smoking. The panacinar form, so named due to a more even pattern of destruction over the acini, is more commonly found in the lower lobes and is usually associated with α1-antitrypsin deficiency (Kim et al. 1991).

Smokers with emphysema exhibit approximately a tenfold increase in lung leukocyte recruitment compared to smokers with normal lung function, particularly neutrophils macrophages and T-lymphocytes (Retamales et al. 2001). It is thought that mediators including elastase and other proteases, released from these inflammatory cell-types, are responsible for the alveolar destruction and structural damage.

1.1.4 Aetiology

The development of COPD is dependent on environmental exposures and genetic susceptibilities. The overwhelming majority of COPD cases (80-90%) are developed as a direct result of smoking tobacco (Sethi & Rochester 2000).

It is frequently quoted that only 15% of smokers will develop or are susceptible to COPD; however this figure is based on out-dated definitions of the disease. In fact it is much more accurate to predict that 50% of lifelong smokers will develop COPD (Hurst et al. 2010), and it is more than likely that this percentage will increase further as life expectancies increase, particularly in less economically developed countries. That said, it is clear that genetic factors also play a major role in determining how likely it is a given individual will develop COPD. It should be noted also that COPD prevalence, morbidity and mortality all increase with age, and a globally ageing population is thus a major contributor to the worldwide increase in COPD prevalence (Mannino & Buist 2007).

The best known, and most extensively researched genetic susceptibility factor, is α1-antitrypsin deficiency, which is directly involved in 1-3% of COPD cases (Stoller and Aboussouan 2012). Several other genes have similarly been implicated in COPD, including TGF-beta, TNF-alpha, microsomal epoxide hydrolase and glutathione-S-transferase among others (L. Zhang et al. 2011; Gingo et al. 2008; Smith & Harrison 1997; Ishii et al. 1999).

Apart from cigarette smoke, exposure to other inhaled irritants has been implicated in certain subsets of COPD patients. In particular occupationally encountered airway irritants including certain dusts, chemicals and vapours were reported by the US Department of Health to be a factor in 19.2% of COPD cases in the USA (Mannino & Buist 2007). It may be inferred that this figure is likely to be even higher in less economically developed countries where there are less stringent laws protecting workers from occupational exposure to inhaled irritants. Another important risk factor is exposure to indoor air pollution from biomass fuels such as coal, wood and animal dung among others. WHO have estimated that in low- and middle-income countries up to 35% of COPD cases may be related to indoor exposure to smoke from biomass fuels.

It is also important to recognise the important role that infection plays in the progression of COPD. Bacterial and viral infections are responsible for the majority of exacerbations, and the number of exacerbations is strongly linked to the rate of lung function decline (Wedzicha & Seemungal 2007).

1.1.5 Exacerbations of COPD

Exacerbations of COPD are defined by GOLD as a period in the natural course of the disease that is characterized by a worsening of the baseline symptoms including dyspnoea, cough and/or sputum production. In the UK alone, COPD exacerbations are responsible for over 90,000 hospital admissions a year, which represents approximately 10% of all hospital admissions (Price et al. 2006). In-patient mortality rate in the UK is 7.4%, and 15.3% within 90 days (Price et al. 2006), and it is therefore of little surprise that frequency of exacerbations is strongly correlated to disease prognosis (Soler-Cataluña et al. 2005)

It is generally accepted that exacerbations of COPD correlate with increased airway inflammation, however the reported surrogate markers and inflammatory infiltrate profile vary between publications (Balbi et al. 1997; Keatings et al. 1996; Saetta et al. 1997; Wedzicha et al. 2000). Bacterial and viral infections are currently thought to cause the vast majority of exacerbations of COPD (Papi et al. 2006), although the 'Air Pollution and Health: a European Approach' (APHEA) study reported a relationship between air pollution and COPD exacerbations across 6 European cities (Hasford and Fruhmann 1998). It has been reported that approximately half of patients admitted for COPD exacerbations test positive for bacteria, of which the most commonly found are *Haemophilus influenzae*, *Streptococcus pneumonae* and *Moraxella catarrhalis* (Sethi and Murphy 2001), although *Pseudomonas aeruginosa* and *Haemophilus parainfluenzae* have also been reported in a separate study (Patel et al. 2002). A further 40-60% of COPD exacerbations are thought to be caused by viral infection (Mallia and Johnston 2005), of which rhinoviruses are reportedly the most common (Seemungal et al. 2000).

On average COPD patients experience 1-2 exacerbation episodes per year, however there is wide variation between patients (Hurst et al. 2010). Given that exacerbations are associated

with rate of COPD progression, understanding the inflammatory pathways involved in these episodes may be crucial to finding effective therapies.

1.1.6 Treatments of COPD

There is no currently available cure for COPD, nor is there any therapy which can reverse disease progression. Stabilization of disease progression and management of symptoms are therefore the only therapeutic options. The current GOLD global strategy for management of COPD comprises four components: 1. Assess and monitor disease, 2. Reduce risk factors, 3. Manage stable COPD, and 4. Manage exacerbations.

1.1.6.1 Smoking cessation therapies

Professional counselling for even extremely brief periods of time has been shown to significantly increase smoking cessation rates. If counselling is not sufficient, there are also a number of pharmacotherapies which are readily available as well as safe and effective.

Nicotine replacement therapy significantly increases long term smoking cessation and comes in many different forms, including nicotine gum, dermal patch, lozenge, inhaler and nasal spray (Lancaster et al. 2000). Anti-depressants, such as *bupropion* and *nortriptyline*, are also effective at increasing long term cessation rates when used in conjunction with counselling in an intervention program (Jorenby et al. 1999; Lancaster et al. 2000). Efficacious nicotinic acetylcholine receptor partial agonists, including *varenicline*, relieve nicotine withdrawal symptoms and reduce reward system activation (Jorenby et al. 2006; Nides et al. 2006; Hajek et al. 2009).

1.1.6.2 Bronchodilators

Bronchodilators are the main tool for symptomatic management in COPD patients and may be used prophylactically or to manage exacerbations. The main types of bronchodilator used are β_2-agonists, anti-cholinergics and methylxanthines. The normal route of administration is by inhalation due to diminished side effects compared to oral administration.

β_2-agonists relax airway smooth muscle by activating β_2-adrenoceptors. A number of studies have shown that regular treatment with long-acting bronchodilators is both more effective and convenient than short-acting (Dahl et al. 2001; Oostenbrink et al. 2004; Vincken et al. 2002).

Anti-cholinergics are muscarinic receptor antagonists, and may help to reduce mucus secretion as well as their main action of bronchodilation. Certain drugs in this class can achieve long duration bronchodilation, tiotropium bromide in particular may last up to 32 hours (Maesen et al. 1995). Long-acting anti-cholinergics have been shown to reduce the frequency of exacerbations in COPD patients (Niewoehner et al. 2005).

Theophylline is the most extensively tested drug in the methylxanthine class. Slow-release preparations have shown bronchodilator effects, however its relative toxicity means other bronchodilators are normally preferable. Theophylline does, however, exhibit anti-inflammatory effects at low concentrations, including decreased neutrophil numbers and TNF-α concentrations in sputum from COPD patients (Chen et al. 2008; Iiboshi et al. 2007).

Although all classes of bronchodilator have been shown to increase exercise capacity, none reduce the rate of long term decline in lung function.

Combining different classes of bronchodilator can increase the efficacy and duration of bronchodilation, removing the need for increasing the dose of individual drugs and thus reducing the risk of side effects. The combination of a β_2-agonist with anti-cholinergic and/or theophylline has been shown to produce improvements in lung function and patient status in some patients (Rabe et al. 2007).

1.1.6.3 Corticosteroids

Chronic inflammation is an important characteristic of COPD, and is thought to drive all main pathologies. Corticosteroids are highly effective at inhibiting inflammatory pathways in a wide variety of diseases including asthma, however they are of little, if any, benefit in COPD patients (reviewed in: Barnes 2010). There have been a multitude of clinical trials examining the effects of inhaled corticosteroids (ICS) on the pathology and progression of COPD. Four relatively large studies all found ICS had no effect on COPD progression as measured by decline in FEV_1 over a 3 year period (Burge et al. 2000; Group 2000; Pauwels et al. 1999; Vestbo et al. 1999). The 'Towards a Revolution in COPD Health' (TORCH) study, involving over 6000 patients, showed no overall effect of ICS (high dose fluticasone) on mortality rates in COPD, although there was in fact a statistically non-significant ~6% increase in death rates in the ICS group compared to controls (Calverley et al. 2007). Corticosteroid resistance has been demonstrated in COPD patients by failure of ICS treatments to reduce inflammatory markers in sputum and bronchial biopsies (Culpitt et al. 1999; Keatings et al. 1997; Loppow et al. 2001). The mechanism for this phenomenon is not fully understood, although there has been some suggestion that it may in part be due to a marked reduction in the expression of histone deacetylase-2 (HDAC-2) in COPD patients (Barnes 2010a).

The only strong evidence for a benefit of ICS in COPD patients, is in fact in the subset which exhibit concomitant asthmatic phenotypes (<10%), where ICS can reduce the number of sputum eosinophils (Brightling et al. 2000). Currently, however, over 70% of COPD patients are prescribed high dose ICS despite extensive side effects including reductions in bone density and increased incidence of pneumonia (Barnes 2010a).

1.1.6.4 Other Pharmacological Treatments

Although long acting bronchodilators and IHCs are the current mainstay of COPD therapy, there are a number of alternative pharmacological treatments which may be of much more clinical relevance to the disease.

Theophylline was used as a bronchodilator for COPD over 70 years ago, but fell out of favour as newer longer acting bronchodilators became available. Currently theophylline is only indicated as an alternative to the first and second choices: β2-agonists and anticholinergics (Vestbo et al. 2012), however its value in severe disease is recognised (Barnes 2006). Theophylline is thought to exert its bronchodilator effects through inhibition of phosphodiesterases, however there are many side effects associated with the therapeutic levels of this mechanism. There is however evidence for a clinical benefit at doses which are sub-therapeutic to bronchodilation, indicating an alternative action. There is now significant evidence that theophylline exerts anti-inflammatory effects in COPD, as evidenced by reduced neutrophils in sputum (Hirano et al. 2006).

Specific phosphodiesterase-4 (PDE4) inhibitors are also now in clinical use, albeit as a third alternative in a restricted subset of patients presenting with only chronic bronchitis (Vestbo et al. 2012). The mucolytic carbocysteine is also recommended as an alternative therapy in certain subsets of COPD patients (Macciò et al. 2009). There are a number of other pharmacological therapies either under development or in clinical trials, including specific chemokine antagonists, lipid antagonists (specifically LTB_4), cytokine inhibitors and antiproteases. These are reviewed in: (Barnes 2010b and Barnes 2012).

What is clear is that pharmacological control of COPD is extremely poor and represents an extensive unmet medical need. This lack of effective pharmacological treatments stems from a poor understanding of the biochemical basis and pathophysiology of COPD. It is hoped that further investment into the understanding of the disease mechanisms will elucidate novel and effective therapies to reduce the growing global burden of COPD. Until then the only options available to physicians and their patients include pulmonary rehabilitation and surgical intervention.

1.1.6.5 Pulmonary Rehabilitation

Pulmonary rehabilitation programs normally involve exercise training, nutrition, counselling and education (Rabe et al. 2007). Exercise training appears to help patients at all stages of COPD, improving exercise tolerance and reducing symptoms including dyspnoea and fatigue

(Nici et al. 2006). Improving patients' nutrition may also improve disease prognosis (Rabe et al. 2007). While rehabilitation may improve a patient's quality of life, it in no way addresses the underlying mechanisms of the disease, or halts its progression.

1.1.6.6 Surgical intervention

There are a number of surgical options to treat COPD, including bullectomy, lung volume reduction surgery and lung transplantation. Bullectomy involves removal of specific bullae (Latin for bubble) from the lung and may reduce patient dyspnoea (Mehran and Deslauriers 1995), whereas lung volume reduction surgery involves removal of larger emphysematous regions of lung tissue. A study of 1,200 patients reported that patients with upper lobe emphysema and low exercise capacity, who received lung volume reduction surgery had a greater survival rate after 4.3 years compared to patients who received pharmacological treatment (Naunheim et al. 2006). However benefits of lung reduction surgery are less certain in patients with other distributions of emphysema, or those with high exercise capacity. This together with the relative expense and inherent risk of surgery, mean lung reduction surgery can be recommended only for very few patients.

In patients with advanced (stage IV) COPD, full lung transplantation may improve patient quality of life and functional capacity (Hosenpud et al. 2000; Trulock 1997). However there are many issues associated with lung transplants, including post-surgical complications such as organ rejection, infection and side-effects to immune-suppressant medications (Patel et al. 2008; Trulock et al. 2007). The costs of lung transplant surgery are also extremely high, reportedly averaging $450,000 including post-operative care in the USA (Anyanwu et al. 2002; Ramsey et al. 1995).

1.2 Inflammation in COPD

1.2.1 Immune defence in the lung

The physiological role of the lungs necessitates a high degree of interaction with the external environment and, as such, the lungs come in to contact with many pathogens, allergens and pollutants. For this reason, the immune system provides a crucial role in maintaining lung function, by detecting and removing potentially harmful pathogens and chemicals.

The immune system is frequently divided into two sub-types: the innate response, and the adaptive response. The innate immune response, as its name suggests, refers to mechanism which protect the host from generic pathogens or particles in a non-specific manner. Specifically, this includes anatomical barriers, such as epithelium, mucus, saliva, tears and the cough reflex, as well as the recruitment of immune cells including neutrophils and macrophages. Increased numbers of neutrophils and macrophages are a hallmark of COPD, and are thought to be integral to the pathogenesis of the disease (Di Stefano et al. 1996; Saetta et al. 1997). The innate immune response is also responsible for activation of the adaptive immune system.

By contrast the adaptive immune system involves highly specialised cell-types and mechanisms, designed to recognise non-self antigens and develop an immunological memory against specific pathogens. Lymphocytes are one of the main conduits of the adaptive immune system, the importance of which to COPD is highlighted by increased numbers in the airways (Di Stefano et al. 1996). Lymphocytes can be classified into a number of subtypes, of which B cells and CD8+ T cells are thought to be of particular importance in COPD (Hogg 2004).

1.2.2 Epithelium

The epithelium is a key cell type in the immunological defence of the lung, as it is the point of first contact with the majority of inhaled pathogens and chemicals. Epithelial cells express a number of innate immune receptors, including toll-like receptors (TLRs). In fact it has been reported that expression of TLR4, a receptor for innate defence against bacteria, is

negatively regulated by CS exposure in the nasal epithelium (MacRedmond et al. 2007). It has further been reported that epithelial cells are involved in initiating the innate immune response to CS by releasing pro-inflammatory mediators including TNFα, IL-1β, and IL-8, leading to the recruitment of leukocytes including macrophages and neutrophils (Hellermann et al. 2002; Mio et al. 1997; Mortaz et al. 2011). As well as releasing pro-inflammatory cytokines, it has been shown that epithelial cells are also sensitive to released cytokines, for example IL-1β stimulation induces IL-8 release (Coulter et al. 1999). For this reason it has been suggested that epithelial cells may play a role in prolonging the inflammatory response in COPD and, indeed, it has been shown that epithelial cells isolated from COPD patients release higher levels of IL-8 compared to those isolated from smokers without obstructive lung disease and non-smokers (Schulz et al. 2003).

1.2.3 Leukocytes in COPD

COPD encompasses three distinct pathologies in three different areas of the lung, the uniting feature of which is inflammatory cell infiltration. Leukocytes are the main effector cells of the innate and adaptive immune systems and, indeed, increased numbers of neutrophils, macrophages and lymphocytes are found in bronchial biopsies, small airways and lung parenchyma from patients diagnosed with COPD, and provide a hallmark of the disease.

1.2.3.1 Neutrophils

Neutrophils are rapidly recruited to the lung by release of specific chemokines, including GROα (CXCL1) and IL-8 (CXCL8), in response to infection or damage. Increased neutrophil levels are found predominantly in the sputum and BALF of COPD patients (Di Stefano et al. 1998). Parenchymal concentrations are not generally found to increase as much, possibly due to the rapid nature of neutrophil infiltration into the airway lumen. A positive correlation has been found between neutrophil levels and severity of disease, indicating a role in disease progression (Keatings et al. 1996; Di Stefano et al. 1998).

This correlation is likely to be due to the mediators released by neutrophils, including neutrophil elastase and reactive oxygen species (ROS), both of which have in fact been shown to induce MUC5AC expression at both the mRNA and protein level (Fischer and Voynow 2000; Voynow et al. 1999), a protein related both to mucus hypersecretion and COPD (Wang et al. 2012). Neutrophil elastase is also known to be an important factor in development of emphysema; mice genetically deficient for neutrophil elastase were protected from CS-induced emphysema (Shapiro et al. 2003), and humans deficient for α1-antitrypsin are extremely susceptible to emphysema (Stoller and Aboussouan 2012).

Chronic neutrophilia is therefore one of the important features associated with the progression and pathophysiology of COPD. In the healthy lung neutrophil recruitment is quickly followed by infiltration of macrophages, which clear away the neutrophils. However despite high numbers of macrophages in COPD, the high levels of neutrophils also persist.

1.2.3.2 Macrophages

Macrophages are derived from monocytes in the circulatory system, which migrate to a specific tissue before differentiating. Once differentiated there is little evidence of proliferation, and they are generally longer lived than neutrophils (Gordon and Taylor 2005). The number of macrophages is significantly increased in COPD patients compared to controls, normally 5-10 fold, in the airways, parenchyma, BALF and sputum (Finkelstein et al. 1995). As with neutrophils, the number of macrophages in the lung tissue was also found to correlate with severity of COPD (Di Stefano et al. 1998), indicating an important role in the pathogenesis of the disease.

One of the main functions of macrophages is their phagocytic activity to remove apoptotic cells as well as inhaled foreign bodies. Decreased phagocytic activity, however, has been found in macrophages isolated from COPD patients (Hodge et al. 2003; Vecchiarelli et al. 1991), and this has been suggested as a possible contributor to increased infection rates in COPD patients (Donnelly & Barnes 2012). Macrophages are also responsible for clearing neutrophils in the lung, so dysfunction may similarly contribute to non-resolving neutrophilia associated with COPD.

Apart from their phagocytic role, macrophages are also known to produce mediators associated with inflammation and COPD (Tetley 2002). Indeed, macrophages are known to produce a number of elastolytic enzymes associated with the development of emphysema, including MMP-1, MMP-2, MMP-9 and MMP-12, as well as cathepsins and elastase (Punturieri et al. 2000; Russell et al. 2002). It is notable that the expression levels of MMP-1 and MMP-9 in particular were found to be increased in macrophages isolated from emphysema patients compared to controls (Finlay et al. 1997).

A number of pro-inflammatory mediators including cytokines and ROS, are also released by macrophages (Schaberg et al. 1992). There have been many reports from a number of groups showing increased cytokine, chemokine and ROS production from macrophages in response to CS stimulation, however there is a huge variability in the responses seen with regard to both mediator profiles and mediator levels (reviewed in: Smith et al. 2010). This variability is most likely due to the lack of a standardised *in vitro* CS exposure protocol between research groups. Despite much research, the mechanisms of CS induced mediator production and phagocytosis deficiency remain poorly understood.

1.2.3.3 Lymphocytes

Lymphocytes are commonly divided into natural killer cells and T-cells and B-cells. In particular CD8+ T-cells and B-cells are thought to play important roles in the development and progression of COPD. Whereas neutrophils and macrophages are normally associated with the innate immune system, T-cells and B-cells are canonically thought of as major effectors of the adaptive immune system. T-cells are so named because they mature in the thymus, and it is the CD8+ subset which are predominantly detected in COPD patient airways (Saetta et al. 1998) and lung tissue (Majo et al. 2001). There is a particular increase in Th1 and Tc1 cells secreting IFN-γ (Barnes 2009).

Named after the 'Bursa of Fabricius' where they were first discovered to mature in birds, B-cells are derived instead from the bone marrow in most mammals, which generally do not have an equivalent organ. B-cells play a crucial role in producing antibodies against specific antigens and developing what is often termed 'immunological memory'. B-cells are notable

for being associated specifically with more severe COPD (Hogg et al. 2004; Barnes 2008). It is thought that B cells are likely to be activated by the repeated and persistent bacterial and viral infection characterising COPD (Barnes 2008). However it has alternatively been suggested that cigarette smoke damaged cells may lead to the development of new antigenic epitopes, leading to an autoimmune element in COPD involving B-cells (Agustí et al. 2003).

1.2.4 Inflammatory mediators in COPD

Mediators are thought to play a key role in both the direct damage and remodelling of lung tissue as well as the orchestration and progression of COPD. The mediators thought to be important in COPD include cytokines, chemokines and growth factors. The most extensively researched of these mediators are discussed below.

1.2.4.1 IL-1β and IL-18

IL-1β and IL-18 are two members of the IL-1 cytokine family, released *in vivo* by the caspase-1 dependent cleaving of their pro-forms. IL-1β activates macrophages, causing them to release other inflammatory cytokines and matrix metalloproteinase (MMP) 9. COPD patients exhibit an increase in IL-1β and IL-18 levels in their sputum, and furthermore these levels correlate with disease severity (Rovina et al. 2009; Sapey et al. 2009). Serum levels of IL-1β are also increased in COPD patients and negatively correlate with FEV_1 (Singh, Arora, and Khanna 2010). COPD patients also exhibit increased IL-18 expression in skeletal muscle, implying a role in the systemic manifestations of the disease, including muscle wasting (Petersen et al. 2011). These findings may suggest a prominent role for IL-1β and IL-18 in COPD progression.

Inducible expression of both human IL-1β and IL-18 have also been shown to induce lung inflammation, emphysema and fibrosis in murine systems (Lappalainen et al. 2005).

1.2.4.2 IL-8 and other chemokines

The chemokine IL-8, also known as CXCL8, is a potent chemo-attractant for neutrophils which exerts pro-inflammatory effects through chemokine receptors CXCR1 and CXCR2. A number of inflammatory agents, including other cytokines, microbes and environmental changes cause the production of IL-8 from a variety of cell types such as epithelial cells, macrophages and neutrophils (Mukaida 2003). IL-8 levels were found to be significantly increased in induced sputum samples from COPD patients, and furthermore the IL-8 levels were found to correlate with neutrophilia (Keatings et al. 1996). IL-8 is further increased during acute exacerbations of COPD, again correlating with increased neutrophilia during these periods (Crooks et al. 2000; Gompertz et al. 2001). It is also noteworthy that patients suffering from α1-antitrypsin deficiency induced emphysema exhibit increased neutrophil activity and correspondingly increased IL-8 levels (Woolhouse et al. 2002).

IL-8 specific antibodies were found to reduce the chemotactic effect of induced sputum obtained from COPD patients on neutrophils (Beeh et al. 2003), however in a clinical trial another monoclonal antibody for IL-8 showed no benefit in health status, lung function, or exercise tolerance, and only a modest effect on dyspnoea (Mahler et al. 2004).

A number of other chemokines have been found at increased concentrations in the induced sputum from COPD patients including CXCL1 (GROα) and CCL2 (MCP-1) (Traves et al. 2002), as well as CXCL9 (MIG), CXCL10 (IP-10) and CXCL11 (I-TAC/IP-9) which are all IFN-γ induced chemokines with affinity for the CXCR3 receptor (Costa et al. 2008).

1.2.4.3 IL-6

Although IL-6 may exert both a pro- and anti-inflammatory effect, it is believed to fulfil a predominantly pro-inflammatory role in COPD. Anti-inflammatory effects are mediated through the inhibition of TNF-α and IL-1 cytokines, as well as activation of IL-10, however it is mainly released from T cells and macrophages in response to pathogen associated molecular patterns (PAMPs). COPD patients exhibit higher concentrations of IL-6 in sputum, BALF and blood which may be further increased during exacerbations (Bhowmik et al. 2000; Bucchioni et al. 2003; Song et al. 2001). Unlike most cytokines, IL-6 is very stable in

circulation, prompting the theory that it may be involved in systemic features of COPD as well as comorbidities (Barnes & Celli 2009).

1.2.4.4 TNF-α

Tumour Necrosis Factor (TNF) –α is involved in immune system regulation and is secreted by a wide variety of cell types, including epithelial, macrophages, T lymphocytes and airway smooth muscle cells. COPD patients exhibit increased levels of TNF-α in their sputum, especially during exacerbations (Aaron et al. 2001; Keatings et al. 1996). Furthermore a murine model of cigarette smoke driven emphysema implicated a particularly prominent role for TNF-α. Despite these findings, anti-TNF therapies, notably the blocking antibody *infliximab*, have so far not been able to reduce symptoms or improve lung function in COPD patients (Rennard et al. 2007). There is also evidence that *infliximab* may increase the risk of pneumonia and respiratory tract cancer (Barnes 2009).

1.2.4.5 Growth Factors

Several cytokines promote proliferation of structural cells and/or survival and differentiation of inflammatory cells. These cytokines are frequently referred to as growth factors, although the term may be used to include hormones as well. Cytokine growth factors may contribute to airway remodelling, a prominent feature of small airways disease and the key process in airway resistance.

Transforming growth factor (TGF) –β can be generated through oxidative stress and provides a multitude of functions including immunoregulation, proliferation of smooth muscle cells and fibroblasts, as well as extracellular matrix formation. TGF-β related genes are up-regulated in the small airways of COPD patients, and are thought to play a key role in small airway fibrosis, which is a major cause airway obstruction in COPD (Wang et al. 2008).

Granulocyte-macrophage colony-stimulating factor (GM-CSF) has been implicated in COPD and may be involved in the increased survival of airway neutrophils and macrophages in the disease (Vlahos et al. 2006). It has further been demonstrated that increased GM-CSF

concentrations in the BALF of COPD patients positively correlate with increased neutrophil burden, particularly during exacerbations (Balbi et al. 1997).

A number of other growth factors have been implicated in COPD, including epidermal growth factor (EGF) and vascular-endothelial growth factor (VEGF), which may be related to MUC5AC expression and vascular leak (Barnes 2009).

1.2.5 Oxidative stress

Due to the oxygen rich environment that supports terrestrial life on our planet, human lungs are constantly exposed to exogenous oxidative stress, and are therefore adapted to dealing with these baseline levels. These adaptations include the synthesis of enzymatic and non-enzymatic anti-oxidants which remove free radical intermediates. Oxidative burden in the lungs however can be dangerously increased by exposure to inhaled pollutants, including cigarette smoke (CS), industrial pollution and smoke from biomass fuels. It has in fact been estimated that CS contains up to 10^{15} free radicals per puff (Pryor and Stone 1993). It has been theorised that persistent exposure to abnormally high levels of inhaled oxidants leads to an oxidant/anti-oxidant imbalance in the lungs, leading to protein and lipid oxidation, endoplasmic reticulum (ER) stress as well as cell death (Babior 2000). Reactive oxygen species (ROS) have also been demonstrated to mediate the release of pro-inflammatory cytokines, including IL-8 from monocytes through activation of transient receptor potential (TRP) M2 (Yamamoto et al. 2008).

These inflammatory mediators can initiate an inflammatory response including the recruitment of neutrophils and macrophages. As previously mentioned, a key function of neutrophils and macrophages is to release ROS as a defence mechanism against inhaled pathogens, and macrophages isolated from smokers release more ROS than those from non-smokers (Schaberg et al. 1992). This may cause further oxidant/antioxidant imbalance which it is thought may be a factor in the self-propagating nature of COPD. It has further been demonstrated that markers of oxidative stress in the lungs are elevated in COPD patients over levels in both smoking and non-smoking controls (Ceylan et al. 2006; Kanazawa and Yoshikawa 2005). Oxidative stress has also been shown to cause stimulation

of mucus hypersecretion and anti-protease inactivation, which may be important features of COPD pathophysiology (Bowler et al. 2004).

1.2.6 Protease/anti-protease imbalance

Protease/anti-protease imbalance has been a long standing theory in the pathogenesis of COPD and, in particular, emphysema development. Oxidative stress in the lung can simultaneously cause both the release of endogenous proteases and the inactivation of endogenous anti-proteases.

The expression of proteases neutrophil elastase and matrix-metalloproteinases (MMPs) -1, -9 and -12 are reported to be up-regulated in the lungs of COPD patients (Damiano et al. 1986; Finlay et al. 1997). The role of these proteases has since been confirmed using animal models. Mice lacking neutrophil elastase or MMP-12 were protected against CS-induced emphysema (Shapiro et al. 2003; Hautamaki et al. 1997). In particular neutrophil elastase is implicated in the degradation of elastin in the parenchyma. This is thought to contribute to both air-space enlargement and destruction of alveolar attachments leading to small airway narrowing. Another important family of proteases in the pathogenesis of COPD are the cysteine proteases, including cathepsins and caspases. Caspase-1 is responsible for cleaving pro-IL-1β to its mature form, IL-1β, a pathway that is key to development of CS-induced inflammation in murine models (Eltom et al. 2011)

Of equal importance to an over-abundance of proteases is a corresponding deficiency of anti-proteases. This is evidenced by the subset of COPD patients which develop the disease due to α1-antitrypsin deficiency, a relationship first documented as early as 1970 (Larson et al. 1970). Another anti-protease strongly linked to COPD is Tissue Inhibitor of MMPs (TIMP)-1 and, it has been reported that, macrophages isolated from the lungs of COPD patients were found to release less TIMP-1 than those of smokers with normal lung function after LPS stimulation (Pons et al. 2005). However, the total levels of TIMP-1 in sputum were found to be increased in sputum from COPD patients compared to both asthmatics and control subjects (Culpitt et al. 2005). Furthermore, these sputum TIMP-1 levels in COPD patients correlated with neutrophil percentage. It is therefore hypothesised that there is a net

deficiency in anti-proteases compared to proteases, leading to damage of the airways and parenchyma, which is thought to play a crucial role in the progressive obstruction of airflow which characterises COPD.

1.3 Models of COPD / airway inflammation

There are no therapies currently available which halt the progression of COPD, or provide any significant reduction in mortality rates associated with this disease (reviewed in section 1.1.6). This is largely due to a lack of understanding in the biological mechanisms which cause and drive the progression of COPD. There is an increasing focus on elucidating these mechanisms, as the increasing global burden of the disease is realised. Developing models of COPD and COPD-like inflammation is proving to be a useful tool in the attainment of this goal, although they are not without limitation. These models can broadly be categorised into two groups: *in vitro* and *in vivo*, and the following section summarises the main models that have been developed and some of the major findings discovered to date.

1.3.1 *In vitro* models

The two main methods employed *in vitro* to assess the effects of cigarette smoke on cells, are the application of single compounds thought to be prominent in the pathological effects (e.g., nicotine), and the generation of tobacco smoke extracts, where whole cigarette smoke is used to condition the cell medium, however both methods have drawbacks. Tobacco smoke is a complex mixture of over 4000 chemicals, so using a single chemical will inevitably miss many of the important effects and interactions that tobacco smoke elicits *in vivo* and which are important for disease manifestation. Cigarette smoke extracts (CSE) frequently exhibit poor reproducibility within experiments (Bernhard et al. 2004), and there is no standardised method, leading to major differences in protocols and results between research groups (Krimmer and Oliver 2011).

One of the benefits of examining the effects of CSE *in vitro* compared to *in vivo* is the ability to differentiate the response of specific cell types. Many different cell types are involved in

the pathogenesis of COPD, including macrophages, neutrophils, epithelial cells, fibroblasts, as well as sub-types thereof, and the interaction between these cell types is likely to play a pivotal role in disease progression.

In human epithelial cells CSE has been shown to decrease cell proliferation and migration (Wang et al. 2001), decrease cilia beat frequency (Cohen et al. 2009) and decrease glutathione levels (Rusznak et al. 2001) but increase many inflammatory mediators including MMP-1 (Mercer et al. 2004), IL-1β (Rusznak et al. 2001), Il-6 and IL-8 (Beisswenger et al. 2004) and growth factors (Rumelhard et al. 2007), as well as increasing mucin production (Kreindler et al. 2005). In A549 cells (a human alveolar type II cell line), CSE was also found to increase apoptosis (Hoshino et al. 2001), as well as increasing ROS production (van der Toorn et al. 2009). In human fibroblasts, CSE has been shown to increase apoptosis (Baglole et al. 2006), production of MMPs (Mercer et al. 2004; Yin et al. 2000), activation of COX-2 (Martey et al. 2004) and consequently release of PGE_2 (Profita et al. 2010).

In primary human macrophages, conversely, CSE has been found to inhibit many pro-inflammatory mechanisms. CSE decreased pathogen clearance (Ryder et al. 2002), and in human macrophage cell line, THP-1 cells, CSE inhibited LPS-induced cytokine release (Birrell et al. 2006).

It is likely that complex cell culture techniques including two or more COPD relevant cell types will further elucidate the inflammatory mechanisms, by providing information on the cross-talk between cell types.

1.3.2 *In vivo* models

COPD is an extremely complex disease with a variety of interacting cell types, all of which are crucial to the development of the disease. Animal models of COPD therefore have the advantage over *in vitro* models in providing a system where these interactions are intact. Furthermore *in vivo* models allow the assessment of more clinically relevant endpoints, including cellular burden in the airways and lung tissue, development of emphysema and lung function assessments. To date a number of disease relevant stimuli have been used to

induce models of COPD, or COPD-like airway inflammation (reviewed in Stevenson & Birrell 2011), of which the most popular are discussed below.

1.3.2.1 Cigarette smoke exposure

A number of predominantly small animals have been used to model COPD induced by exposure to cigarette smoke (CS), including guinea-pigs, rats and mice. Mice have become the most popular choice, due to the availability of an extensive range of 'knock-outs'. Knock-out (KO) mice have a specific gene either deleted or modified to become inactive, allowing the function of said gene to be determined in both normal physiological conditions and under specific stimulations.

The link between CS exposure and development of COPD was first demonstrated in animal models in 1990 when, Wright and Churg, demonstrated progressive emphysematous changes and associated lung function decline in CS exposed guinea-pigs (Wright & Churg 1990). Since then, many groups have demonstrated other COPD relevant phenotypes after CS exposure, including leukocyte recruitment, mucus cell metaplasia and fibrotic remodelling (Stevenson and Birrell 2011).

Many *in vivo* CS exposure models have now shown two distinct phases of inflammation. An initial phase is characterised by the acute neutrophilic infiltration, peaking after between 3 and 5 days of CS exposures. After this initial phase a more chronic phase begins, normally after 2-4 weeks of exposure, involving macrophages and lymphocytes as well as neutrophils (Eltom et al. 2011; Stevenson et al. 2007). These three cell types are all increased in the lungs of COPD patients, and are thought to be central to the pathogenesis. Furthermore the inflammation has been shown to be slowly resolving, which suggests a COPD-like phenotype. A time-course study where rats were exposed to CS for six months, showed that inflammatory markers were still elevated a further two months after the final CS exposure (Stevenson et al. 2007). Many models have also reported steroid-insensitivity, possibly due to oxidative stress, which has been widely advocated as a mechanism in steroid-insensitivity in COPD (Marwick et al. 2004; Ito et al. 2005).

Various groups have reported the importance of many mediators in these CS-induced small animal *in vivo* models, including cytokines TNFα, IL-1β, IL-18, IFNγ, chemokine receptors CXCR2, CXCR3, and enzymes including MMPs, PDE4 and serine proteases among others (reviewed in: Churg et al. 2008). Data obtained from these models has provided a great deal of information about the mediator pathways that are involved in CS induced inflammation. The mechanism(s) responsible for the initiation of these responses however remain at present unknown (Stevenson and Birrell 2011).

1.3.2.2 LPS

Lipopolysaccharide (LPS) is an integral component of the cell membrane of gram-negative bacteria which activates the innate immune system through TLR4. When administered to the lungs, LPS induces a robust neutrophilic infiltration, which peaks between 6 and 24 hours after challenge (Ferretti et al. 2003). The neutrophil is the main source of neutrophil elastase in COPD which was classically regarded as the most important contributor to the development of emphysema. For this reason the LPS model of acute lung injury has previously been used in drug discovery for broad spectrum anti-inflammatories to treat chronic inflammation (Stevenson & Birrell 2011).

It is now widely recognised that the inflammatory phenotype induced by *in vivo* LPS challenge differs significantly from that in COPD. LPS induced inflammation is neither progressive nor slowly resolving. It is in fact rapidly resolving and steroid sensitive (Birrell et al. 2005). The LPS model of airway inflammation is therefore no longer used as a COPD-like model, but does provide an innate mechanistic setting for contrasting with more disease relevant models. LPS has also been used in conjunction with CS exposure to model bacterial exacerbations and the effect of CS on the innate immune response.

1.3.2.3 Elastase

As mentioned previously, elastase fulfils an important pathophysiological role in the development of emphysema. Instillation of elastase into the lungs *in vivo* has been long used as a model of emphysema, but can also lead to alveolitis, mucus cell metaplasia, pulmonary oedema and consequently changes in pulmonary function consistent with COPD patients (Birrell et al. 2005b). The model does have a number of drawbacks however, in that inflammation does not drive the physiological changes but, rather, is a secondary transient effect which resolves quickly. Indeed the inflammation only occurs for one or two weeks after elastase administration while the emphysematous changes are progressive for up to 8 weeks. The rapid onset of alveolar destruction after elastase instillation does, however, provide a useful model for testing therapies aimed at reversing emphysema (Massaro & Massaro 1997).

1.4 TRP Channels

Transient Receptor Potential channels are a superfamily of cation channels, of which there are 28 known mammalian members (Banner et al. 2011). The mammalian members of the TRP family are frequently divided into six subfamilies based predominantly on sequence homology (Clapham 2003). The six families are TRPA, named after the high number of ankyrin repeats, TRPC the canonical family, TRPM (melastatin), TRPML (mucolipin), TRPP (polycystin) and TRPV (vanilloid). The families are often divided into two groups, based on sequence and topological differences. Mammalian Group 1 channels include those of the TRPC, TRPA, TRPM and TRPV families; while Group 2 includes those of the TRPML and TRPP families (Venkatachalam and Montell 2007). This thesis addresses only members of Group 1.

Although there are many differences between TRP subfamilies, as well as between members of the same family, all members of the six subfamilies have a number of features in common. The TRP channels are so named for the well conserved TRP domain common to all the channels and consisting of 23-25 amino acids. All members are six-transmembrane spanning proteins with N- and C- termini on the intracellular side and have a loop between the fifth and sixth transmembrane segments, thought to facilitate pore formation to allow cation transport (Ramsey et al. 2006). Current evidence suggests that TRP channels are formed by four subunits and active channels could assemble as homo- or hetero-tetramers (Latorre et al. 2009).

A single TRP channel can be activated through many seemingly disparate mechanisms (Venkatachalam and Montell 2007), and so they are often thought of as multiple signal integrators. Activation mechanisms include exogenous stimuli, endogenous stimuli, extracellular mediators, intracellular mediators, membrane depolarisation and others, as shown in Figure 1.1. Many TRP channels are also known to be involved or implicated in disease processes which characterise COPD, as well as in other inflammatory diseases and normal inflammatory processes.

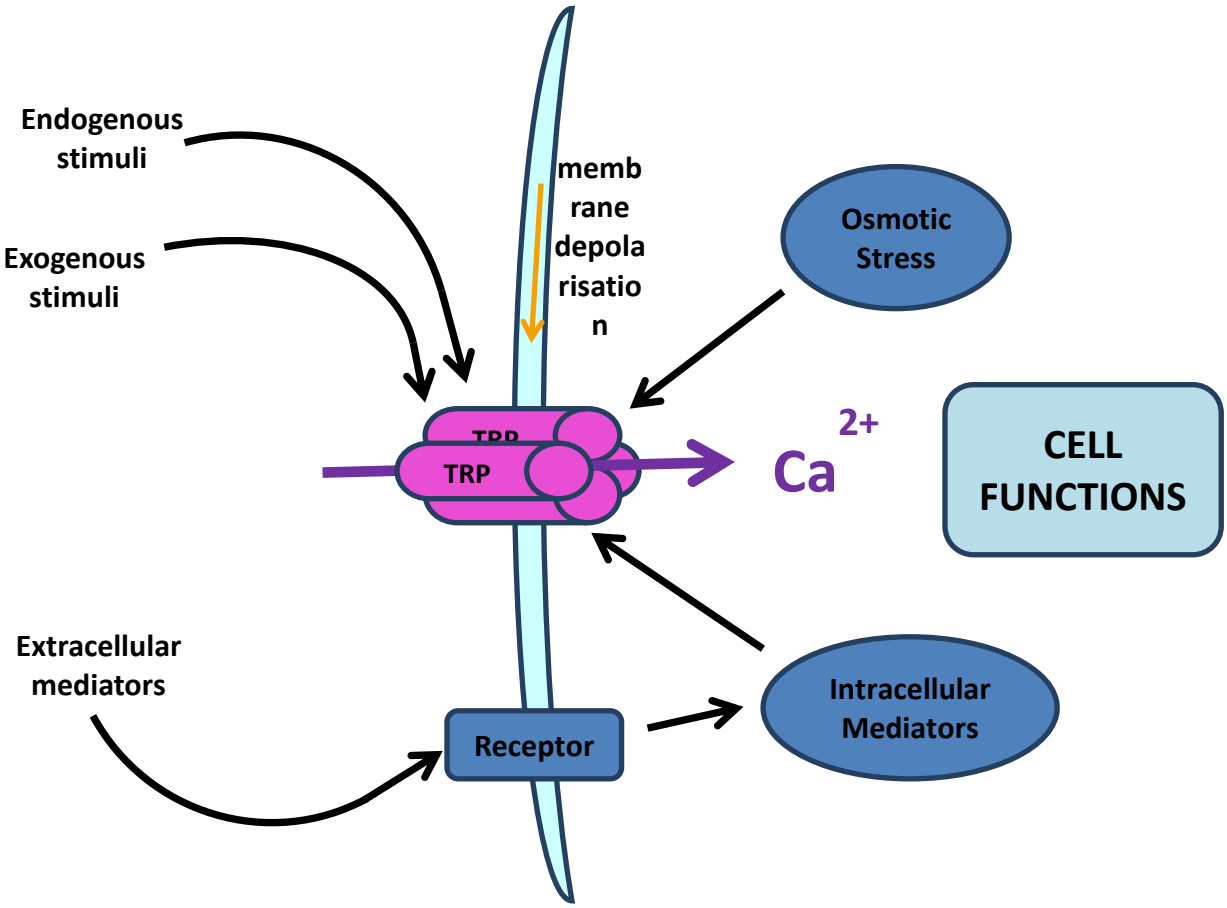

Figure 1.1 Diagram showing the range of mechanisms and stimuli by which individual TRP channels may be activated. *TRP channels arrange as tetramers in the cell membrane. Their activation may be modulated by endogenous stimuli, exogenous stimuli, extracellular mediators, intracellular mediators, membrane depolarisation and physical stimuli such as osmotic stress and temperature. Different members of the TRP channel family are activated by different chemical and physical stimuli. Once activated, the tetrameric TRP channel forms a pore through which cations such as Ca^{2+} may pass, thus eliciting a wide range of cellular functions.*

1.4.1 Regulation and Activation of Mammalian Group I TRP channels

TRPC channels are so named as they are the most homologous to the first identified member of the superfamily, *Drosophila TRP* (Montell and Rubin 1989). There have so far been seven mammalian TRPC members identified, numbered 1-7, all of which are expressed in mice, although, TRPC2 is a pseudo-gene in humans (Wes et al. 1995). TRPC1, 4, 6 and 7 are expressed fairly widely, whereas TRPC3 and 5 are more highly expressed in the central nervous system (CNS). TRPC6 is notably highly expressed in human lungs (Corteling et al. 2004). Activation of all TRPC channels is dependent on phospholipase C (PLC), although there are thought to be some variations in coupling mechanisms (Montell 2005; Venkatachalam and Montell 2007). For example only TRPC3, C6 and C7 require DAG for activation (Hofmann et al. 1999). Other mechanisms for activation of canonical TRP channels include store depletion, conformational coupling and exocytosis. There is evidence for all channels apart from C6 being activated by Ca^{2+} store depletion, whereas exocytosis only modulates the activity of C3, C4 and C5 channels.

TRPV (Vanilloid) subfamily was so named for the reactivity of the founding member (TRPV1) to the inflammatory vanilloid compound capsaicin (Caterina et al. 1997). The rest of the TRPV subfamily members are so categorized for sequence homology. A prominent feature of TRPV channels 1-4 is activation by specific temperature ranges. TRPV1 and TRPV2 are activated by noxious temperatures of >43°C and >52°C respectively (Caterina et al. 1997, 1999). TRPV1 and TRPV2 are however expressed in distinct subsets of dorsal root ganglion neurons allowing individual responses to different temperatures (Caterina et al. 1999). Activation of TRPV3 and TRPV4 is increased at warm temperature ranges of 33-39°C and 27-34°C respectively (Montell 2005). TRPV channels 1-4 are all activated by many other mechanisms as well as temperature, and are thought to play a role in integrating these often disparate signals. TRPV1 is known to be activated by many chemicals including endocannabinoids (Zygmunt et al. 1999), anandamide and many pungent chemicals such as piperine (McNamara et al. 2005). The activation threshold of TRPV1 to its ligands can be lowered by low pH, ethanol, nicotine and proinflammatory cytokines (Caterina et al. 1997; Trevisani et al. 2002). TRPV1 may also be potentiated by bradykinin and nerve growth factor (NGF), through PLC activation leading to PKC-mediated phosphorylation of the channel (Montell 2005). TRPV2 activation is potentiated by phosphatidylinositol-3-kinase (PI3K).

TRPV3 is activated by PLC, arachidonic acid and by compounds found in many common cooking spices including oregano and cloves (Xu et al. 2006). TRPV4 is notably gated by extracellular hypotonicity causing cell stretch, as well as epoxyeicosatrienoic acids formed by cytochrome P450 (Watanabe et al. 2003).

TRPV5 and TRPV6 are unlike the other members of this subfamily as they are not thermoceptors. They are also the most selective for Ca^{2+} over Na^{2+} of any of the mammalian TRP channels (Montell 2005).

TRPV1, 2 and 3 expression is highest in the CNS, particularly the vagal ganglia and dorsal root ganglia (DRG). TRPV4 is relatively more widely expressed, including kidney, lung, heart, liver and endothelial tissues, as well as DRG. TRPV5 and 6 are both expressed in the intestines, pancreas and placenta, and TRPV5 only is expressed in the kidneys (Venkatachalam and Montell 2007).

Members of the TRPM subfamily of channels do not possess the N-terminal ankyrin domains which characterise both the Canonical and Vanilloid families.

TRPM1 and TRPM3 are frequently referred to as a subset of the TRPM family due to high amino acid sequence similarity. TRPM1 was the first mammalian TRPM channel identified and was named *melastatin* due to an inverse correlation with the metastatic potential of certain melanoma cell lines (Duncan et al. 2001). High levels of TRPM3 expression have been found in brain and kidney tissues, and TRPM3 transfected HEK293 cells were found to exhibit constitutive Ca^{2+} and Mn^{2+} entry using patch-clamping (Grimm et al. 2003).

TRPM4 and TRPM5 are highly similar in terms of amino acid sequence homology and channel function. They are both monovalent cation selective channels and are voltage modulated as well as activated by PIP_2 (Hofmann et al. 2003; Launay et al. 2002). TRPM4 and TRPM5 do however have different expression patterns, with highest levels of TRPM4 found in the prostate, colon, heart, kidney and testis, whereas highest levels of TRPM5 are found in the intestines, liver and lungs.

TRPM6 and TRPM7 channels are frequently referred to as chanzymes, because as well as being cation channels, they also possess C-terminal protein kinase domains (Schlingmann et

al. 2002; Walder et al. 2002). Both are selective for divalent cations, normally Ca^{2+} and Mg^{2+}, but also trace metals such as Ni^{2+} (Monteilh-Zoller et al. 2003).

TRPM2 and TRPM8 are most similar to each other and not particularly similar to any of the subsets mentioned above. High levels of TRPM2 expression are found in the brain, bone marrow, spleen, and leukocytes. Key activators of TRPM2 include ADP-ribose, cADP-ribose, pyrimidine nucleotides, arachidonic acid, NAD and oxidative stress (H_2O_2). It should be noted that TRPM2 is also a chanzyme, possessing a C-terminal ADP-ribose pyrophosphatase domain (Perraud et al. 2001; Sano et al. 2001). TRPM2 is frequently thought of as a redox sensor, owing to its activation by both oxidative and nitrative stress (Hara et al. 2002).

TRPM8 is unlike any of the other melastatin sub-family channels in activation and function, in that it is a thermoceptor, activated by physiologically cool temperatures in the range of 23-28°C (McKemy et al. 2002). TRPM8 is also activated by compounds such as menthol, icilin and eucalyptol, which all thus evoke a cooling sensation (Peier et al. 2002). The mechanism of action of cold is known to be separate to that of compounds as several characterised mutations affect one but not the other (Bandell et al. 2006). TRPM8 is also regulated by PIP_2 (Liu and Qin 2005). The highest levels of TRPM8 expression are found in the DRG and trigeminal ganglia as well as the prostate and liver (Bidaux et al. 2005; Nealen et al. 2003).

TRPA1 is the only mammalian member of its sub-family, and was first identified in a screen for down-regulated genes after oncogenic transformation of fibroblasts (Jaquemar et al. 1999). TRPA1 is characterised by an exceptionally high number of N-terminal ankyrin repeats. TRPA1 is activated by a number of pungent chemicals and environmental irritants, including isothiocyanites found in mustard oils, cinnamaldehyde found in cinnamon, and acrolein the active ingredient in tear gas. TRPA1 is also activated by bradykinin through PLC stimulation and subsequent metabolites, such as DAG and poly-unsaturated fatty acids (Bandell et al. 2004). There is also evidence that TRPA1 is gated by noxious cold (<17°C), although this is controversial in (Bandell et al. 2004; Bautista et al. 2006; Jordt et al. 2004; Kwan et al. 2006).

1.4.2 Hetero-multimerisation of TRP channels

An important feature of TRP channels relevant to their function is that the more closely related members can and, frequently, do form functional heterotetramers. This hetero-multimerisation is thought to modulate the function, localisation and biophysical properties of pore forming channels (Venkatachalam and Montell 2007).

TRPC1 may form heterotetrameric channels with TRPC5 when co-expressed in cultured cells. These TRPC1/5 channels possess different conductances and biophysical properties than either TRPC1 or TRPC5 homotetramers (Strübing et al. 2001). Hetero-multimerisation has also been evidenced *in vivo*; for example biochemical analyses have identified heterotetramers involving TRPC1, C4 and C5 in rat embryonic brains (Strübing et al. 2003). There is further evidence of TRPC1 interacting with TRPC3/6 in rat embryonic brain as well (Strübing et al. 2003). The variety of different combinations of TRPC channel multimers provides scope for an extensive diversity in cation channels with distinct characteristics and biological functions, the true depth of which is not well understood.

Members of other mammalian TRP subfamilies exhibit similar hetero-multimerisation properties, including the Vanilloid and Melastatin classes. For example TRPV5/6 may coassemble (Hoenderop et al. 2003), as may TRPM6/7, again forming channels with distinct characteristics such as conductance larger than either homomultimer (Chubanov et al. 2004). Hetero-multimerisation may also be important in the localisation of channels, as specific mutations which disrupt the interaction of TRPM6 and TRPM7 channels prevent trafficking of TRPM6 to the plasma membrane (Chubanov et al. 2004).

1.5 TRP Channels and Airway Inflammation

Of the 21 members of the mammalian group I TRP channel family currently known to be functionally expressed in humans, a number have been implicated in the generation of airway inflammation, which is the underlying pathological mechanism driving the progression of COPD. These channels most notably include TRPA1, TRPC6, TRPM2, TRPM8, TRPV1 and TRPV4. These six channels are discussed in detail in the following section, and their expression in key cell-types which are important in the development and progression of COPD is highlighted in Figure 1.2.

1.5.1 TRPA1

TRPA1 is a good candidate for a role in COPD as it is activated by some of the key inflammatory components of the primary cause of disease, cigarette smoke, including acrolein and crotonaldehyde. Bronchial rings from guinea-pigs were shown to contract to cigarette smoke extract (CSE), acrolein and crotonaldehyde, and this contraction was inhibited by specific TRPA1 antagonist HC-030031, but not by TRPV1 antagonist capsazepine or by reactive oxygen scavengers, suggesting that TRPA1 is the predominant channel sensing these inflammatory agents (Andrè et al. 2008). TRPA1 can also be directly activated by prostaglandins by covalent modification of the N-terminus cysteine residues (Grace et al. 2012; Takahashi et al. 2008) as well as by products of lipid peroxidation (Macpherson et al. 2007; Trevisani et al. 2007), which may be mediators in cigarette smoke induced inflammation.

TRPA1 is known to be an important mediator of cough, a prominent and problematic symptom of COPD. TRPA1 agonists including acrolein, cinnamaldehyde and crotonaldehyde produced dose-dependent cough responses in guinea-pigs, which were inhibited by HC-030031 (Birrell et al. 2009). There is also evidence that TRPA1 has a role in a murine model of OVA-induced airway response to allergen. TRPA1-/- mice exhibited reduced levels of BALF eosinophils and inflammatory mediators, including TNF-α, a prominent drug target in COPD (Caceres et al. 2009). Further to this HC-030031 was shown to significantly reduce late asthmatic response in OVA challenged rats (Raemdonck et al. 2012).

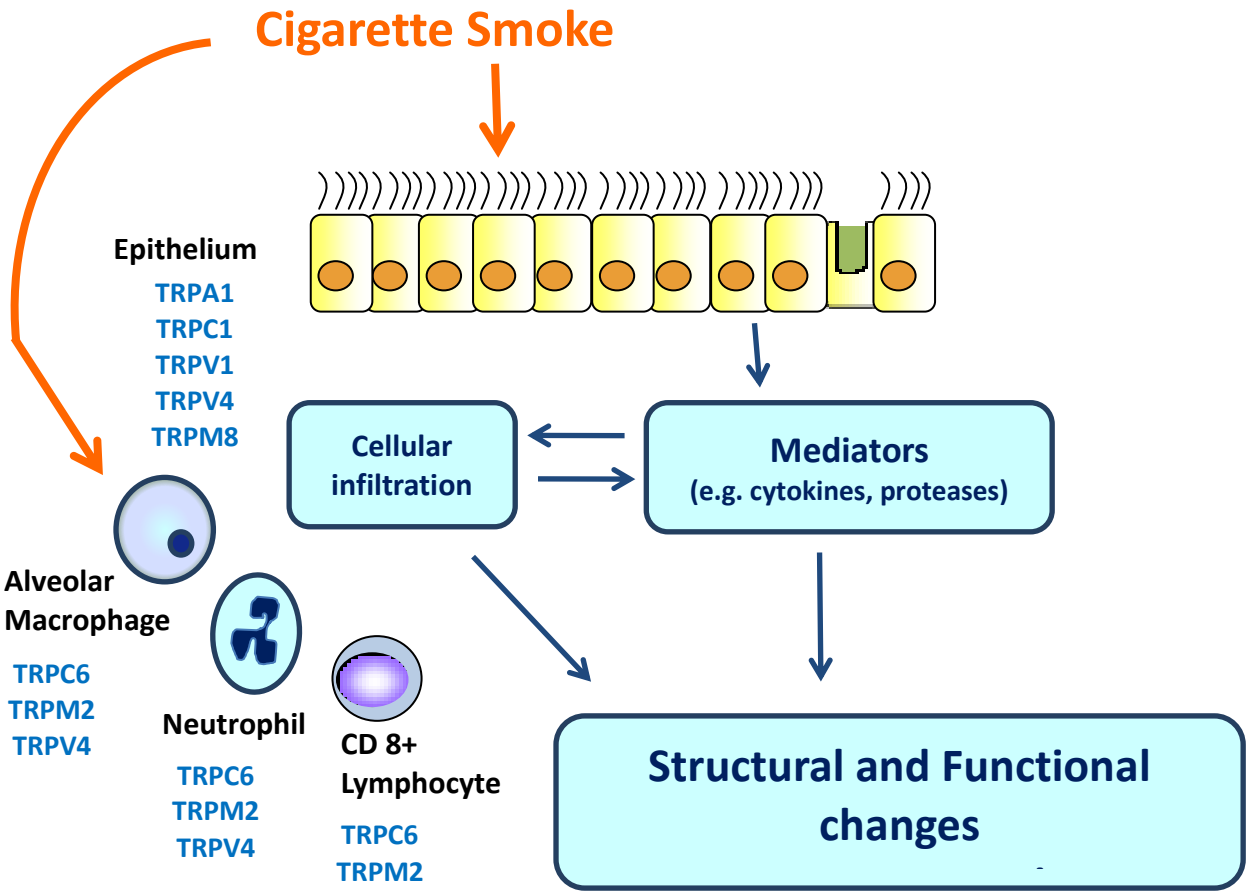

Figure 1.2 Diagram showing the current dogma for the pathogenesis of COPD and the expression of TRP channels in key cell types. *Cigarette smoke (CS) exposure is the most important risk factor for the development of COPD. CS is thought to act on both epithelial cells and leukocytes, causing inflammatory mediator release. Inflammatory mediators induced inflammatory cell infiltration, which in turn leads to the release of more mediators, thus propagating the inflammation. It is this cellular infiltration and mediator release that is thought to drive the structural and functional changes observed in COPD. Various members of the TRP channel family expressed in each of the relevant cell types are listed on the diagram.*

It should also be noted that TRPA1 is known to play a prominent role in neurogenic inflammation in rodents. Indeed, CSE or aldehydes increased Ca^{2+} influx in HEK293 cells transfected with TRPA1 but not in controls, and caused neuropeptide release from isolated guinea pig tissue (Andrè et al. 2008), however, the importance of neurogenic inflammation in human diseases, including COPD, is controversial. There is also increasing evidence for a role of TRPA1 in non-neurogenic inflammatory mechanisms as well, and CSE when introduced into the trachea of mice caused plasma protein extravasation in wild-types but not in TRPA1-/- mice (Nassini et al. 2012).

1.5.2 TRPC6

Elevation of $[Ca^{2+}]$ is central to the activation of macrophages and other inflammatory cells, implicating a pivotal role for cation channels in diseases such as COPD. Of the canonical family of TRP channels, TRPC6 and TRPC7 are found most abundantly expressed in the lung tissue (Venkatachalam & Montell 2007). TRPC6 specifically, is the predominantly expressed TRPC channel in macrophages, lymphocytes and neutrophils (Banner et al. 2011) and is also found in the airway epithelium, making it a reasonable candidate for involvement in inflammatory lung diseases. In fact TRPC6, but not TRPC3 or TRPC7 mRNA was shown to be up-regulated in macrophages form COPD patients compared to control subjects (Finney-Hayward et al. 2010).

It should also be noted that PLC activation, the major modulator of TRPC channels, including TRPC6, is up-regulated by activation of CXC receptors, as is the case in COPD (Baggiolini 2001). This suggests a possible role for TRPC6 in CXC receptor directed inflammation, which is backed up by the findings that bone marrow derived neutrophils deficient in TRPC6 exhibited reduced MIP-2 and OAG (DAG analogue) induced migration (Damann et al. 2009; Lievremont et al. 2005). Further evidence suggests TRPC6 is involved in cytoskeletal arrangements during neutrophil migration (Damann et al. 2009).

There is also evidence for TRPC6 involvement in other inflammatory airway conditions, including TRPC6 mediated Ca^{2+} influx increased in cystic fibrosis patients (Antigny et al. 2011) as well as attenuated allergic inflammatory response in TRPC6-/- mice compared to wild-types (Sel et al. 2008).

1.5.3 TRPM2

Reactive oxygen species (ROS) from both exogenous sources, such as cigarette smoke, and endogenous sources such as oxidative burst from activated inflammatory cells are thought to play a key role in the pathogenesis of COPD (Adcock and Barnes 2008). Oxidant/antioxidant imbalance is one of the key features of the disease and may be responsible for many of the functional changes, so receptors involved in maintaining the oxidant balance could be an important therapeutic target. Elevation of intracellular [Ca^{2+}] is an early response to oxidative stress suggesting a role for cation channels, and it has been shown that TRPM2 can be activated by oxidative extracellular stimuli including H_2O_2 (Hara et al. 2002). TRPM2 can also be activated by TNF-α and LPS, further implicating a mediating role in oxidative disease (Wehage et al. 2002). TRPM2 is widely expressed on inflammatory cells, including those responsible for endogenous production of ROS, and TRPM2 mRNA is up-regulated in primary human monocytes following LPS or TNFα challenge (Wehrhahn et al. 2010). The same study showed that LPS induced increases in [Ca^{2+}] and release of cytokines including TNF-α, IL-6 and IL-10 from human monocytes was reduced by TRPM2-targetting siRNA.

In Vitro it has been shown that H_2O_2 stimulates Ca^{2+} influx in a human monocyte cell line (U937) through TRPM2, leading to IL-8 production (Yamamoto et al. 2008). It was further shown that monocytes from TRPM2-/- mice exhibited reduced Ca^{2+} influx and reduced MIP-2α production in response to oxidative stress than monocytes from wild-type mice. There is also evidence that TRPM2 may be involved in neutrophil chemotaxis in humans (Heiner et al. 2005) and in mice (Yamamoto et al. 2008).

TRPM2 has been implicated in inflammatory responses to oxidative stress *in vivo* as well. A DSS-induced model of colitis showed reduced levels of neutrophilia, cytokine release and

ulceration in TRPM2-/- mice compared to wild-type controls (Araki et al. 2006; Blackburn et al. 1998). These are inflammatory processes which also characterise COPD.

1.5.4 TRPM8

TRPM8 is thought to predominantly function as a thermoceptor for noxiously cold temperatures, although this remains a controversial issue. TRPM8 expressing vagal afferent neurons were shown to be activated by both cold (21°C) and TRPM8 ligand menthol (Xing et al. 2008), and cold air is known to initiate autonomic respiratory responses including bronchial constriction, cough, mucosal secretion and plasma protein extravasation, which are notable processes which may contribute to the symptoms of inflammatory airway disease (Yoshihara et al. 1996; Carlsen & Carlsen 2002). A TRPM8 functional variant expressed in human epithelial cells, has also recently been reported, which promotes endoplasmic reticulum Ca^{2+} release leading to increased inflammatory cytokine transcription (Sabnis et al. 2008a; Sabnis et al. 2008b).

1.5.5 TRPV1

TRPV1 can be activated or sensitised by a particularly diverse range of seemingly disparate stimuli, including heat, protons, voltage, endogenous chemicals (including lipoxygenase products), and exogenous chemicals (including capsaicin and resiniferatoxin) (Reviewed in Venkatachalam & Craig Montell 2007).

TRPV1 is also activated by members of many prominent signalling cascades, indeed direct phosphorylation of TRPV1 by Protein Kinase A (PKA), Protein Kinase C (PKC) and other kinases thought to be important in regulating immune function, has been shown to increase TRPV1 sensitivity (Premkumar & Ahern 2000; De Petrocellis et al. 2001). TRPV1 is also thought to mediate Phospholipase C (PLC) controlled effects. PLC hydrolyses membrane PIP2 into DAG and IP3 which have been shown to stimulate PKC activity. Endogenous chemicals including histamine are thought to elicit their effects on TRPV1 through stimulation of PLC and PKC (Kajihara et al. 2010). Furthermore PAR2 activation has been

shown to sensitise TRPV1 via a pathway involving PLC, PKA and PKC as well as cyclo-oxeganse (COX) (Amadesi et al. 2004; Gatti et al. 2006). Other endogenous mediators are known to indirectly activate TRPV1 including PGE_2, which stimulates sensory nerve depolarisation in a TRPV1 and TRPA1 dependent manner (Grace et al. 2012). Interestingly, the pro-inflammatory cytokine TNF-α potentiated the response of sensory neurons to TRPV1 activators. This may indicate a direct role for TRPV1 in the modulation of the effects of this pro-inflammatory cytokine.

The endogenous levels of a number of TRPV1 activators and sensitisers are known to be elevated in the lungs of COPD patients, especially lipoxygenase mediated arachidonic acid products and mediators involved with the PKA, PKC and PLC pathways. Low pH, another important activator of TRPV1, is also a key feature of COPD, as measured in the exhaled breath condensate of sufferers (MacNee et al. 2011). Notably, it has also been reported that, COPD patients exhibit an augmented cough response to inhaled capsaicin, indicating an increased sensitivity of airway TRPV1 channels (Doherty et al. 2000). When taken together, these facts support the hypothesis that enhanced TRPV1 sensitivity plus increased levels of TRPV1 activators in the lung lead to TRPV1 dependent inflammatory processes in the COPD lung. This theory is supported by evidence reported in an *in vivo* rat model, where pulmonary myelinated primary afferents were more sensitive to capsaicin following airway inflammation, and there was a significant increase in the proportion of neurons expressing TRPV1 (Zhang et al. 2008).

TRPV1 is classically viewed as a sensory nerve channel, and indeed high levels of TRPV1 expression are found on pulmonary sensory afferents. The vast majority of TRPV1 research to date has focussed on its role in sensory nerves and, especially, in mechanisms of cough which is itself a prominent symptom of COPD (Grace and Belvisi 2011; Maher et al. 2011).

Neuronal TRPV1 channels are often co-localised with sensory neuropeptides including calcitonin gene-related peptide (CGRP) and tachykinins. TRPV1 is therefore thought to be important in mechanisms of “neurogenic inflammation” first described in Holzer 1988. Neurogenic inflammation is the process where release of inflammatory neuropeptides from stimulated neurons leads to localised inflammatory responses including increased vascular permeability, extravasation of plasma proteins, airway constriction and mucus

hypersecretion. These processes are also seen in COPD but the importance of neurogenic inflammation in human disease phenotypes, however, remains a contentious issue (Barnes 2001).

Increasingly, however, the importance of non-neuronal TRPV1 to many cellular functions is becoming recognised, particularly in inflammatory processes. TRPV1 expression has been found in a variety of lung cell types, including epithelial cells (Agopyan et al. 2003).

Airborne particulate matter induced apoptosis was completely attenuated by capsazepine in human airway epithelial cells, and sensory nerves from TRPV1-/- mice were similarly protected (Agopyan et al. 2004). In similar studies it has also been shown that TRPV1 agonists cause endoplasmic reticulum stress and cell death in airway epithelial cell lines BEAS-2B and A549. It should be noted however that these processes were not inhibited by TRPV1 antagonists (Reilly et al. 2003; Thomas et al. 2007). Furthermore it has been shown that TRPV1 stimulation causes the release of pro-inflammatory cytokines including IL-6 from airway bronchial epithelial cells (Seki et al. 2007). This advocates a role for non-neuronal TRPV1 in airway inflammatory processes, thus negating the issues around neurogenic inflammatory mechanisms in COPD.

To date few *in vivo* studies have looked to examine the role of either neuronal or non-neuronal TRPV1 in inflammatory processes of the lung, although it was first reported in 1984 that rats treated with neo-natal capsaicin to ablate neuronal TRPV1 expression abolished Cigarette Smoke Extract (CSE) induced increase in vascular permeability in the airways. More recently it has been shown that TRPV1-/- mice were protected against airway inflammation and bronchial hyperactivity induced by LPS (Helyes et al. 2007), and that pre-treatment with TRPV1 agonist SA13353 attenuated both neutrophil influx and increases in cytokines TNFα and CXCL1 (Tsuji et al. 2010).

TRPV1 has also been implicated in other inflammatory diseases, particularly allergic rhinitis and rheumatoid arthritis (Alenmyr et al. 2009; Valdes et al. 2011). TRPV1 antagonist SB-705498 (GSK) is currently in phase 2 clinical trials for treatment of non-allergic rhinitis (Alenmyr et al. 2012).

1.5.6 TRPV4

Both TRPV1 and TRPV4 are thermo-sensitive cation channels however, TRPV4 is sensitive to much lower temperatures, and is in fact closed at temperatures below 30°C (Watanabe et al. 2002). TRPV4 can also be activated by chemical stimuli including, 4α-phorbol 12, 13-didecanoate (4αPDD) (Watanabe et al. 2002), GSK1016790A (Willette et al. 2008), 5', 6'-epoxyeicosatrienoic acid (EET) (Watanabe et al. 2003). As with TRPV1, a role for TRPV4 has been discovered in neurogenic inflammation, where hypotonic solutions and 4αPDD induced release of neuropeptide release from isolated murine airways (Vergnolle et al. 2010). TRPV4 is expressed in a wider variety of tissues than TRPV1, including heart, lung, kidney, CNS and skin (reviewed in J. Yin & Kuebler 2010). Within these tissues, the highest levels of TRPV4 are normally found in the endothelial and epithelial cell types, and within the lungs the highest levels are found in the epithelial linings of the trachea, bronchi and lower airways, and in the alveolar septal walls (Alvarez et al. 2006; Willette et al. 2008). These observations conform with the current dogma that TRPV4 is important in the sensing of osmotic (Strotmann et al. 2000) and mechanical (Liedtke et al. 2000) stimuli, and the subsequent control of epithelial and endothelial barrier function, especially in response to increased vascular pressure and stretch.

Given this role in mechanical stress sensing and endothelial barrier function, it is unsurprising that TRPV4 has been majorly implicated in the development of ventilator induced injury. A murine *ex-vivo* model of ventilator-induced acute lung injury induced by high peak inflation pressure ventilation caused Ca^{2+} entry and increased vascular permeability, resulting in oedema in lungs of wild-type but not TRPV4-/- mice (Hamanaka et al. 2007). Heat activation (40°C) of TRPV4 further potentiated this increase in vascular permeability, whilst ruthenium red (non-selective TRP inhibitor), methanandamide (inhibitor of arachidonic acid production) and miconazole (inhibitor of P450 epoxygenases) reduced the increase in vascular permeability in wild-type mouse lungs (Hamanaka et al. 2007). TRPV4 expression has also been described on mononuclear cells (Delany et al. 2001) and alveolar macrophages (Liedtke et al. 2000) and, intriguingly, it has been shown that instillation of TRPV4+/+ macrophages into the lungs of TRPV4-/- mice restored the susceptibility of isolated mouse lungs to ventilation induced increases in permeability (Hamanaka et al. 2010). This not only implies that macrophages may be important to the

development of ventilator induced acute lung injury, but also raises the possibility that TRPV4 exerts effects on the lung by release of pro-inflammatory mediators. Indeed, TRPV4 activated inflammatory signals have been identified in a murine model of colitis (D'Aldebert et al. 2011).

TRPV4 has also been shown to contribute to mild temperature and ATP-induced increase in ciliary beat frequency *in vitro*, and may therefore play a key role in the transduction of physical and chemical stimuli into a Ca^{2+} signal which regulates mucociliary clearance (Lorenzo et al. 2008). TRPV4 activation has further been shown to release ATP from human bronchial epithelial cells via pannexin channels, in a RhoA and myosin light chain dependent mechanism, in response to osmotic stress (Seminario-Vidal et al. 2011). It is worth noting that ATP is increased in BALF from COPD patients (Mortaz et al. 2009). Furthermore ATP is an activator of purinergic receptors, including $P2X_7$ which was shown to be involved in the induction of inflammasome activation, and subsequent development of airway inflammation after cigarette exposure in a murine model (Eltom et al. 2011).

Other strong evidence for the role of TRPV4 in the pathogenesis of COPD includes two separate genome-wide association studies, which highlighted the association of up to seven single-nucleotide polymorphisms (SNPs) with disease susceptibility (Obeidat et al. 2011; Zhu et al. 2009). The phenotypic implications of these TRPV4 SNPs are not clear, however, the authors suggest that the associated variants may affect TRPV4 splicing, resulting in deletions in the ANK domains, leading to TRPV4 retention in the endoplasmic reticulum, and therefore dysfunction (Zhu et al. 2009).

1.6 Thesis Aims

COPD is one of the largest unmet medical burdens in world. Of the common causes of death worldwide, COPD is arguably the most poorly controlled. There are, indeed, no currently available therapies which can halt the progression or reduce the mortality rate of this disease. This lack of effective therapies stems largely from a deficit in understanding of the pathological mechanism responsible for disease development and progression. Continued focus on hypothesis-based mechanistic research in COPD is therefore vital to development of effective therapies in the future.

The main purpose of this thesis is to assess the possible involvement of specific TRP channels in the pathogenesis of COPD. TRP channels are frequently thought of as signal integrators, making them an attractive therapeutic target for mechanistically complex diseases such as COPD. Furthermore a number of TRP channels have been implicated in inflammatory mechanisms as well as in various inflammatory diseases and lung diseases, as discussed in the previous section (1.5).

In particular, TRP channels A1, C6, M2, M8, V1 and V4 have been identified as candidates for a mechanistic role in the processes underlying COPD. The main aim of this thesis will be to test the following hypothesis:

'TRP channels A1, C6, M2, M8, V1 and V4 are involved in the pathogenesis of COPD'

In order to test this hypothesis I will use *in vivo* techniques to model the disease processes in a murine system, as well as *in vitro* techniques to examine the similarity between TRP functions in murine and human systems.

Previously characterised murine models of airway inflammation induced by CS and LPS will be initially used to profile the expression of the specified TRP channels during airway inflammation. Lung tissue from human COPD patients, 'healthy' smokers and non-smokers will be collected for TRP channel expression which can then be compared with that of the murine models. Subsequently, knock-out colonies of mice, (deficient for specific TRP genes), will be developed to test in the *in vivo* models, in order to determine the physiological importance of these channels in the relevant inflammatory processes. Cell lines will also be

used to further elucidate the involvement of TRP channels in CS induced physiological mechanisms.

The overall aim is that the experiments performed in this thesis will elucidate the role of the specific TRP channels identified in the pathological mechanisms of COPD and, thus, indicate the validity of these specific TRP channels as therapeutic targets.

Chapter 2

Methodologies

2. Methods

This general methodologies section describes all the technical information and details of reagents and materials pertaining to the experimental procedures and techniques employed in this thesis. There are further methods sections within each results chapter, which describe specific experimental protocols as well as the model development information relevant to the techniques used within that chapter.

2.1 Animals

Colonies of C57/BL6 (wild type) mice, as well as TRPA1-/-, TRPC6-/-, TRPM2-/-, TRPM8-/-, TRPV1-/- and TRPV4-/- bred on a C57BL/6 genetic background, were maintained in Central Biomedical Services at Imperial College London. Food and water was supplied *ad libitum*. All protocols were approved by a local ethical review process and strictly adhered to the Animals (Scientific Procedures) Act 1986 UK Home Office guidelines. Two breeding pairs each of TRPA1-/- and TRPV1-/- mice were purchased from The Jackson Laboratory. Breeding pairs of TRPC6-/- mice were kindly donated by Professor Alexander Dietrich of Phillips University, Marburg. TRPM2+/- breeding pairs were provided by Dr. Yasuo Mori of Kyoto University. TRPM8+/- breeding pairs were kindly donated by Dr. Papapoutian from Scripps Research Institute. TRPV4+/- breeding pairs were purchased from Riken Bioresource Centre, Japan. The genetic integrity of all animals was monitored throughout the course of the studies as described below.

2.2 Genotyping

Polymerase Chain Reaction (PCR) is a technique used to amplify small amounts of a specific DNA sequence in to an easily detectable and measurable quantity. PCR was used in this thesis to monitor the genotype of all animal strains throughout breeding programs to ensure the respective genes remained disrupted in all generations. All animals involved in each of the studies were also genotyped to ensure the validity of the data obtained.

The genotyping protocol involved DNA Extraction, followed by gene amplification using PCR and finally product visualisation using Gel Electrophoresis.

2.2.1 DNA extraction

DNA was extracted from tail tips of mice using Quanta Biosciences Extracta DNA prep for PCR kit, according to the manufacturer's instructions. 75µl of extraction reagent was added to mouse tail tips in 0.5ml 'eppendorf' tubes, before heating to 95°C for 30 minutes. Samples were allowed to cool to room temperature before addition of 75µl of stabilisation reagent.

The purity and integrity of the DNA samples was assessed by A_{260}/A_{280} spectrophotometry on the GeneQuant RNA/DNA quantifier (Amersham Pharmacia Biotech, U.K.). DNA samples were subsequently diluted to 10ng/µl, and stored at -20°C until needed for PCR.

2.2.2 Polymerase Chain Reaction

PCR was used to exponentially amplify regions of DNA in between specifically designed primers. Primer sequences were provided by the suppliers of the genetically modified animals, and were designed across the modified exon of the specifically disrupted gene. Two sets of primer pairs were used for each Knock-out strain, the first which would anneal across the wild type gene, and the second to anneal across the disrupted site. The primer pairs were designed so that each would yield a different amplicon size, enabling the varying products to be separated by gel electrophoresis, differentiating whether animals contained copies of the wild type gene, the disrupted gene, or both. Primer sequences are listed in table 3.1.

Each PCR reaction contained, 1x Green GoTaq Flexi Buffer, 0.2 mM dNTPs, 2 mM $MgCl_2$, 1.25 µl enzyme, 10 pmol forward and 10 pmol reverse primers, and a volume of DNA (50 ng) or Nuclease-free water for control, made up to 25 µl with Nuclease-free water (Promega U.K. Ltd). Samples were heated to 95°C for 2 minutes followed by 40 cycles of denaturing, annealing and extension phases. The temperature and length of these phases was tailored

to the total number of base pairs and proportion of guanine/cysteine to adenine/thymine of the relevant primers (see table 3.2 for optimised reaction conditions). After the 40 cycles samples were maintained at 72°C for 10 minutes to ensure all single-stranded DNA fragments were completely extended, before cooling the samples to 4°C, ready for electrophoresis.

2.2.3 Gel Electrophoresis

The PCR products and DNA ladder (Hyperladder IV, Bioline Ltd, London) were run on a 2% agarose gel in Tris Borate EDTA (TBE) buffer containing 0.05 µl/ml Safeview (NBS Biologicals Ltd, Huntingdon, U.K.) at 80V for 1-2 hours. The gel was then visualised under ultra-violet light and photographed.

2.3 *In Vivo* Models of Disease

Animal models have proven to be a crucial tool in the scientific understanding of many human diseases, and the discovery of treatments and therapies. The general methodologies for each of the *in vivo* disease models are described below. The model development and exposure protocols are described in more detail within the relevant results chapters.

2.3.1 Tobacco Smoke-Induced models of inflammation

The exposure protocol for tobacco smoke (TS) was previously characterised and refined (Eltom et al. 2011; Morris et al. 2008). This determined the amount of TS, the ratio of TS to air and the flow rate into the smoke chambers, in order to elicit a sub-maximal level of inflammation.

Mice were exposed to either cigarette smoke (CS) or room air (control) twice daily for three (acute CS model) or fourteen (sub-chronic CS model) consecutive days. Mice were contained in air tight chambers (volume = 136 litres) and TS/room air was drawn through the chambers using a negative pressure system. Research cigarettes, UKY Research Cigarettes

3R4F cigarettes (without filters) were used to generate TS. A pinch valve system was used to control the ratio of TS to room air which mice were exposed to. The ratio was 2 seconds of TS to 4 seconds of air. Control mice were only exposed to room air. The flow rate into the chamber was set at 1.5 L/min and total smoke particulate levels inside the smoke chambers were measured after 35 minutes of exposure. Exposure periods were 50 minutes followed by 10 minutes of ventilation at max airflow. The two daily exposures were separated by 4 hours.

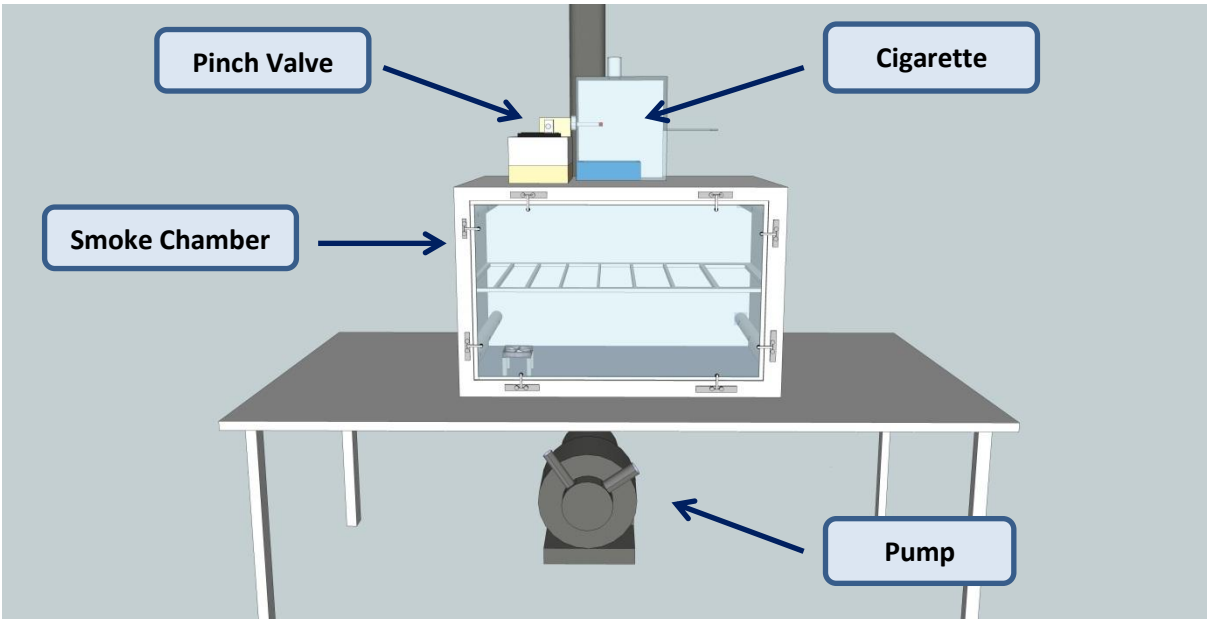

Figure 2.1 Diagram of smoke exposure system*. Mice are placed in metal cages inside the smoke chamber. The pump creates a negative flow system, drawing air through the pinch valve and subsequently through the smoke chamber, with a flow rate of 1.5 L/min. a cigarette is placed into the pinch valve, which controls the ratio of room air to cigarette smoke drawn through the system.*

A fan was used to ensure a continually even distribution of CS within the chamber. Total smoke particulate (TSP) levels were measured using a TSP sampling kit (Teague Enterprises, CA, USA) after 30 minutes of each exposure, in order to monitor variation between exposures. The TSP sampling kit consisted of an extractor fan connected to a port in the exposure chamber with a disposable filter built into the circuit. The filter was weighed before and after the 1 minute sampling period, and the volume of gas drawn from the exposure chamber was measured. The TSP level was then calculated as the change in the

weight of the filter divided by the volume of gas drawn from the exposure chamber. The same process was employed for both CS and room air chambers.

2.3.2 LPS Induced model of lung inflammation

Mice were challenged with a previously determined sub-maximal dose of 1mg/ml aerosolised LPS (*Escherichia coli*, serotype 0111:B4, Sigma-Aldrich Ltd. Poole, UK) or endotoxin free saline (Fresenius Kabi, Warrington, UK) in a Perspex treatment box (600 x 240 x 350mm) for 30min using a System 22 nebuliser (Medic-Aid Ltd., Pagham, Sussex) driven by a high-flow-rate compressor (Medic-Aid Ltd., Pagham, Sussex). This dose of LPS was previously shown to elicit a sub-maximal broncho-alveolar lavage fluid (BALF) neutrophilia response (Eltom et al. 2011).

2.4 Sample collection and processing

At a specific time point after exposure to either cigarette smoke or LPS, mice were euthanized with an overdose of intraperitoneal (i.p.) sodium pentobarbitone (200mg/kg) before BALF and lung tissue was collected for total and differential cell counts, cytokine analysis, ATP assay and Caspase-1 activation assay.

2.4.1 Bronchoalveolar lavage

The trachea was cannulated and the lungs were instilled with 0.3ml Roswell Park Memorial Institute 1640 medium + GlutaMAX-I (RPMI, Invitrogen, Paisley, UK, 61870-157). The media was removed from the lungs after 30 seconds. This was repeated three times and the lavage fluid from each animal was pooled for total and differential cell counts (see section 4.5.3). Remaining BALF was stored at -20°C for cytokine analysis.

2.4.2 Tissue digest

After the Bronchoalveolar lavage, the rib cage was removed and the lungs were taken out then weighed. Subsequently they were chopped finely using a McIlwain tissue chopper (Campden Instruments Ltd, Loughborough, UK), and transferred to 1ml RPMI 1640/ 10% FBS (Gibco, Invitrogen Ltd., Paisley, UK). 4ml of collagenase (1 mg/ml, Roche diagnostics, Mannheim, Germany) with DNAse (0.025 mg/ml, Roche diagnostics, Mannheim, Germany) in RPMI/ 10% FBS was added to the samples, and they were incubated in a water bath (37°C for 1 hour) with gentle agitation. The samples were then filtered through a cell sieve (70 µm mesh size) and washed twice with RPMI (including 10% FBS and penicillin/streptomycin) by centrifuging for 10 min at 1900 rpm. After the second wash, the supernatant was discarded and the cells re-suspended in 1 ml RPMI (10% FBS with penicillin/streptomycin). This was then used for the total tissue leukocyte cell count. To perform the differential cell counts the samples underwent a further 1:5 dilution in RPMI (10% FBS, penicillin and streptomycin).

2.4.3 Total leukocyte counts and differential cell counts

The total number of leukocytes in BALF, blood and tissue samples was determined using a Sysmex F820 haematology analyser (Sysmex UK Ltd, Milton Keynes, UK) according to the manufacturer's instructions. Slides were prepared for differential cell counts by cytocentrifuging 100ml samples of either BALF or tissue digest in a cytospin (Shandon, Runcorn, UK) at 700 rpm for 5 min at standard room temperature and pressure. The slides (including blood smears) were then fixed and stained on a Hema-tek 2000 (Ames Co., Elkhart, IN, USA) using modified Wright-Giemsa stain (Sigma-Aldrich Ltd., Poole, UK, WG128). Differential cell counts were carried out on a minimum of 200 cells per slide using standard morphological criteria under light microscopy. The differential cell counts for the whole sample were then calculated as a percentage of the total counts.

2.4.3.1 Differential cell counts

Differential cell counts from all sample types were performed using light microscopy at 40x magnification. For all LPS experiments in this thesis, 3-way counts were performed, differentiating neutrophils, eosinophils and lymphomononuclear cells. The lymphomononuclear group included all lymphocytes, macrophages and monocytes. For all CS-exposure experiments in this thesis, 4-way cell counts were performed, differentiating lymphocytes, neutrophils, monocytes/macrophages and eosinophils. Lymphocytes are the smallest of these cell types and were identified by dark blue staining of the nucleus and the presence of very little or no cytoplasm. Monocytes/macrophages again have a dark blue staining of the nucleus, but are distinctly larger than lymphocytes with a much larger proportion of cytoplasm. Neutrophils are 'medium' sized cells with reference to lymphocytes and macrophages, and are easily identifiable by their polymorph multi-lobed nucleus. Eosinophils by contrast possess a bi-lobed nucleus, forming a distinctive 'figure-8' shape, and their cytoplasm stains pink provided they have not de-granulated. Figure 2.4 shows typical example pictures of each cell type.

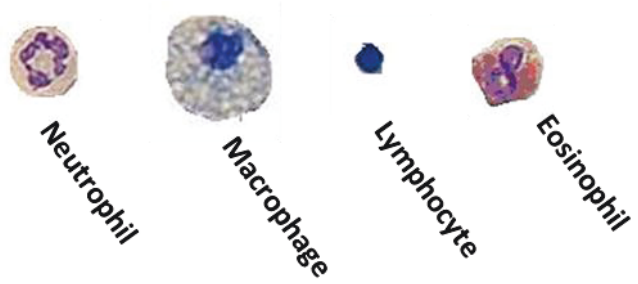

Figure 2.2 Inflammatory cell staining for differential counts. *Images were taken from actual differential cell count slides during model characterisation.*

2.4.4 Cytokine measurement by ELISA

After taking enough BALF sample for total and differential cell counts, the remaining BALF was frozen (-20°C) for future cytokine analysis. Cytokines were measured using R&D systems ELISA kits, according to the manufacturer's instructions. First the capture antibody was diluted in PBS to working concentration, and 100µl per well was added to a 96-well plate. The plate was sealed with an adhesive plastic strip and incubated overnight at room temperature. The following day the plate was washed three times, by first aspirating the wells then adding 400µl of wash buffer (Sigma-Aldrich) using a manifold dispenser, and repeating this twice. Plates were then blocked with 300µl of reagent diluent (1% BSA in PBS) and incubated at room temperature for 1-2 hours before being washed 3 times as described above.

Standards were serially diluted in reagent diluent as per the specific instructions for the relevant assay. 100 µl of each standard was then added in duplicate to the previously coated and blocked ELISA plate. Samples, uniformly diluted to an appropriate concentration in reagent diluent, were added to the plate concurrently, and the plates were incubated for two hours at room temperature. Plates were then washed three times before addition of detection antibody in reagent diluent was added to wells, which was incubated on the plate for a further two hours at room temperature. After washing the detection antibody off, 100µl of working concentration streptavidin-HRP in reagent diluent, and the plate was covered to avoid direct light and incubated at room temperature for 20 minutes. The plate was subsequently washed for a final time, before 100µl of substrate solution was added to each well. Direct light was avoided whilst the substrate colour change developed. The reaction was stopped when the colour had developed (less than 20 minutes) by addition of 50 µl of 1M sulphuric acid. The optical density, at 450 nM, of each well was determined immediately, and the cytokine concentration of each sample was thus determined using the standard values.

2.4.5 ATP assay

ATP levels were measured in BALF samples and cell supernatants using ATPlite® luminescence detection system (Perkin Elmer, Cambridge, UK) according to the manufacturer's instructions. Luciferase catalyses the reaction of D-luciferin with the ATP present in the samples, to produce Oxyluciferin and light (see reaction scheme below). The amount of light emitted is proportional to the concentration of ATP in the sample.

$$\text{ATP} + \text{- Luciferase} + \text{O}_2 \xrightarrow[\text{Mg}^{2+}]{\text{Luciferase}} \text{Oxyluciferin} + \text{AMP} + \text{PP}_\text{i} + \text{CO}_2 + \text{Light}$$

2.4.6 Measurement of Caspase-1 activation

Caspase-1 is an enzyme which can be found in cell cytosol. For this reason it is first necessary to isolate the cytosolic fraction from the nuclear fraction of the cells of flash frozen tissue in order to measure Caspase-1 activation. The protocol used for each step is described below.

2.4.6.1 Isolation of Cytosolic and Nuclear Cell Fractions

NXTRACT CelLytic NuCLEAR Extraction kit (Sigma-Aldrich Ltd. Poole, UK) was used to prepare cytosolic and nuclear fractions according to the manufacturer's instructions. Frozen tissue samples (>100mg in size), were crushed in liquid nitrogen then transferred to a 2ml eppendorf. Samples were washed twice by addition of 0.5 ml wash buffer (PBS + protease inhibitors) followed by centrifugation at 10,000 x g for 5 minutes at 4°C and removal of the supernatant. 0.5ml of lysis buffer (containing DTT and protease inhibitor cocktail) was added to the washed samples, which were then homogenised for 40 seconds. Subsequent centrifugation at 11,000 x g for 20 minutes at 4°C separated the nuclear fraction into the pellet, leaving the cytosolic fraction in the supernatant. The cytosolic containing supernatant was transferred to a clean tube and stored at -80°C, until needed for future analysis. Total protein concentration was determined by Bradford Assay.

2.4.6.2 Caspase-1 Activation Assay

A commercially available Colourimetric Assay Kit (Enzo Life Sciences, Exeter, UK) was used to measure Caspase-1 activation in cytosolic extract samples, according to the manufacturer's instructions. Cytosolic fraction samples were incubated with reaction buffer, DTT and YVAD-*p*NA (200 µM final concentration) at 37°C for 1 hour. Caspase-1 present in the samples cleaves the YVAD-*p*NA substrate, releasing the chromophore *p*NA. The amount of caspase-1 in the sample was thus quantified using a spectrophotometer at 405 nM.

2.4.7 Bradford Assay

Bradford assay was used to quantify the amount of protein in the cytosolic fraction extracted from lung tissue. A seven-point protein standard curve was made by serial dilution with saline from a top concentration of 1 mg/ml down to 0.0156 mg/ml. Cytosolic tissue samples were diluted 1:10 in saline containing 1 mg/ml BSA. 10 µl of each standard or sample was pipetted in duplicate into a 96-well plate. Bradford's reagent (Bio-Rad) was diluted 1:5 with de-ionised H_2O, then, 190 µl was added to each well of the plate. The plate was incubated at room temperature for 5 minutes then, absorbance at 620 nM was measured using a plate-reader.

2.5 *In Vitro* Models of Disease

In vitro models of disease and non-disease biological systems have been a crucial tool in the scientific pursuit to understand and characterise protein functions. *In vitro* models allow for the isolation of specific cell types from complex living organisms, enabling detailed analysis of the interaction between exogenous and endogenous mediators.

2.5.1 Cell Culture

A549, LA-4, J-774 and THP-1 cells were obtained from the European Collection of Cell Cultures (ECACC). All cell types were passaged 3 times a week. After passage 50, cells were

discarded and fresh cells were grown up. All cell cultures were incubated at 37°C in humidified air, 5% v/v CO_2. Individual cell culture conditions are listed below.

A549 cells (ECACC Cat no: 86012804) were cultured in DMEM growth medium (Sigma, D6546), supplemented with 2mM L-Glutamine (Sigma, G7513), 10% foetal calf serum (FCS) (Sigma, F96665), 100 µg/mL penicillin, 100 µg/mL streptomycin (Sigma, PO781) and 0.25 µg/mL Amphotericin B (Sigma, A9528) (37°C, 95% air, 5% v/v CO_2). A549 cells were grown in 75cm^2 flasks, and passaged by trypsinisation followed by centrifugation (13,000xg, 5 min, 4°C), and re-suspension in fresh media with a split ratio of 1:2. The day before an experiment cells were plated at 100,000 cells per well in 500 µl of normal growth medium in 24-well plates. The following day the cell growth medium was aspirated from the wells, and replaced with growth arrest medium (DMEM containing the same supplements, except 3% FCS). The cells were then allowed to equilibrate for 1 hour before treatment.

LA-4 cells (ECACC Cat no: 90040512) were maintained in F-12 Ham medium (Sigma N4888), supplemented with 10% foetal calf serum (Sigma, F96665), 2mM L-Glutamine (Sigma, G7513), 100 µg/mL penicillin, 100 µg/mL streptomycin (Sigma, PO781), 0.25 µg/mL Amphotericin B (Sigma, A9528), and 1% Non-essential amino acids (Sigma M7145). As with A549 cells, LA-4 cells were passaged by trypsinisation, followed by centrifugation (13,000 x g, 5 min, 4°C), and re-suspension in fresh media with a split ratio of 1:2. The day before experiments the cells were plated in 24-well plates at 100,000 cells per well, then growth arrested the following day in medium containing 5% FCS.

THP-1 (ECACC Cat no: 88081201) and J774 cells (ECACC Cat no: 61870-010) were maintained in RPMI 1640 medium plus GlutaMAX-I (Invitrogen, Cat no: 61870-010), supplemented with 10% FCS (Invitrogen, Cat no: 10108-157) 100 µg/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL Amphotericin B.

J774 cells are semi-adherent, and therefore in order to passage the cells a cell-scraper was used to detach cells from the bottom of the flask, before centrifugation (13,000 x g, 5 min, 4°C) and re-suspension in fresh medium. THP-1 cells are non-adherent and were passaged by centrifugation (13,000 x g, 5 min, 4°C) and re-suspension in fresh media.

In all experiments THP-1 and J774 cells were seeded in growth arrest medium (3% FCS) at 400,000 cells per well in 24-well plates 2 hours before treatment, to allow time to equilibrate.

2.5.2 Cigarette Smoke Conditioned Medium

Cigarette smoke-conditioned medium (CSM) was generated according to the protocol published by Birrell *et al* 2008 (Birrell, *et al.* 2008). A custom-made apparatus was constructed: four cigarettes were 'plugged' by their filters into four holes in a container, which was connected through the centre of a rubber bung plugged into the top of a conical flask. The side arm of the conical flask was then connected to an air extraction pump which could be turned on and off repeatedly to generate a mock 'puff' on the cigarettes. Smoke generated in this way was therefore drawn through the conical flask; a small rubber pipe was plugged into the bottom of the bung and angled into 50 ml of growth arrest media so that smoke entering the conical flask was directed through the media. The extractor pump was then turned on for 2s (at 11.5 L/min) at a time, with 10s between each 'puff' of the lit cigarettes, to maintain the temperature of the burning tobacco, and therefore the constituents of the smoke. Cigarettes were 'smoked' in this way until burned to just above the filters. The resultant 'smoked' media was then removed from the conical flask, and filter-sterilised, using a 2 µm filter, before dilution and use for cell treatments.

2.6 Measurement of Gene Expression

Measurement of gene expression was carried out on human lung samples, mouse lung samples and cultured cells. Human and mouse lung samples were flash frozen in liquid nitrogen prior to RNA extraction. Cells were cultured in 6 well plates until confluent before removal by trypsinisation (A549/LA-4), scraping (J774) and then pipetting, before immediate RNA extraction.

2.6.1 RNA Extraction

Frozen tissue was crushed using a pestle and mortar then transferred to a clean, autoclaved 2ml eppendorf. Cells were transferred immediately to a 2 ml eppendorf before centrifugation at 13,000 x g, 5 min, 4°C to pellet the cells, and removal of the supernatant by pipetting. 1 ml of TRI Reagent was added to the samples and mixed. TRI Reagent contains phenol, and guanidine thiocyanate, which lyse the sample and dissolve DNA, RNA and protein. Samples were then immediately centrifuged at 15,000 x g for 15 minutes at 4°C. The clear supernatant was transferred to a fresh autoclaved 2 ml tube and left to stand at room temperature for 5 minutes to ensure the complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform (free from isoamyl alcohol) was subsequently added before the tubes were shaken enthusiastically for 15 seconds. The samples were then left to stand for a further 10 minutes at room temperature followed by centrifugation at 15,000 x g for 15 minutes at 4°C. Centrifugation separated the samples into 3 phases: an organic phase (red) containing proteins, interphase containing precipitated DNA, and the upper phase (aqueous, colourless) containing RNA. The RNA containing aqueous phase was transferred to an autoclaved 1.5 ml tube and 1/10 the volume of isopropanol was added then mixed. After 5 minutes at room temperature samples were centrifuged at 12,000 x g for 10 minutes at 4°C. The supernatant was again transferred to a clean 1.5 ml tube before the remaining volume of isopropanol was added to precipitate the RNA. The RNA was pelleted by centrifugation at 12,000 x g for 10 minutes at 4°C, and the supernatant was discarded before washing the RNA with 1 ml of 75% ethanol. The samples were centrifuged for a final time at 12,000 x g for 5 minutes at 4°C, then the ethanol was removed and the RNA was re-dissolved in 50 µl of RNAse-free water.

RNA samples were quantified using a GeneQuant RNA/DNA quantifier (Amersham Pharmacia Biotech, U.K.), and the purity was assessed by A_{260}/A_{280} spectrophotometry analysis. A ratio of approximately 1.8 was expected.

2.6.2 cDNA Synthesis by Reverse Transcription

RNA samples were diluted to 0.1 µg/µl prior to cDNA synthesis. 5µl of diluted RNA was mixed with 1x TaqMan RT Buffer, 5.5 mM $MgCl_2$, 2 mM dNTPs mixture, 2.5 µM random hexamers, 0.4 U/µl RNase inhibitor and 1.25 U/µl Multiscribe reverse transcriptase, made up to a final volume of 50µl per sample. The mixed samples were then incubated in a Perkin Elmer 480 thermal cycler (Boston M.A., U.S.A.) at 25°C for 10 minutes, 48°C for 30 minutes and 95°C for 5 minutes (equilibration, reverse transcription, denaturation). Samples were allowed to cool, producing 10 ng/µl cDNA, which was stored at -80°C, until needed for real time PCR.

2.6.3 Real Time PCR

Real Time PCR was performed using TaqMan reagents (Applied Biosystems). 3µl of cDNA (diluted to 2.5ng/µl) was added to 12.5µl TaqMan Universal Master Mix, 1.25µl 18s (internal control) fluorescent probe, 1.25µl of the assay on demands (AoD) and 7µl of RNase-free H_2O, making a total reaction volume of 25µl containing 7.5ng of cDNA template. Mixed samples were placed in a 96-well TaqMan plate and PCR was performed using a thermal cycler (Applied Biosystems). The PCR protocol of an initial 10 minute dissociation phase at 95°C followed by 40 cycles of 95°C for 15 seconds then 60°C for 60 seconds.

TaqMan assays contain forward and reverse primers for a specific gene, and a probe with a fluorescent reporter dye (FAM) at the 5’ end and a quencher dye (TAMRA) at the 3’ end. The probe is designed to anneal to a DNA sequence in between the primers on the gene of interest. When the probe is intact the quencher dye is in sufficient proximity to the reporter dye to quench the fluorescence. As the section of cDNA is amplified by successive PCR cycles, the amount of template for the probe to anneal to increases exponentially. During each cycle the exonuclease action of the polymerase enzyme (contained in the universal master mix) degrades any probe annealed to the cDNA sequence, and consequently the quencher dye is taken out of proximity of the reporter dye, allowing the latter to fluoresce. The level of fluorescence is thus proportional to the amount of template that has been produced, and increases with successive PCR cycles.

18s is measured simultaneously to the gene of interest in each sample, as a 'house-keeping' gene. House-keeping genes are constitutively expressed genes necessary for cellular maintenance. Measuring 18s provides an internal control for the total amount of cDNA present in each sample, allowing for deviations between samples to be corrected. The 18s probe is designed in the same way as the assay probe, except it uses VIC as a reporter dye instead of FAM. All assays were validated for multiplex reactions with 18s, to ensure there was they had equivalent reaction efficiencies. For more detail on assay validation see section 3.3.3.

2.6.4 Analysis of RT-PCR Results

RT-PCR was quantified using ABI Prism 7000 software. Fluorescence of VIC and FAM was measured during the extension phase of each PCR cycle. Critical thresholds (ct) were set for VIC and FAM (representing 18s and target gene respectively) fluorescence during the exponential phase of cDNA replication. The cycle number where the level of fluorescence reaches the relative critical threshold for 18s and AoD was recorded for each sample. The relative difference between 18s and AoD ct (Δct) was then calculated. To work out the relative original amount of template for the relevant gene the following formula was used for each sample: $2^{-\Delta ct} \times 10^{-6}$.

2.7 Human Tissue Samples

Human lung tissue parenchymal samples were obtained from a transplant programme, run by Royal Brompton and Harefield hospitals. Ethical approval for the study was obtained from the Royal Brompton and Harefield ethics committee (09/H0708/72). Lung samples, upon becoming available, were immediately transported from the hospital to the laboratory and incubated in Kreb's solution for dissection and processing. Samples relevant to this thesis were flash frozen in liquid nitrogen and stored at -80°C until required for RNA extraction as described section 2.6.1, and subsequent gene expression using TaqMan. Patient details are reported where necessary in the relevant chapters hereof.

2.8 Statistical Analysis

Unless otherwise stated in the appropriate section, all results are expressed as mean ± S.E.M. of *n* observations. Statistically significant differences between independent sets of data were assessed by Mann-Whitney U-test for non-parametric data. For multiple comparisons tests, a Kruskal-Wallis test with Dunn's multiple comparisons post-test was utilised for non-parametric data. A *p*-value of less than 0.05 was considered statistically significant for all tests. Statistical analyses were performed using PRISM 5 software from GraphPad.

Chapter 3

Model Characterisation

3.1 Rationale

A number of members of the TRP channel family have been implicated for roles in the pathogenesis of COPD and, more specifically, in the inflammatory pathways which are thought to drive disease progression. If TRP channels can be identified which have a role in the airway inflammation thought to drive COPD, then they may represent effective therapeutic targets. Animal models of airway inflammation induced by disease relevant stimuli are currently some of the most effective ways to study the disease as well as the legitimacy of potential therapeutic targets. One way to study the role of a particular protein in an *in vivo* model is to examine the response of animals which are genetically deficient (''knock-outs'') for that protein compared to animals expressing the gene normally (''wild-types''). For these reasons, mice deficient for specific TRP channels were sought for study in previously characterised models of murine airway inflammation. The agents chosen for inducing airway inflammation were cigarette smoke (CS) and Lipopolysaccharide (LPS). As discussed in section 1.1.4, CS exposure is responsible for the vast majority of COPD cases, making it the ideal stimulus to model COPD-like inflammation in murine models. As discussed in section 1.3.2.2, aerosolised LPS induces acute airway inflammation through innate inflammatory pathways, providing a model of a 'normal' airway inflammatory response.

The first aim of this chapter is to establish colonies of TRPA1, C6, M2, M8, V1 and V4 knock-out mice, bred on a C57BL/6 genetic background. C57BL/6 is the strain of mice with which the 3-day cigarette smoke, 14-day cigarette smoke and LPS induced airway inflammation models have been previously characterised (Eltom et al. 2011). Markers of airway inflammation relevant to COPD can then be compared in wild type and TRP channel KO mice in order to indicate the roles of specific TRP channels in these inflammatory pathways.

The second aim of this chapter is to measure the expression of various TRP channels in the murine models of inflammation. Transcriptional regulation of genes is often altered when the proteins they code for are activated. Changes in the transcriptional expression of TRP channels, therefore, may indicate their involvement in the inflammatory pathways activated in the murine models. The TRP channel expression levels will be measured at successive

time-points after exposure to either 3-day cigarette smoke, 14-day cigarette smoke, or acute LPS, in what is termed a time-course study.

TRP channel expression will also be measured in human lung parenchymal samples from non-smokers, smokers and COPD patients. Differences in the transcriptional regulation of these channels between the three patient groups may help to identify whether these channels are involved in the generation of the disease, and therefore whether they represent possible therapeutic targets. Furthermore, measurement in human samples will allow a comparison to be made between the role of TRP channels in the murine models and in the human disease, providing translational data on the validity of the models.

3.2 Methods

3.2.1 Animals

Animals were maintained in CBS as described in section 2.1. The 'wild-type' mice strain used for characterisation of the murine models of airway inflammation was C57BL/6. The animals used in the time-course experiments were all male, 8-10 weeks old and housed in individually ventilated cages (IVCs).

Two breeding pairs each of TRPA1-/- and TRPV1-/- mice were purchased from The Jackson Laboratory. Breeding pairs of TRPC6-/- mice were kindly donated by Professor Alexander Dietrich of Phillips University, Marburg. TRPM2+/- breeding pairs were provided by Dr. Yasuo Mori of Kyoto University. TRPM8+/- breeding pairs were kindly donated by Dr. Papapoutian from Scripps Research Institute. TRPV4+/- breeding pairs were purchased from Riken Bioresource Centre, Japan.

3.2.2 Genotyping

Genotyping reagents including 5 x buffer, $MgCl_2$, nucleotides, polymerase and RNase/DNase free water were purchased from Promega. Primers and probes specific for wild-type and knock-out genes were purchased from Invitrogen. Genotyping was performed on genomic

DNA extracted from tail-tips, amplified by polymerase chain reaction (PCR). PCR products were visualised after electrophoresis on a 2% agarose gel containing safeview. For further details of genotyping protocols please refer to section 2.2.

3.2.3 RT-PCR

RT-PCR was performed using TaqMan assays and reagents from Applied Biosystems. Human and Murine assays were validated as described in section 3.3.3.1. All gene expression levels were normalised against 18s internal control and data are represented as relative values calculated using the formula $2^{-\Delta ct}$ x (10^6). TaqMan RT-PCR protocols are detailed in section 2.6.

3.2.4 Murine LPS and cigarette smoke-induced models of airway inflammation

LPS exposure model was previously characterised in C57BL/6 (wild-type) mice (Eltom et al. 2011). Dose responses were performed and a dose which elicited submaximal neutrophilia (1 mg/ml) was selected. A time course was then used to establish the best time point at which to collect samples. Animals were challenged with 1 mg/ml LPS or Saline (vehicle) then groups were culled at 2, 6, 24, 48, hours after exposure. Lung tissue was collected and snap-frozen in liquid nitrogen at each of these time points for future RNA extraction and gene expression.

Similarly, a cigarette smoke (CS) dose response was initially used to determine a dose of 500 ml/min would elicit a sub-maximal neutrophilic response. The 3-day and 14-day smoke models were then established as the acute CS and the sub-chronic CS exposure models respectively. Time-courses were performed to monitor the inflammation after the last exposure in each model. Again, lung tissue was taken and snap-frozen from air and smoke exposed groups at specific time-points for gene expression. The development of these models is further discussed in chapters 4, 5 and 6.

3.3 Results

3.3.1 Characterisation of genotyping technique for TRP channel KO mice

Genotyping was necessary to establish colonies of homozygous KO mice from the heterozygous breeding pairs originally obtained. Once stable KO colonies were attained, and fertile offspring were being regularly born, these colonies could then be used for *in vivo* experiments. Genotyping was then used to confirm the genetic integrity of all animals used for *in vivo* experiments.

All genetically modified mice strains were provided with genotyping primer sequences and suggested optimised reaction conditions. Primer sequences for all primers are listed in table 3.1 below.

All KO mice were bred on a C57BL/6 genetic background, with the exception of TRPA1-/- mice, which were bred on a mixed B6;129 genetic background. For this reason TRPA1 were backcrossed onto a pure C57BL/6 genetic background to allow the use of previously characterised C57BL/6 mice as the wild-types for in vivo experiments. This is discussed in more detail in section 3.3.2.1.

In order to establish a reliable genotyping method for each of the KO strains, it was necessary to optimise the PCR reaction conditions for all of the primers. Genomic DNA was extracted from the tail tips of the first litter of each of the respective breeding pairs. Where breeding pairs were provided as heterozygous animals, the offspring were a mix of homozygous wild-types, homozygous knock-outs and heterozygous animals, meaning all the primers could be optimised using these offspring. Where the breeding pairs were already homozygous knock-outs, all the offspring are also knock-outs. Therefore genomic DNA from in house wild type animals was used to check the wild-type primers worked. Optimised primer reaction conditions are listed in Figure 3.2.

Primer	Sequence
TRPA1 wt Forward	5’-TCA TCT GGG CAA CAA TGT CAC CTG CT-3’
TPRA1 wt Reverse	5’-TCC TGC AAG GGT GAT TGC GTT GTC TA-3’
TRPA1 KO for	5’-GAG CAT TAC TTA CTA GCA TCC TGC CGT GCC-3’
TRPA1 KO rev	5’-CCT CGA ATC GTG GAT CCA CTA GTT CTA GAT-3’
TRPC6 wt Forward	5’-CAG ATC ATC TCT GAA GGT CTT TAT GC-3’
TRPC6 wt Reverse	5’-CAT CAG GAC CCC GAG CAC CAC ATA C-3’
TRPC6 KO for	5’-ACG AGA CTA GTG AGA CGT GCT ACT TCC-3’
TRPC6 KO rev	5’-GGG TTT AAT GTC TGT ATC ACT AAA GCC TCC-3’
TRPM2 wt Forward	5’-CTT GGG TTG CAG TCA TAT GCA GGC-3’
TRPM2 wt Reverse	5’-GCC CTC ACC ATC CGC TTC ACG ATG-3’
TRPM2 Neo	5’-GCC ACA CGC GTC ACC TTA ATA TGC G-3’
TRPM8 wt Forward	5’-CCG GGT GCT GCC CAT AGT ACC ATT TC-3’
TRPM8 wt Reverse	5’-GGG ATG TCA TAG TGC TGA AAG GCA GA-3’
TRPM8 Neo	5’-GGT GCA GAT GAA CTT CAG GGT CAG CT - 3’
TRPV1 wt Forward	5’-CCT GCT CAA CAT GCT CAT TG-3’
TRPV1 wt Reverse	5’-TCC TCA TGC ACT TCA GGA AA-3’
TRPV1 Neo	5’-CAC GAG ACT AGT GAG ACG TG-3’
TRPV4 wt Forward	5’-TGT TCG GGG TGG TTT GGC CAG GAT AT-3’
TRPV4 wt Reverse	5’-GGT GAA CCA AAG GAC ACT TGC ATA G-3’
TRPV4 Neo	5’-TGG ATT GCA CGC AGG TTC TC-3’

Table 3.1 Primer sequences for genotyping TRP channel knock-out mice

3.3.2 Expanding TRP channel Knock-out colonies

Once the PCR reaction conditions had been optimised for the primers, and genetically modified offspring could be reliably genotyped, the colonies could be expanded for *in vivo* experiments. Figure 3.3 shows an example gel where DNA has been extracted from the tail tips from the offspring of a TRPM8 heterozygous breeding pair, amplified by PCR with TRPM8 primers and then visualised on an Agarose gel. As listed in table 3.2, the expected amplicon size for the wild-type TRPM8 gene is 275 base pairs, while the amplicon size for the disrupted TRPM8 gene is 350 base pairs. Therefore samples in figure 3.3 with only the 275 base pair product are from homozygous wild-type animals, samples with only the 350 base pair product are from animals homozygous for the disrupted TRPM8 gene (i.e. "full knock-outs"), and samples where both product sizes are visible are from heterozygous animals. Figure 3 shows 10 heterozygotes, 3 wild-types and 5 KOs, broadly conforming to mendelian genetics. Given these data, male and female animals which were homozygous for the disrupted gene could be selected as a breeding pair, and all the resultant litters would also be full knock-outs.

Receptor	Primers	Annealing		Extension		Product Size (Base pairs)
		°C	Secs	°C	Secs	
TRPA1	wt for/rev KO for/rev	68	30	72	60	wt = 317 KO = 184
TRPC6	wt for/rev KO for/rev	55	60	75	60	wt = 245 KO = 310
TRPM2	for/rev/neo	65	30	75	60	wt = 500 KO = 700
TRPM8	for/rev/neo	65	30	75	60	wt = 275 KO = 350
TRPV1	for/rev/neo	64	60	72	60	wt = 984 KO = 600
TRPV4	for/rev/neo	60	30	72	80	wt = 740 KO = 1490

Table 3.2 Optimised PCR conditions for genotyping TRP channel knock-out mice

The same process was undertaken for the TRPC6, TRPM2, TRPV1 and TRPV4 colonies, with each of their respective primers, to identify male and female knock-outs. In order to produce enough animals of a similar age for the studies in chapters 4, 5 and 6, four to five knock-out breeding pairs were set up for each of the colonies.

TRPM8

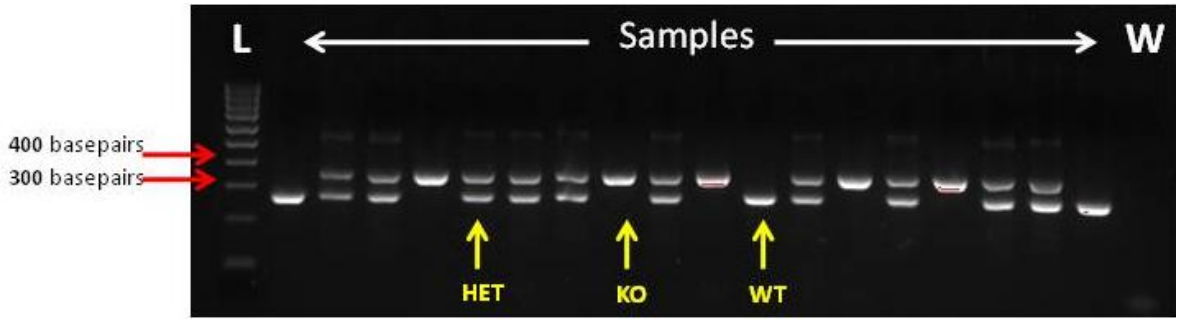

Figure 3.1 Example visualised gel from genotyping of TRPM8 colony offspring*. L = DNA ladder (hyperladder V, Bioline). DNA ladder is used to check the size of PCR products. Amplicon size for wild-type TRPM8 = 275 base pairs; amplicons size for knock-out TRPM8 gene = 350 base pairs. W = water control: Nuclease-free water was used as a negative control instead of extracted DNA. No band is visible in the water control lane, confirming there is no DNA contamination in the PCR mastermix.*

3.3.2.1 TRPA1 colony backcrossing

TRPA1-/- mice purchased from Jackson Laboratory were developed by modifying an embryonic stem cell with a targeting vector to replace the endogenous TRPA1 encoding exons, thus disabling the gene. Successfully modified embryonic stem cells were injected into C57/BL6 blastocysts. Chimeric males were bred to C57BL/6 or B6129P1/F2J females and the resulting heterozygotes were bred together and maintained on a mixed B6;129 background by Jackson Laboratory.

Unlike other KO strains in this project, these mice were not subsequently backcrossed onto C57BL/6 genetic background before purchase. Therefore a backcrossing program was undertaken to bring the genetic background of the TRPA1-/- colony closer to that of the

C57BL/6 control mice which were used to characterise the LPS and cigarette smoke exposure models. TRPA1-/- mice were bred with C57BL/6 mice to create a heterozygous litter. The original TRPA1-/- colony was already 50% C57BL/6, therefore the heterozygous litter were 75% C57BL/6 genetic background. Full KO animals were then retrieved by breeding the heterozygotes together, producing a mixed litter of heterozygotes, homozygous knock-outs and homozygous wild-types with respect to the TRPA1 gene. Litters broadly conformed to mendelian genetics, producing a respective ratio of 2:1:1. Tail tips were taken from the litter and genotyped to identify homozygous KOs, which were subsequently bred with another C57BL/6 to repeat the process (please see figure 3.2).

After three backcrosses, the animals were approximately 93.625% C57BL/6 genetic background. It was decided at this point to use this colony to breed littermate controls for *in vivo* experiments, as time restraints would not permit the 9 backcrosses recommended before direct comparison with wild-type controls. Breeding pairs of generation 3 (3 backcrosses) heterozygotes were established to produce littermate wild-types and knock-outs.

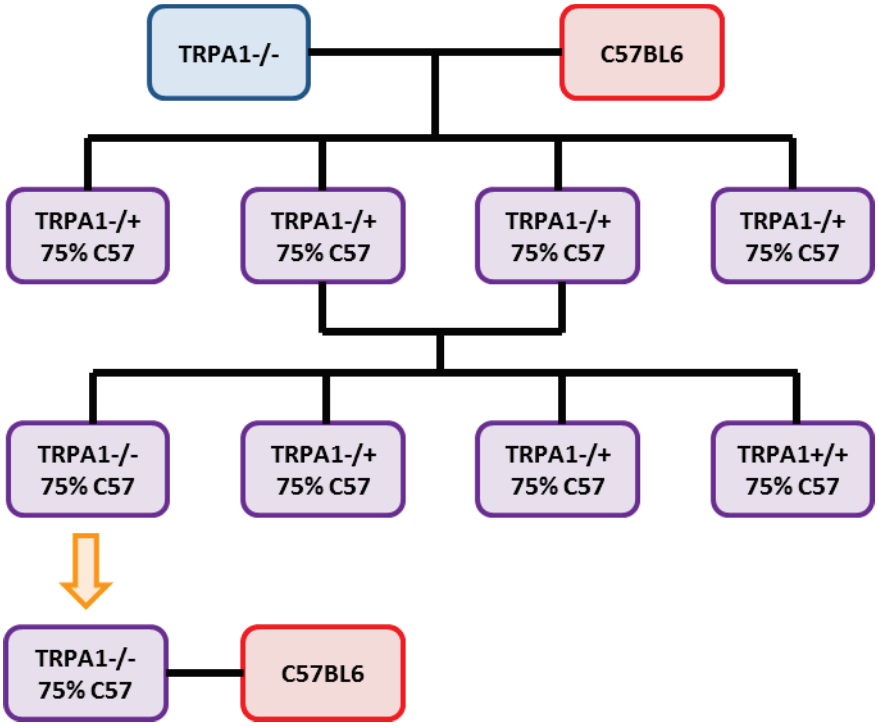

Figure 3.2 ***TRPA1-/- mouse colony backcrossing diagram****. TRPA1-/- (50% C57BL/6 genetic background) purchased from Jackson labs were bred with wild-type (C57BL/6) mice to produce heterozygous animals with increased wild-type genetic 'background'. The heterozygotes (75% C57BL/6 genetic background) were then bred together to produce homozygous wild-types, heterozygotes and homozygous knock-outs. The knock-outs were then identified and the process repeated to further increase the wild-type genetic background of the colony.*

3.3.3 Measurement of TRP Channel Gene Expression

Part 2 of this chapter assesses the transcriptional expression of TRPA1, TRPC6, TRPM2, TRPM8, TRPV1 and TRPV4 in murine models of airway inflammation and in human samples from non-smokers, "healthy" smokers and emphysema sufferers. Changes in the transcriptional levels of these TRP channels may indicate involvement in the inflammatory pathways in the murine models and the human disease. Further to this it will allow a comparison of the roles of these TRP channels in murine models and human disease, providing translational information.

The TaqMan gene expression system provides an accurate and quantitative way to measure the amount of mRNA for a given gene, and allows for the use of internal controls. An internal control refers to the measurement of a 'house-keeping' gene, concurrently with measurement of the gene of interest. House-keeping gene expression should remain uniform across cells regardless of activation or stress, and thus allows correction for RNA concentration variations between samples. TaqMan assays must be validated as multiplex reactions with an internal control prior to target measurement in samples, to ensure assays do not interfere with each other. Separate reporter dyes, VIC and FAM, are used to facilitate the reading of 18s and AoD respectively, in the same sample.

3.3.3.1 Validation of TRP channel TaqMan Assays

A TaqMan assay (AoD) must be validated for use in multiplex reactions to ensure it has the same efficiency as the internal control assay. An assay for 18s (ribosomal RNA) was used as the internal control for all gene expression experiments in this thesis. Initially assays were run in singleplex against a panel of tissues or cell types (human or murine depending on the assay target), in order to find a sample with medium to high expression levels of the target sequence. An 8-point 1/3 serial dilution of this cDNA sample from 8.33ng/µl down to 0.03ng/µl and multiplex RT-PCR reactions were performed in triplicate on each of the serial dilution concentrations, using the AoD and the 18s assay. A critical threshold was then set for both assays across all the samples.

The critical threshold is manually set on the linear part of the amplification plot for each assay, and above the background noise, to ensure the most accurate possible readings between samples. An example amplification plot of 18s measured in a serial dilution of human lung samples is shown in figure 3.3. The horizontal green line shows the manually set critical threshold; values for each sample are taken as the cycle number where the curve intercepts the critical threshold. A critical threshold was similarly set for the AoD amplification plot in the same samples. The lower the critical threshold, the higher the concentration of that specific mRNA target in the sample, as shown in figure 3.3 where the samples with a higher concentration cross the critical threshold at a lower cycle number.

Critical threshold values for 18s and the AoD were plotted as a graph, and a line of best fit was applied, in order to test the linear regression of each of the assays. In order for an assay to be considered validated, the R^2 value for each line was above 0.98, and the coefficients of *x* had a difference of less than 0.1, as specified by Applied Biosystems. The R^2 value defines how well the data fit to a linear regression, and hence whether the concentration range of cDNA used, has a linear relationship to amplification. The coefficient of *x* determines the rate of amplification; it is advised by Applied Biosystems that a difference between the internal control (18s) and the AoD of 0.1 is acceptable in order to predict relative concentrations of target. The coefficient of x, or rate of amplification should be close to 3 for both assays, demonstrating a high reaction efficiency, as the dilution between samples was 1/3.

Figure 3.4 shows an example validation graph, in this case for the murine TRPV4 AoD, with the R^2 values and coefficients of *x* displayed for both 18s and the AoD. The validation graph satisfies the criteria described above, therefore the AoD was deemed validated for measuring murine TRPV4 given an input cDNA concentration between 8.33 and 0.03 ng/µl. The same process was undertaken to validate AoDs to measure murine and human TRPA1, TRPC6, TRPM2, TRPM8, TRPV1 and TRPV4.

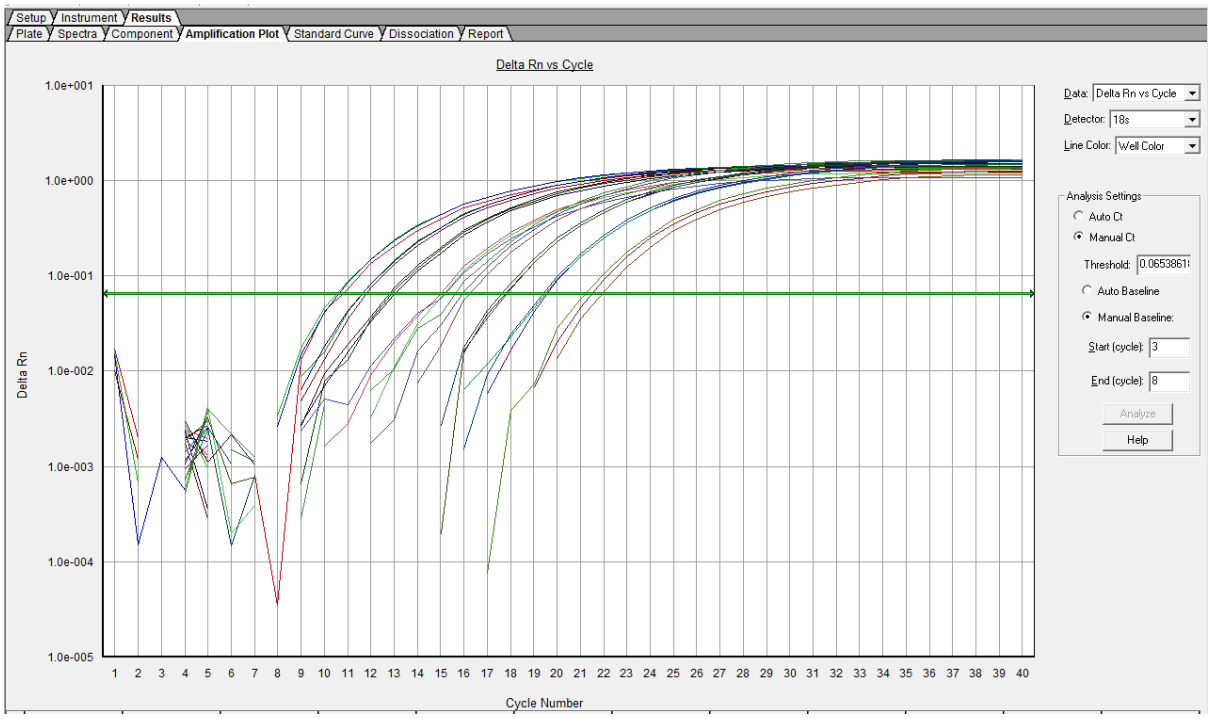

Figure 3.3 Example amplification plot for 18s mRNA in human lung serial dilution. *RNA was extracted from lung samples, transcribed to cDNA then serially diluted 1/3 from 8.33ng/µl down to 0.03ng/µl (8-point dilution). Samples from each of the 8 dilution points were plated with 18s and TRPV4 TaqMan assays in triplicate. Fluorescence (Delta Rn) increases proportionally as cDNA signals are amplified. Only the 18s amplification plots are shown. The critical threshold was manually set (green horizontal line), in the exponential phase of cDNA replication, and critical threshold values for each sample were calculated as the point where each curve intercepts the threshold.*

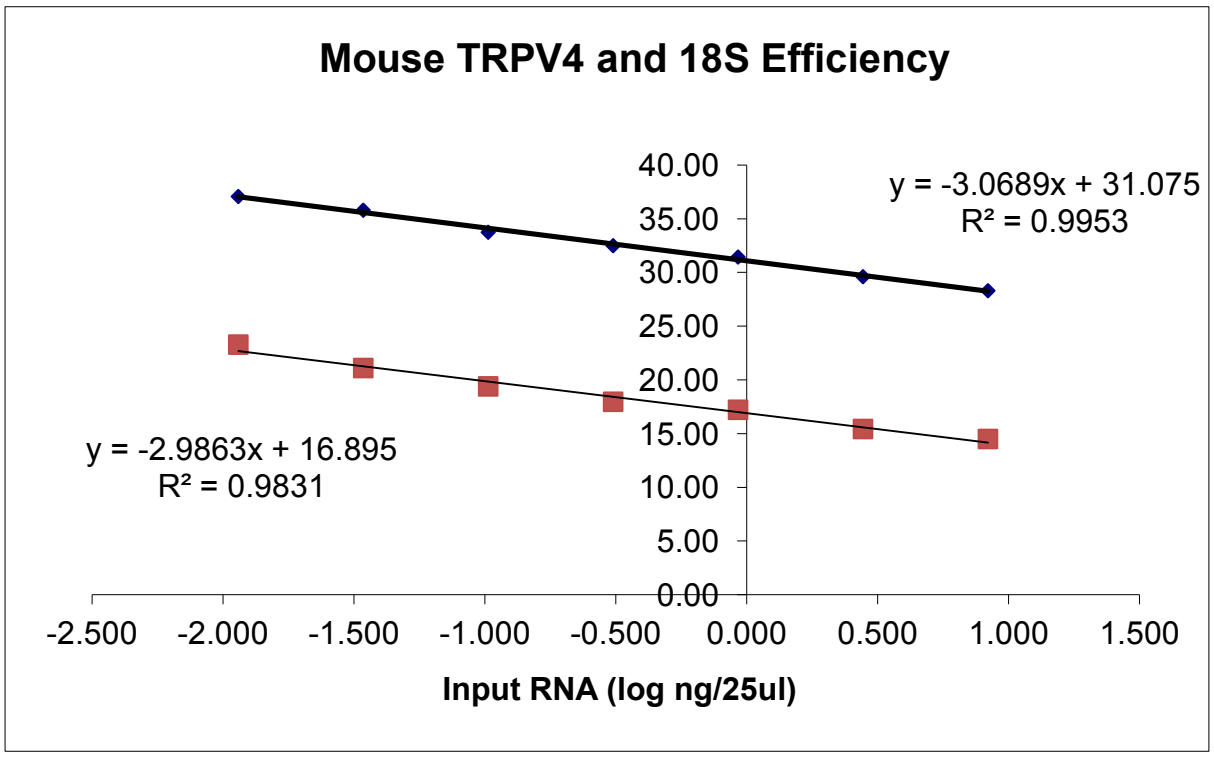

Figure 3.4 Example Multiplex Validation Graph for TaqMan AoD. *This figure shows the validation data for murine TRPV4 AoD. AoD critical threshold values are plotted with diamonds and 18s values are plotted as squares. The AoD was deemed validated as the R^2 value was >0.98, and the coefficient of* x *was less than 0.1 different than that of the 18s assay.*

3.3.4 Expression of TRP channels in 3-day smoke, 14-day smoke and LPS models

Time-course studies were previously carried out on three models of murine airway inflammation: LPS exposure, 3-day cigarette smoke exposure and 14-day cigarette smoke exposure. The main endpoints for these time courses were BALF cellular recruitment, tissue cellular recruitment and BALF cytokine analysis. Lung samples from some of the mice in each time-point were flash frozen in liquid nitrogen for expression analysis. TaqMan Assays for TRPA1, C6, M2, M8, V1 and V4 were used to measure mRNA levels in frozen lungs from each of the models. In Figures 3.2 and 3.3 relative expression of mRNA is measured at 2, 6 and 24 hours after the last exposure to cigarette smoke or time-matched control (room air). In Figure 3.4 relative expression of mRNA is measured at 2, 6, 24 and 48 hours after LPS exposure or time-matched control (saline).

TRPA1 and TRPM8 mRNA levels were below the detection limit of their respective assays when measured in the whole lung samples. TRPA1 channels are thought to be most highly expressed in neurons, including vagal airway neurons in mice (Nassenstein et al. 2008). Proteins found on airway neurons are transcribed in the cell bodies before becoming localised along the neuronal projections into the lungs. The cell bodies of the TRPA1 expressing neurons are thought to be mainly found in the vagal ganglia which are located in the cranium (Nassenstein et al. 2008). Genetic expression of these neuronal TRPA1 channels can therefore not be detected in the lung tissue. Similarly, TRPM8 is thought to be mostly expressed in a sub-population of primary sensory afferents, the cell bodies of which reside within the dorsal root and trigeminal ganglia (Nassenstein et al. 2008; Peier et al. 2002). Transcriptional neuronal TRPM8 can therefore not be detected in the lung tissue either. Non-neuronal TRPA1 and TRPM8 has, however, been reported in lung-relevant cell-types and is discussed in section 3.4.2.

TRPM2 expression is significantly increased after 2 hours in all three models. In the 3-day smoke exposure model, TRPM2 expression is significantly increased by approximately 40% after 2 hours compared to time-matched controls. This increase is maintained at 6 and 24 hours after the last smoke exposure, albeit statistically non-significant. In the 14-day smoke exposure model TRPM2 expression is increased more than 3-fold compared to time

matched control. Figure 3.4 shows that TRPM2 expression is more than 6-fold increased, 2 hours after LPS exposure, compared to time-matched controls. The level of TRPM2 expression increase correlates with the relative severity of inflammatory markers between these models, including cellular recruitment and cytokine release in BALF (Figure 4.1).

There are no significant differences in TRPV1 expression between smoke exposed animals and time-matched control at any time point in either smoke exposure model. However there does appear to be a slight trend of TRPV1 mRNA suppression at 2 hour and 6 hour time points in both models.

Similarly with TRPV4 there are no significant differences in mRNA expression in the smoke exposure models at any of the time-points, however there is a slight increase in smoke exposed animals after 2 hours in both models. This is in contrast to the data from the LPS model, where there is a slight decrease in TRPV4 expression 2 hours after LPS exposure, and a significant 60% decrease at 6 hours compared to the time-matched control.

TRPV1 expression levels were below detection at the 2, 6, and 24 hour time-points after LPS exposure, indicating that LPS exposure attenuates the transcription of this channel. At the 48 hour time-point TRPV1 was measurable after LPS, but at lower levels than the time-matched saline control. It should be noted however that one or two of the samples from the saline exposed time-points, also had TRPV1 levels below the detection limit of the assay. Despite this the trend seems clear, that LPS down-regulates the production of TRPV1 mRNA.

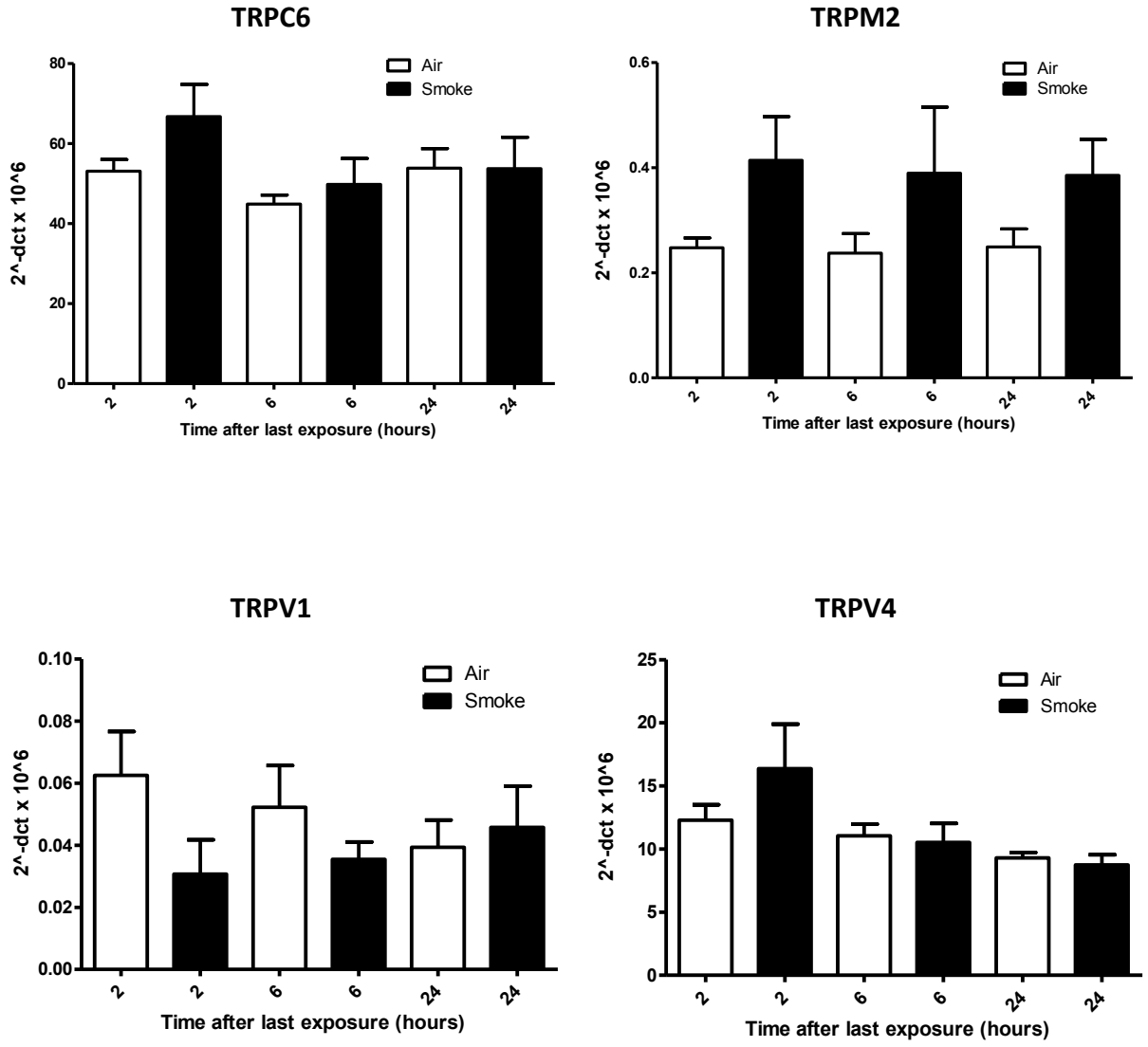

Figure 3.5 Temporal analysis of TRP channel expression in lung tissue after acute cigarette smoke exposure. *Relative expression levels of TRPC6, TRPM2, TRPV1 and TRPV4, at 2, 6 and 24 hours after last exposure of 3-day smoke exposure model. Frozen lung samples were collected at the relevant time-point and flash frozen in liquid nitrogen. RNA was extracted and transcribed to cDNA for RT-PCR analysis of gene expression. Data are expressed as mean ± SEM for n=6. * denotes statistical significance of LPS exposed expression levels compared to time-matched control. P < 0.05 was established using Kruskal-Wallis test, with Dunn's multiple comparisons post-test.*

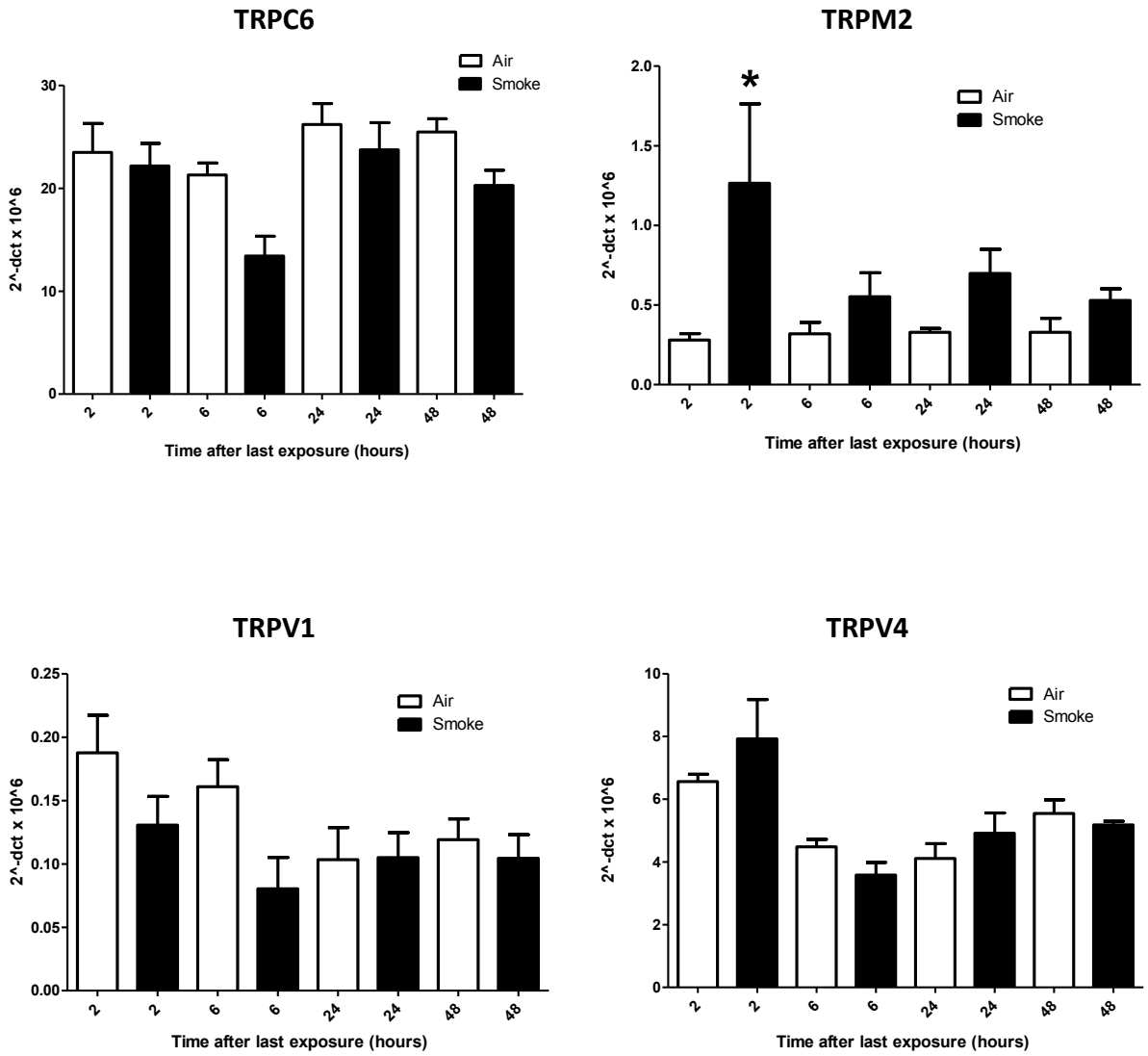

Figure 3.6 Temporal analysis of TRP channel expression in lung tissue after sub-chronic cigarette smoke exposure. *Relative expression levels of TRPC6, TRPM2, TRPV1 and TRPV4, at 2, 6, 24 and 48 hours after last exposure of 14-day smoke exposure model. Frozen lung samples were collected at the relevant time-point and flash frozen in liquid nitrogen. RNA was extracted and transcribed to cDNA for RT-PCR analysis of gene expression. Data are expressed as mean ± SEM for n=6. * denotes statistical significance of LPS exposed expression levels compared to time-matched control. P < 0.05 was established using Kruskal-Wallis test, with Dunn's multiple comparisons post-test.*

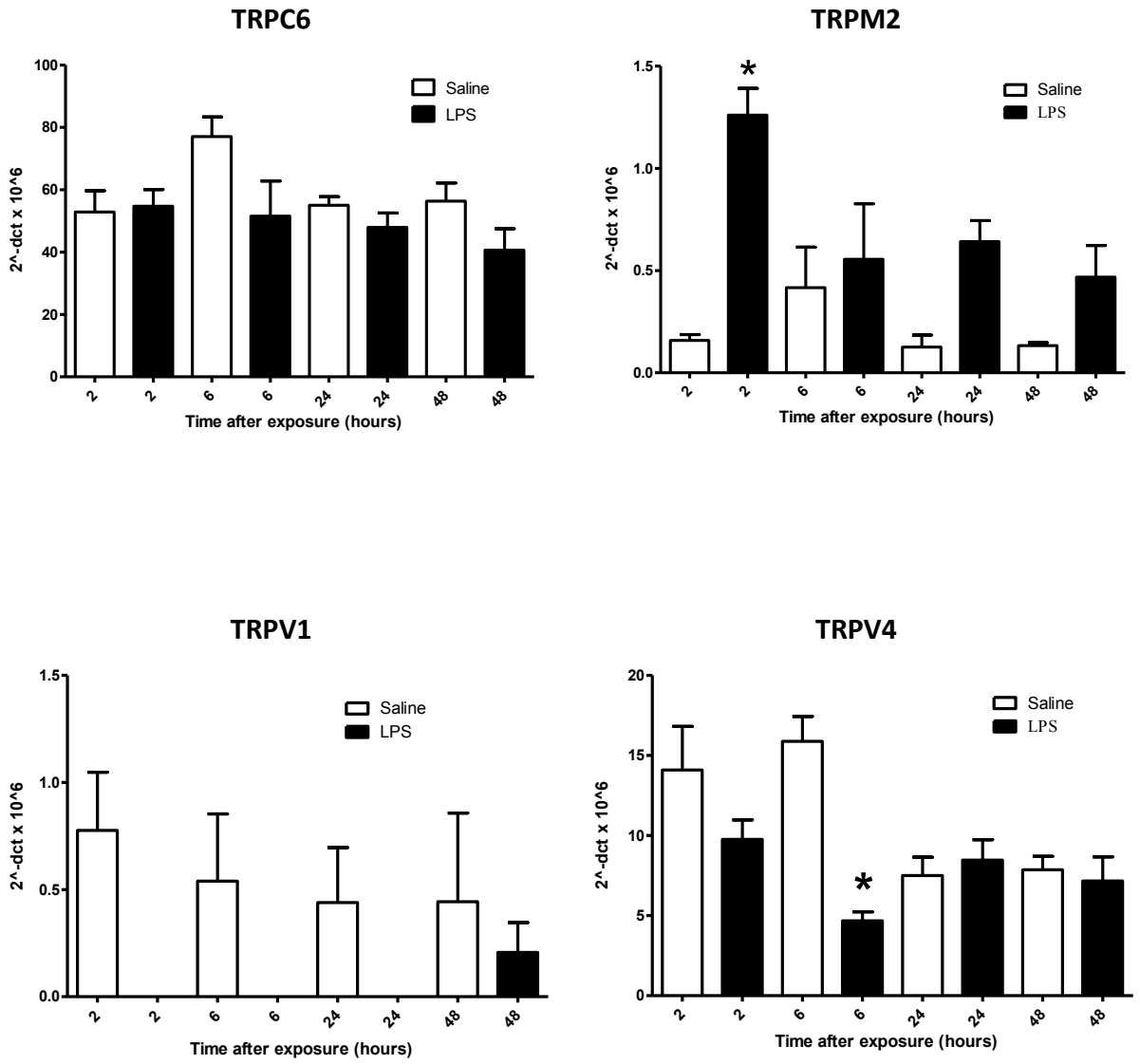

Figure 3.7 Temporal analysis of TRP channel expression in lung tissue after LPS exposure. *Relative expression levels of TRPC6, TRPM2, TRPV1 and TRPV4, at 2, 6, 24 and 48 hours after exposure to LPS or control (saline). Frozen lung samples were collected at the relevant time-point and flash frozen in liquid nitrogen. RNA was extracted and transcribed to cDNA for RT-PCR analysis of gene expression. Data are expressed as mean ± SEM for n=6. * denotes statistical significance of LPS exposed expression levels compared to time-matched control. P < 0.05 was established using Kruskal-Wallis test, with Dunn's multiple comparisons post-test.*

3.3.5 Expression of TRP channels in human lung samples

Human lung samples were collected from transplant programs as detailed in section 2.7. According to patient notes and medical history, the lung samples were divided into three categories: Donor (non-smoker), 'healthy' smoker, and emphysema patients. The term 'healthy' in the smoker group must be regarded with some caution, as the donor may have died from a non-respiratory disease or trauma, however there are no signs or symptoms of inflammatory respiratory disease in these patients despite smoking.

Table 3.3 below, lists the average age, standard error of the mean (SEM) for the ages, and the ratio of male to female patients for each of the groups of patients from which tissue was collected. Emphysema patients were on average 8.9 years older than donors, and 16.3 years older than smokers. Both the donor and emphysema groups had a majority of females, whereas the smoker group had a majority of males.

	Number	Age	SEM (age)	Sex (M:F)
Donor	**10**	**47.4**	**2.0**	**3 : 7**
Smoker	**9**	**40.0**	**4.7**	**6 : 3**
Emphysema	**15**	**56.3**	**1.4**	**6 : 9**

Table 3.3 Table of age and gender ratio for human lung tissue samples*. SEM = standard error of the mean. Lung samples, collected from a transplant programme, were flash frozen in liquid nitrogen for RNA extraction and future gene expression assays. Patients' details are summarised in the above table.*

Figure 3.5 shows the expression data for TRPC6, TRPM2, TRPV1 and TRPV4 in each of the three patient groups. In contrast to the murine models of inflammation, there is no difference in TRPM2 expression between smokers and non-smokers. There is also no difference between emphysema patients and either of the other two groups. In the murine models, the increased TRPM2 expression is a rapid response to airway challenge, so it is possible that during the time between the last cigarette of the patient and the processing of the lung tissue, any increase in TRPM2 expression is resolved. However this data does not suggest that a change in TRPM2 expression is an underlying cause for emphysema development in response to cigarette smoke. Similarly there is no statistical difference between the expression levels of TRPC6 in any of the 3 patient groups. It is noticeable however that TRPC6 expression appears to be higher on average in the emphysema group compared to both the smoker and non-smoker groups. It is possible that this is a real difference but did not reach statistical significance due to the high levels of variability within the groups.

The expression of both TRPV1 and TRPV4 is significantly increased in emphysema patients compared to non-smokers. There is approximately a 100% increase in TRPV1 expression and a 70% increase in TRPV4 expression between these two groups. There is, however, no difference in the expression of either V1 or V4 in smokers compared to non-smokers. This suggests that up-regulation of V1 or V4 may be an integral process in disease development.

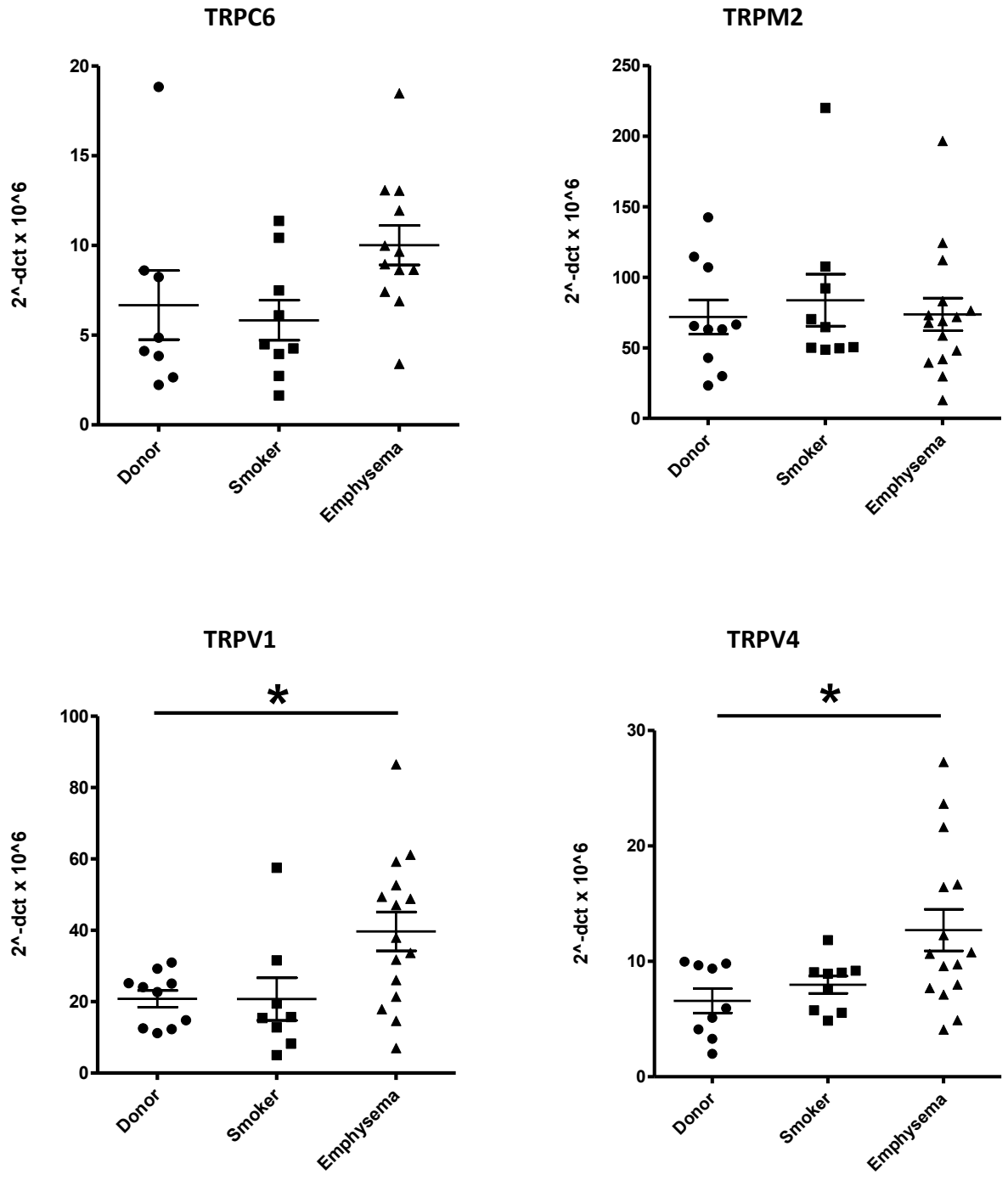

Figure 3.8 Analysis of TRP channel expression in human lung tissue. *Relative expression levels of TRPC6, TRPM2, TRPV1 and TRPV4 in human tissue samples. Patients were divided into three groups based on medical history: Donor – No evidence of airway disease and no smoking history; Smoker – No evidence of airway disease but a current smoker at time of death; Emphysema – Clinically diagnosed emphysema. Individual data points are shown as a scatter plot. Averages and SEM are shown for each group. * denotes statistical significance, p<0.05 using Kruskal-Wallis test with Dunn's multiple comparisons post-test. Data points with unusually high 18s values were excluded.*

3.4 Discussion

3.4.1 Characterisation and development of knock-out mouse colonies

Part one of this chapter established and optimised genotyping techniques for each of the knock-out colonies of mice, which are to be used as tools for investigating the role of TRP channels in the *in vivo* murine models of airway inflammation. Robust and reliable genotyping protocols were developed for the wild-type and disrupted version of each of the relevant TRP channel genes.

The species *Mus musculus* was selected for use, as this was the first laboratory animal to have its complete genome published, thus making it possible to develop targeting vectors to disrupt specific genes in mouse blastocysts, and therefore breed knock-out mice. Whilst the genomes of other animals have now been published, the mouse is still the main animal used for genetic knock-out studies. Mice have a similar number of genes to humans, and 80% of human genes directly correspond to an equivalent mouse gene, making them a good *in vivo* model of the function of most human genes. It should be noted, however, that there are some disadvantages to using developmentally 'knock-out' animals for *in vivo* studies. In particular it is possible that certain genes perform different functions in the developing embryos than they do in the adult animal. Thus knocking-out a particular gene may have un-foreseen developmental effects which could change the physiology of the adult mouse in ways that do not necessarily pertain to the functions of the protein in the mature animal. The risk of this is minimised by observing the knock-out animal's behaviour and development compared to the wild-type.

Genotyping techniques were established in this chapter as a quick and efficient way to breed colonies of knock-out mice where enough age-matched animals were produced alongside wild-types for the *in vivo* experiments in this thesis. Genotyping will also be used to check the integrity of knock-out mice used in each of the studies in the following chapters. Phenotypic analysis was also performed on mice from the TRPA1-/-, TRPV1-/- and TRPV4-/- colonies by other members of the Respiratory Pharmacology group, using isolated vagus nerve preparations, which is a tissue expressing high levels of both TRPA1, TRPV1 and TRPV4 protein. These studies confirmed that TRPV1-/- mice did not respond to specific

agonist capsaicin, TRPA1-/- mice did not respond to specific agonist acrolein (Grace et al. 2012), and that TRPV4-/- mice did not respond to specific agonist GSK1016790A.

Colonies of TRPC6, M2, M8, V1 and V4 negative mice were obtained already backcrossed 9 or 10 times onto a pure C57BL/6 genetic background, resulting in genetically modified mice with a more than 99.9% wild-type genetic background. This allows direct comparison to be made between the genetically deficient mice and the C57BL/6 wild-type colony in *in vivo* models, with a high degree of certainty that any phenotypic differences are caused only by the disrupted gene, and not by other genetic differences which may occur between strains.

TRPA1-/- mice, however, were purchased with a mixed genetic background of B6;129. As the LPS and smoke *in vivo* models of airway inflammation were previously characterised in C57BL/6 mice, it was decided to backcross these mice onto that genetic background, rather than re-characterise the models with wild-types possessing the TRPA1-/- genetic background. Backcrossing is a time-consuming process, with every cycle increasing the genetic background by half of the previous cycle, i.e. 1 backcross = 50%, 2 = 75%, 3 = 87.5%, 4 = 93.75% and so on. When the background of the mice was 93.75%, it was decided to breed littermate controls for LPS and cigarette smoke *in vivo* studies. A higher number of back-crosses would have been desirable however this was not possible within the time-constraints of a PhD project. Heterozygous TRPA1-/- were therefore bred together, to yield homozygous knock-out and homozygous wild-type offspring for profiling *in vivo* against models of airway inflammation. These wild-type mice provide the perfect controls for their knock-out littermates, and their genetic background is close enough to C57BL/6 that they should behave similarly to the wild-type mice previously used to characterise the models, when exposed to the inflammatory challenges.

3.4.2 Measurement of TRP channel expression in murine models and human tissue

RT-PCR is an excellent tool for accurately measuring the relative amount of a specific gene transcript, and TaqMan is one of the most accurate methods of quantitative RT-PCR. The main RT-PCR competitor to TaqMan is SYBR-Green, and it has been shown that TaqMan is more accurate of a broader range of input cDNA concentrations (Matsenko et al. 2008).

The TaqMan method was first reported in 1991 by researchers at Cetus Corporation, and utilises primers and a probe designed with high specificity for individual genes. The forward and reverse primers anneal to the 5' and 3' ends of the relevant gene respectively, facilitating the amplification of the gene transcript during successive PCR cycles. The oligonucleotide probe, which has a fluorophore covalently attached to its 5'-end and a quencher dye at its 3' end, anneals within the amplified region of cDNA, i.e. between the two primers. When the probe is intact the quencher is sufficiently close to the fluorophore to quench the fluorescence emitted by fluorescence resonance energy transfer (FRET) after excitation by the TaqMan machine's light source. However once annealed to the cDNA sequence, the probe will be degraded by the exonuclease action of the Taq-polymerase enzyme during the next PCR cycle. Thus the fluorophore is freed from its quencher and begins to emit fluorescence when stimulated, which is detected by the TaqMan machine. The amount of fluorescence is directly proportional to the amount of genetic template created, and therefore increases with each successive PCR cycle. A critical threshold for fluorescence can therefore be set during the exponential phase of cDNA amplification, and the cycle number at which the fluorescence crosses this threshold is inversely proportional to the amount of cDNA template, for a given gene, that was present in the original sample.

TRPC6 is the predominantly expressed canonical TRP channel on human neutrophils and may be involved in cytokine induced neutrophil migration (Damann et al. 2009; Heiner et al. 2005). TRPC6 expression was shown to be significantly increased in macrophages from COPD patients compared to control subjects (Finney-Hayward et al. 2010). TRPC6 is also highly expressed in lung tissue in both humans and mice. There was however, very little difference in TRPC6 expression between smoke exposed mice and time-matched air-exposed controls at any of the measured time-points, in either the 3 or 14 day smoke exposure model. This observation also holds true for the LPS model. There does seem to be, however, a small trend towards increased TRPC6 expression in human emphysema patients compared to both non-smokers and smokers. It must be stressed however that there were no statistically significant differences in TRPC6 expression between any of the groups. This may be due to an outlier in the 'donor' group. An increase in TRPC6 expression in the emphysema group would conform to the observations of Finney-Hayward *et al* 2010 in

macrophages, although it should be regarded with some caution as the changes are not statistically significant when using Dunn's multiple comparisons post-test.

TRPM2 is predominantly expressed in inflammatory cells, including macrophages, lymphocytes and neutrophils which are thought to be integral to driving the progression of COPD. The only study to look at the expression of this channel in relation to COPD and cigarette smoke, showed that neither disease nor smoking status affected TRPM2 expression (Finney-Hayward et al. 2010). This observation is paralleled by the data presented in Figure 3.5, where TRPM2 expression measured in lung parenchyma was not affected by inflammatory disease (emphysema) or smoking status. These observations, however, are in marked contrast to the data obtained in the murine models, where TRPM2 was significantly increased from the first time-point (2 hours) in the 3-day smoke, 14-day smoke and LPS exposed animals compared to their time-matched controls. The level of TRPM2 up-regulation compared to control also correlates with relative severity of BALF cellular infiltration in each of the models. However the amount of TRPM2 expression in these models cannot be attributed to the increased numbers of TRPM2 expressing cells, as the up-regulation occurs before the cellular infiltration.

TRPV1 is a heat activated thermoceptor, expressed highly in neurons, but also widely in other tissues and cell-types, including lung alveolar epithelial cells. Finney-Hayward *et al.* measured TRPV1 expression in macrophages, and found no difference between COPD patients, smokers and controls. However another study looking at human primary bronchial fibroblasts found that TRPV1 was induced by three separate inflammatory stimuli: TNFα, LPS, and IL-1α. The fibroblasts which predominantly did not express TRPV1 before treatment, consistently expressed the channel at 24 and 48 hour time points after inflammatory stimulation (Sadofsky et al. 2012). This TRPV1 mRNA was shown to be functional as the specific TRPV1 agonists, capsaicin and resiniferatoxin, stimulated Ca^{2+} signalling in the fibroblasts treated with inflammatory mediators.

However in the current experiments, LPS exposure of mice *in vivo* did not induce increased TRPV1 expression in lung tissue samples, but in fact appeared to ablate the low levels of expression measureable in the saline exposed animals at the 2, 6, 24 and 48 hour time-points (Figure 3.4). The expression also appeared to be decreased, albeit slightly and non-

significantly, in the smoke exposed animals (3-day and 14-day). A number of the inflammatory mediators induced by LPS and cigarette smoke are known to activate TRPV1, both directly and indirectly. It is feasible that these mediators may induce a down-regulation of TRPV1 message, which is proportional to the severity of inflammation. However this would be in conflict with the *in vitro* data presented by Sadofsky *et al.* 2012.

In contrast to TRPV1, TRPV4 is appeared slightly (but non-significantly) increased at 2 hours after both the 3-day and 14-day smoke model. There is however a significant down-regulation of TRPV4 expression at the 6 hour time-point after LPS expression. TRPV4 was significantly increased in emphysema patients compared to donors. A search of the literature yielded no relevant published documents detailing expression changes of TRPV4 in models of lung inflammation, either *in vitro* or *in vivo*, however TNF-α was shown to cause up-regulation of TRPV4 (and TRPV1) mRNA in synoviocytes (cells found in the synovial membrane) (Denadai-Souza et al. 2012). It is also worth noting that up to seven single nucleotide polymorphisms of TRPV4 were identified in a genome-wide association study, as being associated with COPD (Zhu et al. 2009). The authors postulate that at least three of the associated polymorphisms may affect TRPV4 splicing and so result in loss of TRPV4 function, while other evidence suggests that TRPV4 may play a pro-inflammatory role, indicating a gain of function mutation may contribute to COPD. The observed increase in TRPV4 expression in emphysema patients in Figure 3.5 suggests that a gain of function mutation is more likely to have a role in the development of COPD than a loss of function.

TRPA1 and TRPM8 were below the detection threshold in all of the above murine models and controls. As previously mentioned, this is not unexpected as these two channels are predominantly expressed in the neuronal cell bodies then trafficked to the lungs. Non-neuronal expression of TRPA1 has however been described in both human and mouse lungs or associated cell types (Kunert-Keil et al. 2006; Stokes et al. 2006). TRPM8 expression is not reported in whole lung tissue, however it has been reported in human bronchial epithelial cells (Sabnis, Shadid, et al. 2008; Tsavaler et al. 2001). Using the reagents and methods described in this chapter, however, non-neuronal expression of these channels was below the detection threshold in all control, LPS-exposed and CS-exposed murine groups. This is not to say that TRPA1 and TRPM8 are not present or active in the non-neuronal murine lung

tissues under control and disease conditions, rather just that the available tools were not sensitive enough under these experimental conditions.

3.4.3 Summary

In summary this chapter has characterised the genotyping techniques necessary to test the role of six TRP channels in murine models of inflammation. TRPA1-/- mice were backcrossed onto a 93.75% C57BL/6 genetic background, to allow littermate controls to be used in the previously characterised murine models. TaqMan RT-PCR was used to measure the expression of TRP channels C6, M2, V1 and V4 in the murine models and human tissue samples, implicating a role for each of the channels, whilst highlighting some similarities and differences between the three murine models, as well as between the murine models and the human disease. TRPA1 and TRPM8 were not expressed at detectable levels in the lung parenchyma in either the murine models or the human examples.

Chapter 4

Acute Cigarette Smoke Exposure Model

4.1 Rationale

The current therapies for COPD, including inhaled corticosteroids, are ineffective at halting disease progression or reducing the mortality rate. Disease progression is thought to be driven by a dysregulated inflammatory response in the lungs, associated with the inhalation of noxious particles, in particular, cigarette smoke (CS) (Sethi and Rochester 2000). The key effectors of this inflammatory response include leukocytes and pro-inflammatory mediators, which are thought to drive the functional changes that lead to airflow limitation in COPD patients (Di Stefano et al. 1998). Understanding how inhaled pollutants, such as CS, initiate this inflammatory response in the lungs, may lead to the identification of novel therapeutic targets which can reduce the inflammation in the COPD lung. It is hoped that if therapies are identified which can control the airway inflammation, they will also be able to control the progression of the airflow limitation.

There is a growing body of evidence implicating a pivotal role for many individual TRP channels in inflammatory mechanisms in the lung. In section 1.5, TRP channels A1, C6, M2, M8, V1 and V4 were identified, for varying reasons, as potential candidates for a role in the inflammation thought to drive COPD.

This chapter aims to elucidate the role of these TRP channels in the acute inflammatory response to CS, the primary cause of COPD.

Murine models of airway inflammation provide a useful tool for understanding and elucidating these inflammatory mechanisms, with the ultimate aim of identifying potential therapeutic targets. Acute CS exposure in mice has been demonstrated to initiate an airway inflammatory response, predominantly characterised by infiltration of neutrophils into the airways (D'hulst et al. 2005; Eltom et al. 2011). In the previous chapter, colonies of mice genetically deficient for each of the six identified TRP channels were bred and genotyping protocols were established. In this chapter, animals from these colonies will be exposed to acute cigarette smoke alongside wild-type controls (strain C57BL/6) in order to elucidate the role of each of these TRP channels in the acute inflammatory response to CS. In the case of the TRPA1-/- backcrossed (generation 4) colony, knock-out mice will be exposed alongside littermate wild-type controls as discussed in the previous chapter.

4.2 Methods

In this section the general protocols for the specific experiments carried out in this chapter are detailed. Details of the characterisation of the acute smoke exposure model are also reported.

4.2.1 Animals

Animals were bred as described in section 2.1. Wild-type and knock-out mice were age matched for each study and were a minimum of 10 weeks old. Mice were housed in individually ventilated cages (IVCs) with littermates, and were moved to a room adjacent to the smoke exposure room a minimum of one week before the first exposure, to allow adequate time for acclimatisation.

4.2.2 Acute Smoke Exposure Model

Animals were separated into two groups, air exposure and smoke exposure. Wild-type and knock-out animals of each group were placed into the same cage, thus ensuring identical exposure conditions, which was then placed into either the smoke exposure or the air exposure chamber. Air and smoke exposures occurred concurrently, twice a day for three consecutive days. Mice were returned to their IVCs in between exposures, with water and food available *ad libitum*. For further details of the smoke exposure system please refer to section 2.3.1.

24 hours after the final smoke exposure the animals were terminally anaesthetised by inter-peritoneal injection of sodium pentobarbitone (200 mg/kg). BALF and lung tissue were collected as described in sections 2.4.1 and 2.4.2 and processed to establish total and differential cell counts. Cytokines and ATP levels were also measured in the BALF as detailed in sections 2.4.4 and 2.4.5 respectively.

4.2.2.1 Characterisation of the Acute Smoke Exposure Model

The acute smoke exposure model was previously characterised by Respiratory Pharmacology, and published by Eltom *et al.* 2011. A sub-maximal dose of cigarette smoke (CS) was identified by measuring BALF neutrophilia after a dose-response. It was also established that two smoke exposures a day elicited higher levels of neutrophilia than one exposure per day. A time-course study was subsequently performed to identify the key time-points for inflammatory markers including leukocytes and cytokines. Neutrophilia, KC release and IL-1β release in the BALF were found to be the key endpoints of the model. Figure 4.1 shows the time-course data for neutrophilia, KC levels and IL-1β levels in the BALF. Neutrophilia is increased compared to time-matched controls at 24 hours after the last exposure, peaks at 48 hours, and is mostly resolved by 96 hours. Levels of KC (a homologue of human IL-8) are increased from 2 hours, peaking at 24 hours and resolving by 72 hours, whereas IL-1β is increased at the 24 and 48 hour time points. A number of other cytokines, including IL-18, are also increased at various time-points in this model (data not shown). Neutrophilia, increased IL-8 and increased IL-1β in BALF are all pathologically relevant features of clinical COPD. Mice exposed to the 3-day cigarette smoke model, exhibited no significant changes in tissue leukocyte numbers, but caspase-1 activation is increased. Based on this time-course data the 24-hour time-point was selected to examine the effects of TRP channels, using the knock-out animals characterised in chapter 3.

4.2.3 Statistical analysis

Data are expressed as mean ± S.E.M in all graphs. All experiments were performed with an *n* of at least 6 (please refer to individual figure legends). Statistical significance of differences between air exposed (control) and CS exposed groups, as well as between KO and wild-type groups was determined by a Mann-Whitney U-test for non-parametric data. P values < 0.05 were taken as significant, and are denoted with a * on each graph

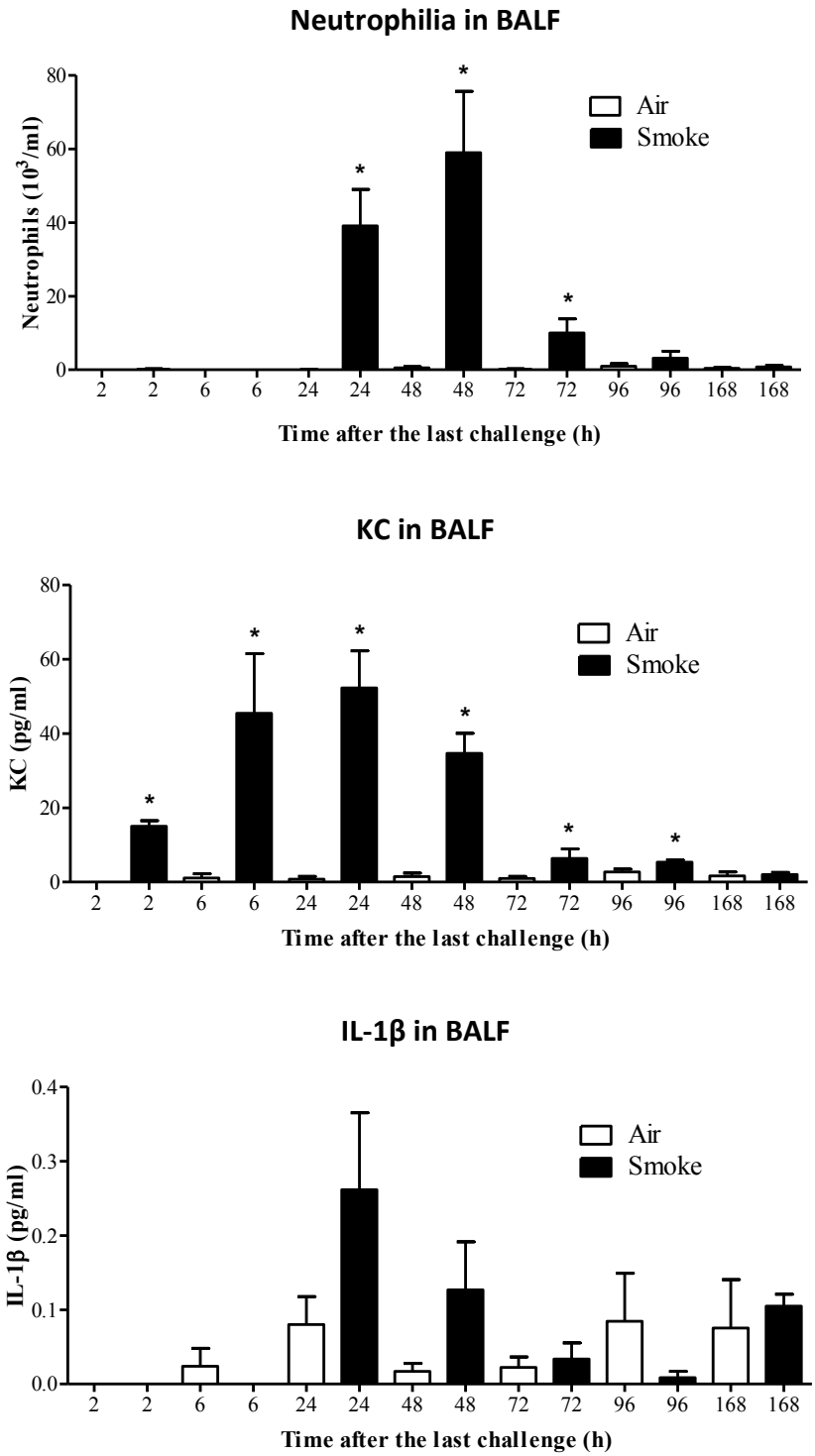

Figure 4.1 Temporal characterisation of inflammation in BALF after acute smoke exposure model. *Wild-type (C57BL/6) mice were exposed to cigarette smoke (grey bars), or control (room air, white bars), for one hour twice daily for three consecutive days. BALF samples were collected at 2, 6, 24, 48, 72, 96 and 168 hours after the final exposure for analysis of inflammatory markers, including neutrophilia and cytokine release. Data are represented as mean ± SEM for n=6 animals in each group. Statistical significance was determined using Mann-Whitney U test. * = P<0.05, denoting a significant difference between the smoke exposed group and the time-matched air exposed control.*

4.3 Results

Two 1-hour exposures a day for 3 consecutive days was shown to induce airway inflammation in C57BL/6 mice, including neutrophilia and cytokine release in the BALF. A time course analysis taking samples at 2, 6, 24, 48, 72, 96 and 168 hours after the last smoke exposure, showed that KC levels in the BALF were increased from 2 hours after the last exposure, peaking at 24 hours. Caspase-1 activation in lung tissue, IL-1β and IL-18 levels in the BALF were all increased at the 24 and 48 hour time-points. Neutrophilia occurred in the BALF from 24 hours and peaked at 48 hours. Macrophage, lymphocyte and eosinophil levels in the BALF were not increased at any time-point, and there was no measurable change in any cell type in the tissue.

The main end points in the acute smoke model therefore are neutrophilia and cytokine release in the BALF, which fits with analysis of COPD patients, where inflammation is thought to be initially driven by neutrophils, before phenotypic change incorporating macrophages, lymphocytes, and chronically $CD8^+$ T cells and B cells (Hogg 2004; van der Strate et al. 2006).

It is also noteworthy that COPD patients exhibit increased concentrations of ATP in BALF (Lommatzsch et al. 2010) and it was previously shown that $P2X_7$-/- mice were protected against 3-day smoke induced BALF neutrophilia (Eltom et al. 2011). For these reasons BALF ATP was also measured in the wild-type and TRP knock-out mice after 3-days smoke.

In all of the following experiments the wild-type mice which were exposed to cigarette smoke exhibited significantly increased levels of neutrophilia, ATP and cytokine release in the BALF. The levels of BALF neutrophilia were determined by 4-way differential counts as described in detail in section 2.4.3.1. Only the BALF neutrophil levels are shown in the figures of this chapter, as this is the only cell type where a significant effect is observed in the acute smoke model. Full data tables of differential cell counts for each experiment including all four cell types – lymphocytes, eosinophils, macrophage/monocytes and neutrophils – are printed in the appendix if required for further reference.

All strains of knock-out mice were viable, fertile and largely indistinguishable from their wild-type controls.

4.3.1 TRPC6

TRPC6 deficient mice exhibited no differences in any of the inflammatory markers measured compared to the wild-type mice after 3 days of cigarette smoke exposure. In particular BALF levels of neutrophilia, IL-1β, KC and ATP were equivalent in mice which expressed TRPC6 and mice deficient for it (Figure 4.2). These data suggest that TRPC6 does not play a role in this acute murine model of cigarette smoke induced inflammation.

4.3.2 TRPM2

TRPM2 KO mice exhibited decreased neutrophil levels in the BALF compared to the wild-type controls. Conversely there was no decrease in cytokine levels or ATP levels (Figure 4.3). This result was deemed unusual as the conventional dogma is that cytokines including KC and IL-1β drive the infiltration of neutrophils into the airways after smoke challenge.

Subsequent analysis of the lung tissue from this experiment revealed that the levels of neutrophilia were significantly increased in the TRPM2-/- mice compared to the wild-types (Figure 4.4). As previously mentioned the leukocyte levels in the tissue do not change measurably in wild-type mice after acute CS challenge. The increase in neutrophils in the tissue of TRPM2-/- mice totals approximately 2 x 10^6 cells per mg, which dwarfs the reduction of neutrophils measured in the BALF. This suggests that in fact absence of TRPM2 may exacerbate the acute cigarette smoke induced inflammatory reaction in mice. Further to this the inflammatory phenotype of the inflammation is different to the wild-type, inasmuch as the number of macrophages is significantly increased. Increased recruitment of macrophages into the airways is a characteristic of more advanced cigarette smoke induced inflammation in murine models and in humans (Stevenson and Birrell 2011).

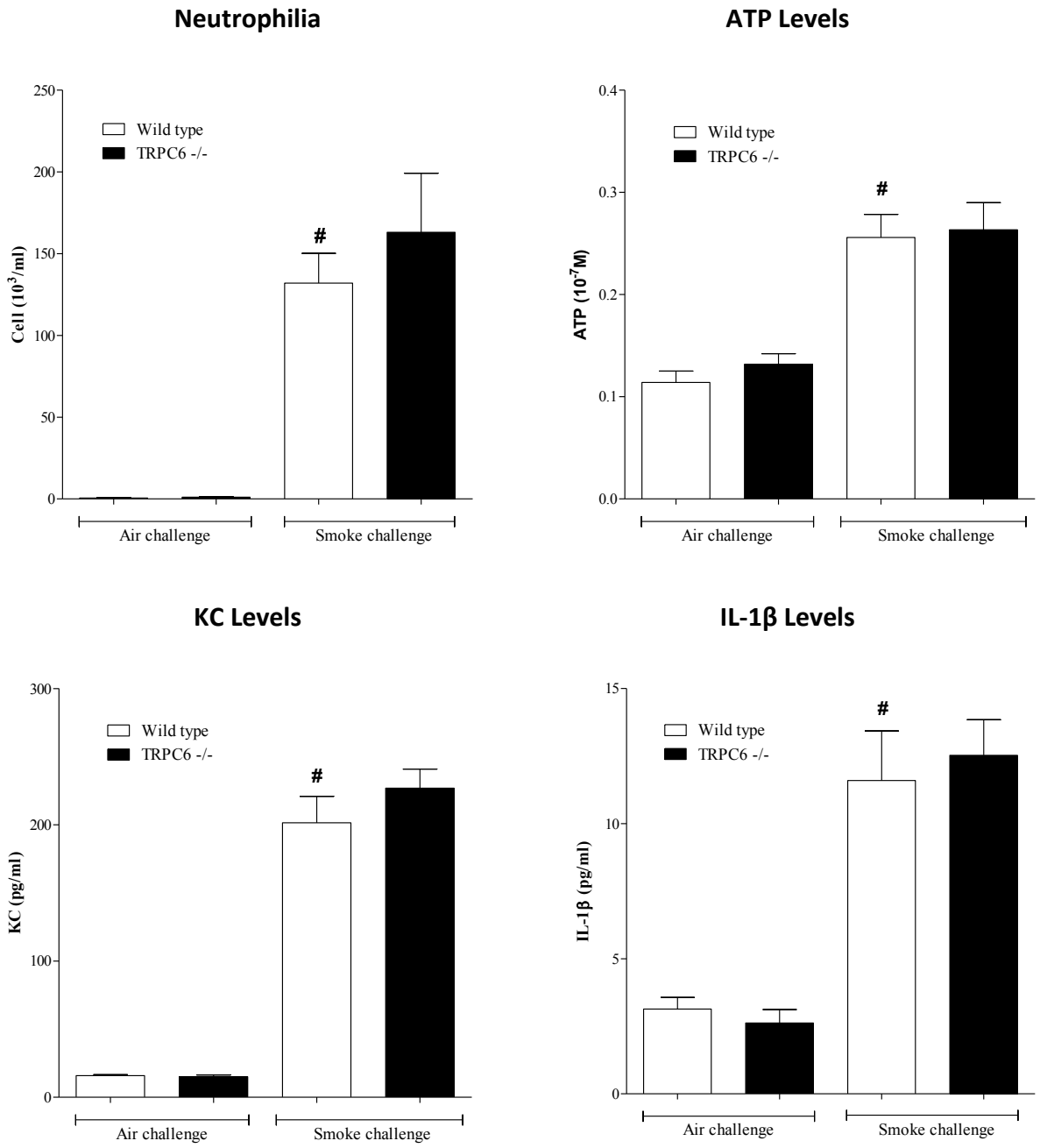

Figure 4.2 The role of TRPC6 on markers of inflammation measured in BALF after acute smoke model. *Wild-type and TRPC6-/- mice were exposed to cigarette smoke, or control (room air), for one hour twice daily for three consecutive days. BALF samples were collected 24 hours after the final exposure for analysis of neutrophilia, ATP levels and cytokine levels. Data are represented as mean ± SEM for n=8 animals in each group. Statistical significance was determined using Mann-Whitney U test. # = P<0.05, denoting a significant difference between the smoke exposed and air exposed wild-types.*

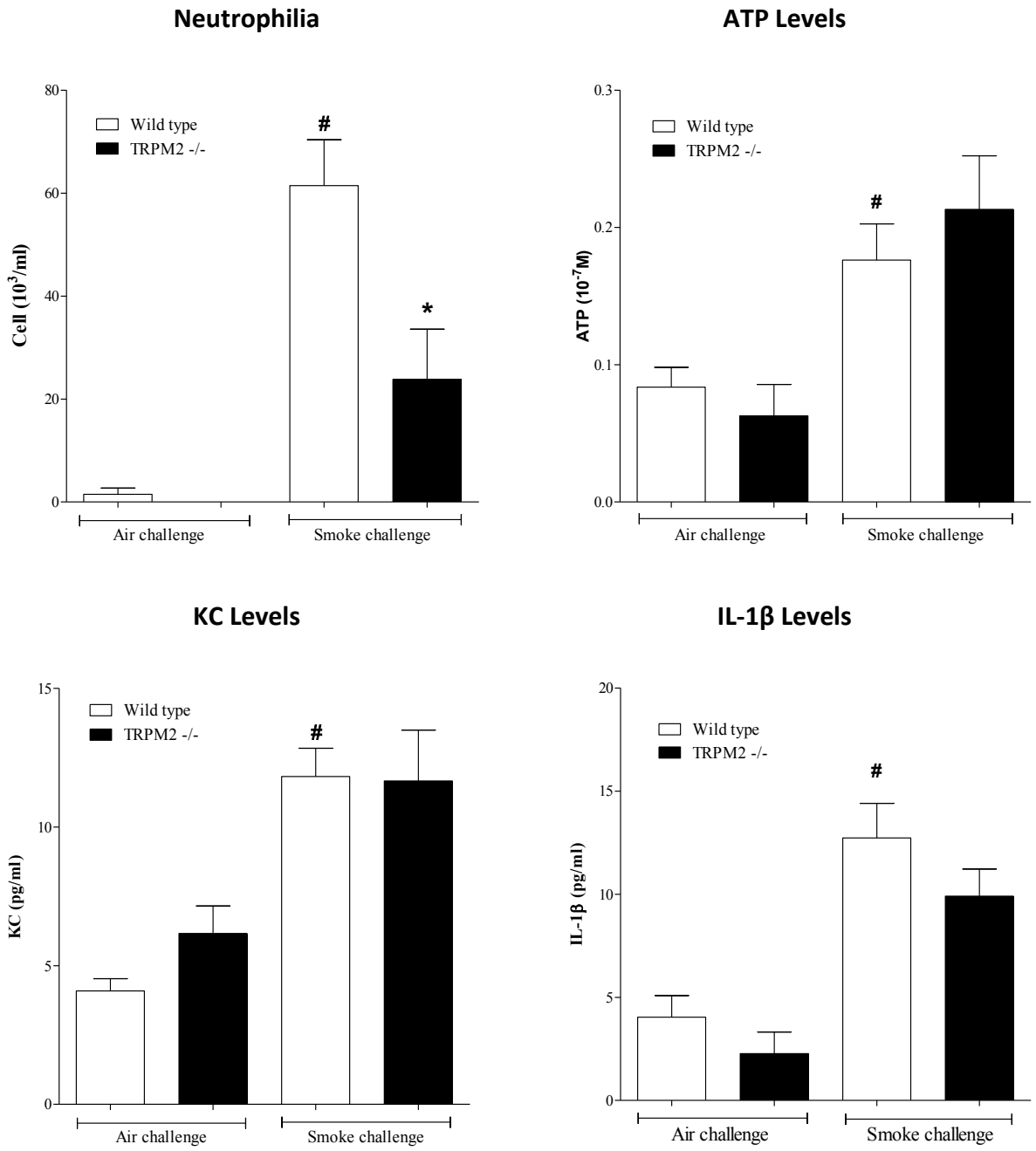

Figure 4.3 The role of TRPM2 on markers of inflammation measured in BALF after acute smoke model. *Wild-type and TRPM2-/- mice were exposed to cigarette smoke, or control (room air), for one hour twice daily for three consecutive days. BALF samples were collected 24 hours after the final exposure for analysis of neutrophilia, ATP levels and cytokine levels. Data are represented as mean ± SEM for n=8 animals in each group. Statistical significance was determined using Mann-Whitney U test. # = P<0.05, denoting a significant difference between the smoke exposed and air exposed wild-types; * = P<0.05, denoting a significant difference between the smoke exposed wild-types and knock-outs.*

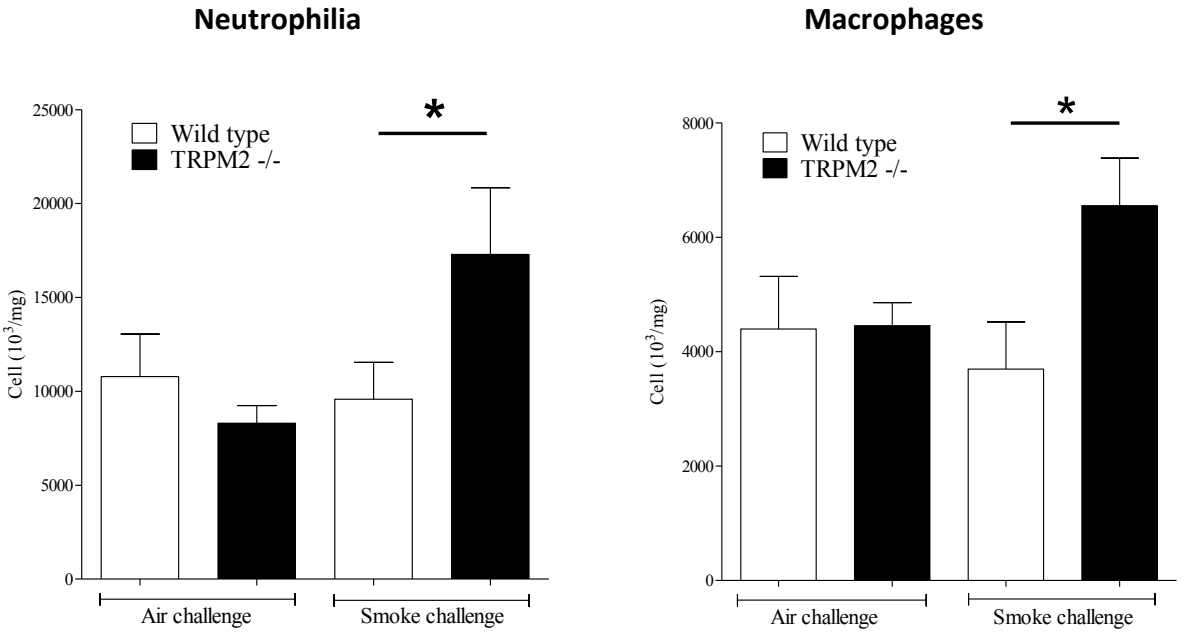

Figure 4.4 The role of TRPM2 on markers of inflammation measured in lung tissue after acute smoke model. *Wild-type and TRPM2-/- mice were exposed to cigarette smoke, or control (room air), for one hour twice daily for three consecutive days. Tissue samples were collected 24 hours after the final exposure for analysis of neutrophilia and macrophagia. Data are represented as mean ± SEM for n=8 animals in each group. Statistical significance was determined using Mann-Whitney U test. # = P<0.05, denoting a significant difference between the smoke exposed and air exposed wild-types; * = P<0.05, denoting a significant difference between the smoke exposed wild-types and knock-outs.*

4.3.3 TRPM8

Genetic ablation of TRPM8 resulted in a statistically significant ($P < 0.01$) decrease in neutrophilia of 90%, compared to the wild-type controls, after smoke exposure. The increase in KC levels in the BALF was similarly significantly inhibited in TRPM8-/- mice. There was no significant difference, however, in the IL-1β levels measured between the smoke exposed wild-types and TRPM8-/- animals, and furthermore there is a significant increase in IL-1β levels in smoke exposed TRPM8-/- mice compared to air exposed TRPM8-/- mice. There also is no decrease in the ATP levels, which would infer that $P2X_7$-inflammasome activation leading to caspase-1 cleavage of pro-IL-1β is likely to be unchanged between the knock-out and the wild-type. These data suggest that TRPM8 may play a role in cigarette smoke induced KC release and neutrophilia, but not necessarily in ATP release or IL-1β levels. There were no changes in any markers measured in the lung tissue.

4.3.4 TRPV1 and TRPV4

TRPV1 and TRPV4 are members of the same protein sub-family, and both act as thermoceptors *in vivo* among other functions, however their expression profiles are relatively distinct from one another. It is therefore interesting to note that the data for mice lacking TRPV1 show a similar pattern to the data for mice lacking TRPV4. Genetic ablation of either channel results in significantly attenuated neutrophilia compared to wild-type mice after 3 days of smoke exposure. Both TRPV1-/- and TRPV4-/- mice had approximately a 50% reduction in ATP levels and IL-1β levels compared to wild-types. However there was little if any attenuation of KC levels in BALF in either TRPV1-/- or TRPV4-/- mice compared to wild-types. As ATP is an activator of the $P2X_7$-inflammasome pathway, which has previously been shown to play an important role in the neutrophilia associated with this model, this suggests that TRPV1 and TRPV4 may be upstream regulators of this CS-induced pathway. There were no changes in any markers measured in the lung tissue.

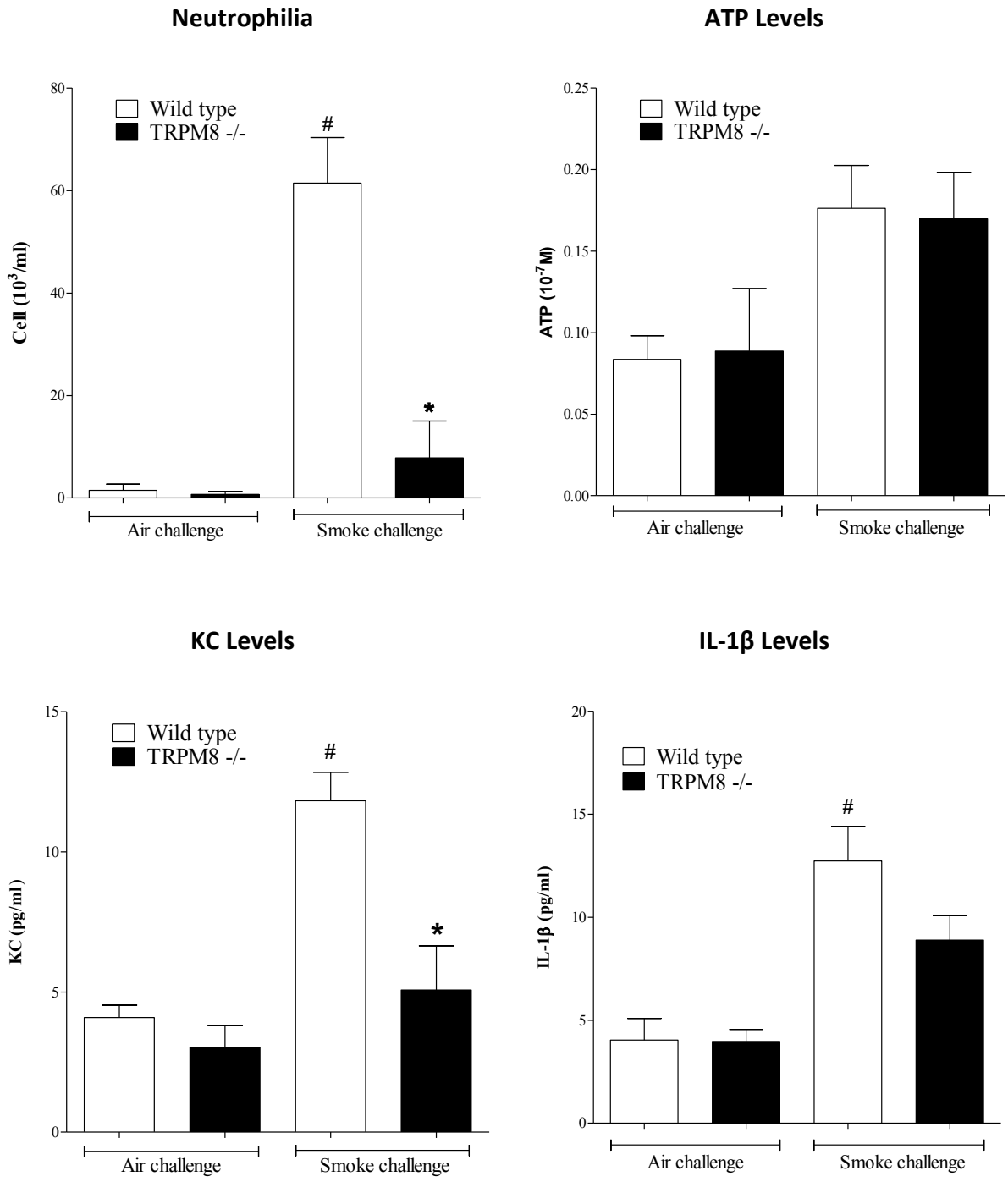

Figure 4.5 The role of TRPM8 on markers of inflammation measured in BALF after acute smoke model. *Wild-type and TRPM8-/- mice were exposed to cigarette smoke, or control (room air), for one hour twice daily for three consecutive days. BALF samples were collected 24 hours after the final exposure for analysis of neutrophilia, ATP levels and cytokine levels. Data are represented as mean ± SEM for n=8 animals in each group. Statistical significance was determined using Mann-Whitney U test. # = P<0.05, denoting a significant difference between the smoke exposed and air exposed wild-types; * = P<0.05, denoting a significant difference between the smoke exposed wild-types and knock-outs.*

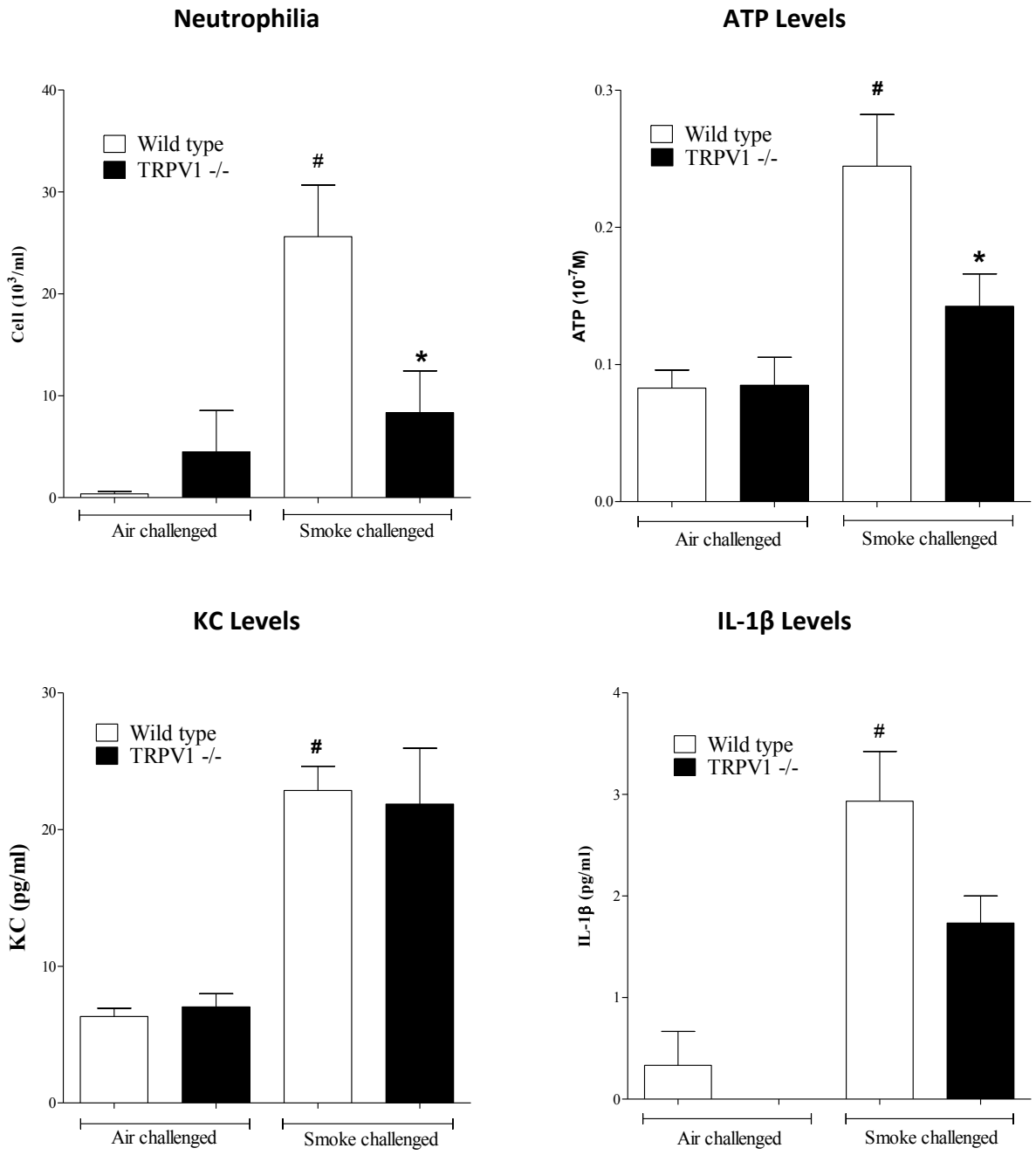

Figure 4.6 The role of TRPV1 on markers of inflammation measured in BALF after acute smoke model. *Wild-type and TRPV1-/- mice were exposed to cigarette smoke, or control (room air), for one hour twice daily for three consecutive days. BALF samples were collected 24 hours after the final exposure for analysis of neutrophilia, ATP levels and cytokine levels. Data are represented as mean ± SEM for n=8 animals in each group. Statistical significance was determined using Mann-Whitney U test. # = P<0.05, denoting a significant difference between the smoke exposed and air exposed wild-types; * = P<0.05, denoting a significant difference between the smoke exposed wild-types and knock-outs.*

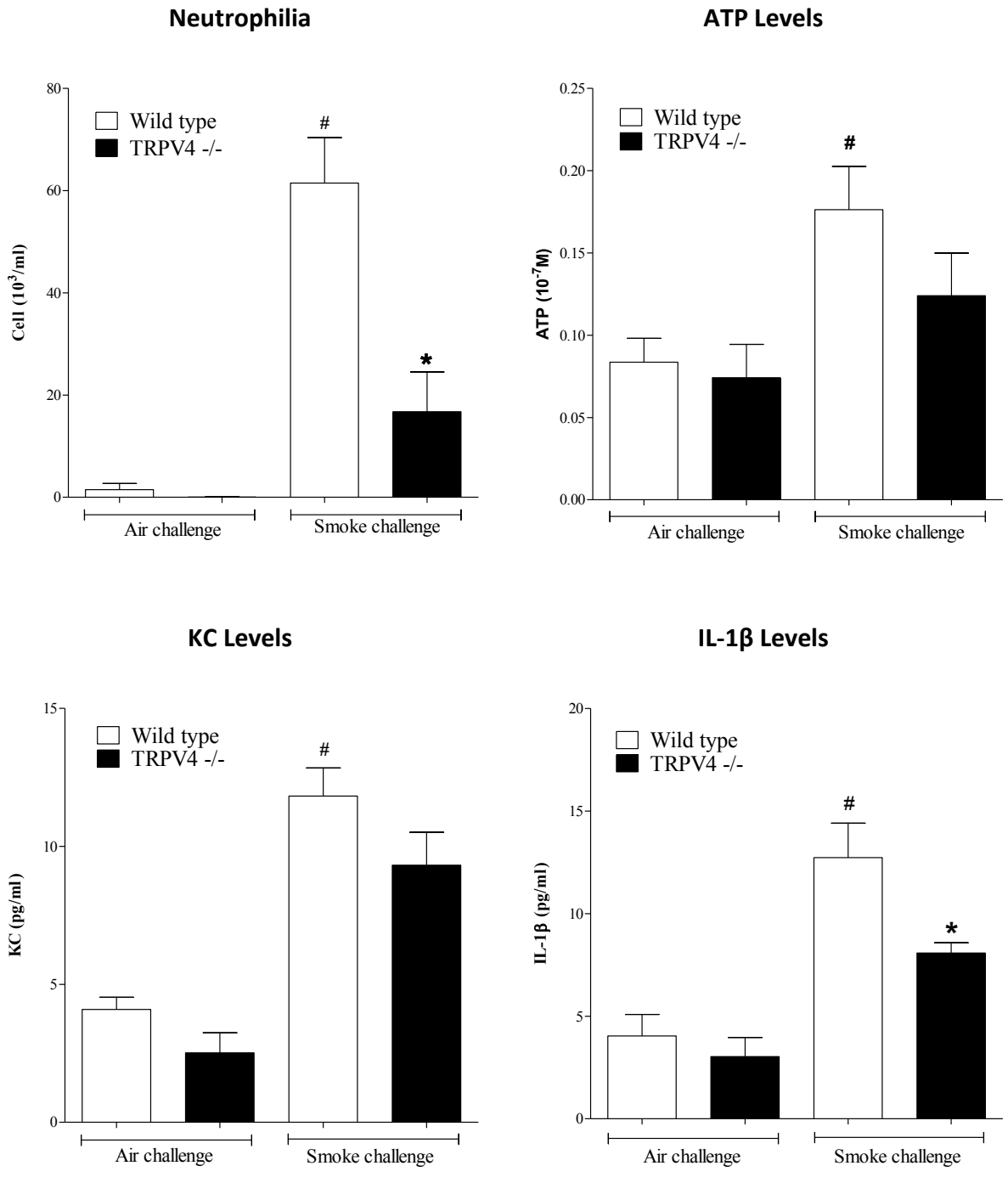

Figure 4.7 The role of TRPV4 on markers of inflammation measured in BALF after acute smoke model. *Wild-type and TRPV4-/- mice were exposed to cigarette smoke, or control (room air), for one hour twice daily for three consecutive days. BALF samples were collected 24 hours after the final exposure for analysis of neutrophilia, ATP levels and cytokine levels. Data are represented as mean ± SEM for n=8 animals in each group. Statistical significance was determined using Mann-Whitney U test. # = P<0.05, denoting a significant difference between the smoke exposed and air exposed wild-types; * = P<0.05, denoting a significant difference between the smoke exposed wild-types and knock-outs.*

4.3.5 TRPA1

There is a growing body of evidence implicating TRPA1 as a pro-inflammatory regulator in a number of *in vitro* and *in vivo* models. Indeed TRPA1-/- mice exhibited reduced levels of leukocytes and cytokines in BALF in an OVA-induced model of asthma (Caceres et al. 2009). TRPA1 is also known to be directly gated by acrolein which is a prominent constituent of cigarette smoke. It has further been shown that mice deficient for TRPA1 are protected against CS and acrolein induced KC release *in vivo* (Nassini et al. 2012).

For these reasons it was strongly suspected that deletion of TRPA1 would inhibit inflammation compared to wild-type littermate controls. As with TRPC6 however, there were no significant differences between the knock-outs and the wild-types in BALF or lung tissue. Neutrophilia, KC and ATP levels in the BALF were the same in the wild-type and the TRPA1-deficient groups. IL-1β levels were increased, although not significantly, in both the air and smoke exposed TRPA1-/- mice compared to the relevant wild-type groups. Overall, this data suggests that TRPA1 does not make a significant contribution to the induction of acute CS induced inflammation, in particular contradiction to the data in the Nassini *et al*. 2012 paper.

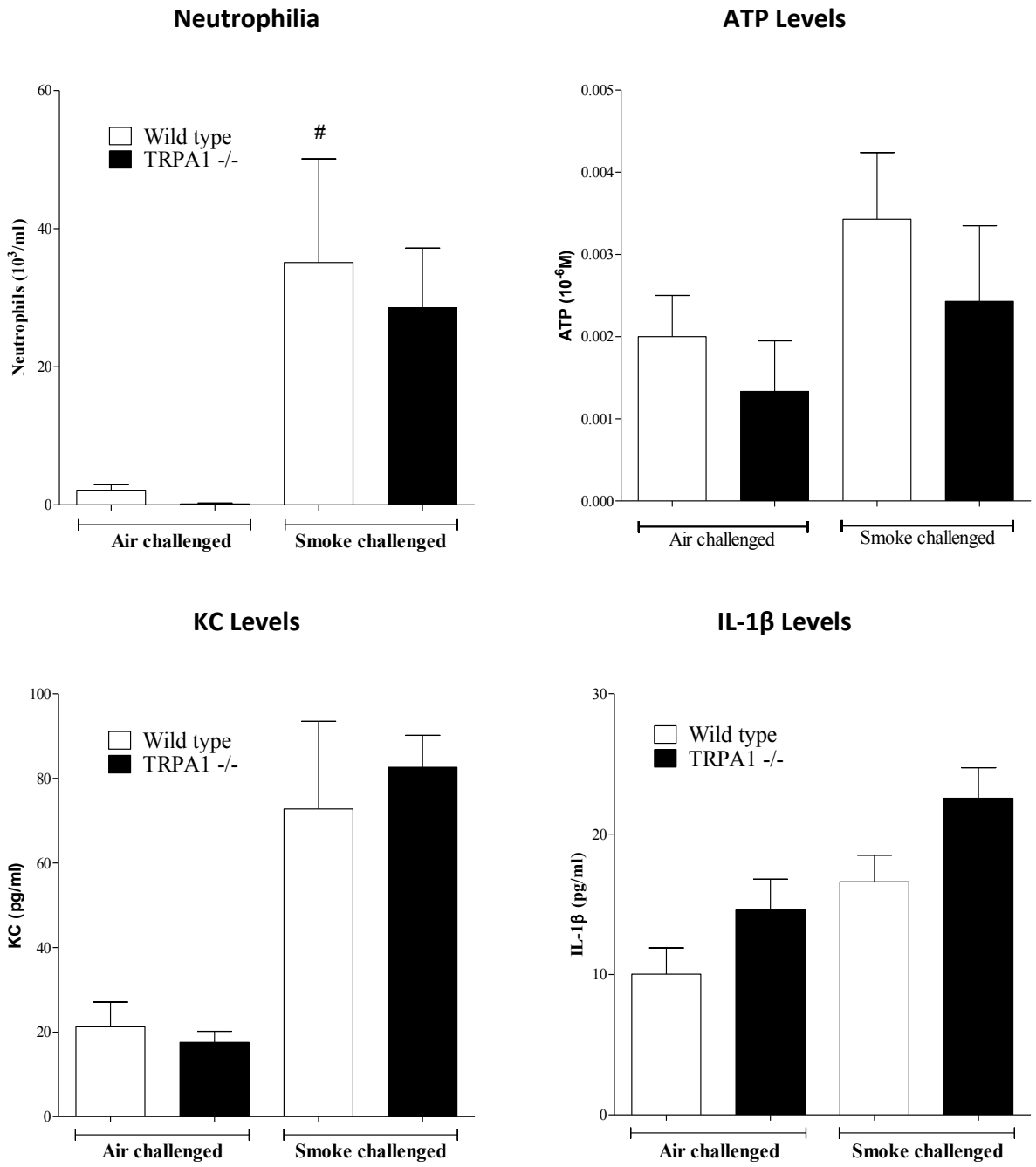

Figure 4.8 The role of TRPA1 on markers of inflammation measured in BALF after acute smoke model. *Wild-type and TRPA1-/- mice were exposed to cigarette smoke, or control (room air), for one hour twice daily for three consecutive days. BALF samples were collected 24 hours after the final exposure for analysis of neutrophilia, ATP levels and cytokine levels. Data are represented as mean ± SEM for n=8 animals in each group. Statistical significance was determined using Mann-Whitney U test. # = P<0.05, denoting a significant difference between the smoke exposed and air exposed wild-types; * = P<0.05, denoting a significant difference between the smoke exposed wild-types and knock-outs.*

4.4 Discussion

The aim of this chapter was to assess whether specific TRP channels were involved in the inflammatory response to cigarette smoke. A previously characterised murine model of cigarette smoke-induced inflammation was used, as colonies of mice genetically deficient for specific TRP channels are available, and are an excellent tool to ascertain the functions of individual proteins. TRP channels which have been identified as possibly having a role in inflammation or airway diseases were identified from the available literature, and colonies of mice deficient for these specific channels were exposed to cigarette smoke exposure twice daily for three consecutive days, alongside wild-type controls.

4.4.1 Discussion of Results

TRPC6 reportedly has a fundamental role in neutrophil migration (Damann et al. 2009), and is known to be up-regulated in macrophages from COPD patients (Finney-Hayward et al. 2010). It is therefore a prime candidate for involvement in the cellular recruitment, which is a primary symptom of the COPD lung, and is thought to drive the functional changes. To test this hypothesis, the effect of 3 days of cigarette smoke on neutrophil recruitment and cytokine levels in the BALF of wild-type and TRPC6-/- mice was compared. However as figure 4.2 shows, genetic ablation of TRPC6 resulted in no significant differences in any of the inflammatory markers measured. This result suggests that TRPC6 is not involved in the propagation of cigarette smoke induced airway inflammation in this rodent system. It is however important to note that COPD is a chronic disease, and although all smokers exhibit lung inflammation, only around 25% develop the disease. It is therefore still possible that TRPC6 may play an important role in the change from a normal lung inflammatory response to a disease phenotype, however longer smoke exposure models would be required to test this.

It has been shown that TRPA1 can be directly gated by chemical components of cigarette smoke, including acrolein and crotonaldehyde, and there is evidence supporting a pro-inflammatory role for TRPA1 in various murine models. In the past it has been strongly suggested that TRPA1 contributes to murine inflammation through neurogenic mechanisms,

involving the release of neuropeptides including substance P and neurokinins, and indeed the highest levels of TRPA1 expression are found on sensory nerves (Story et al. 2003). It has been suggested however that this mechanism is not directly relevant to COPD as no evidence has been found linking neurogenic inflammation to human airway diseases, despite much research (Boot et al. 2007; Fahy et al. 1995). However increasing evidence supports a crucial role for TRPA1 in non-neuronal inflammation as well. TRPA1 was found to mediate IL-8 release from primary human airway and lung cells in response to acrolein and CSE and, furthermore an NK1 antagonist did not reduce CSE induced inflammation after tracheal instillation in mice (Nassini et al. 2012). This new evidence provides reasonable mechanisms for TRPA1-mediated cigarette smoke induced inflammation, through non-neuronal tissues, which could conceivably contribute to the pathogenesis of COPD. It has also been reported that CSE causes contraction of guinea-pig bronchial rings, and Ca^{2+} mobilisation in cultured jugular ganglia neurons through TRPA1 but not through TRPV1 (Andrè et al. 2008; Grace et al. 2012). However genetic deletion of TRPA1 did not affect any of the inflammatory markers measured in the present study compared to littermate controls, whereas TRPV1-deficient mice had significantly reduced neutrophilia and IL-1β levels in BALF.

TRPV1 is frequently co-expressed with TRPA1 on a subset of airway sensory neurons, and is dogmatically thought to cause airway inflammation through neurogenic mechanisms. As with TRPA1, however, there is increasing evidence for TRPV1 expression on a wide range of other cell types. There are many endogenous activators of TRPV1 increased in the lung of COPD patients, including low pH of exhaled breath condensate, lipoxygenase mediated arachidonic acid products, as well as PKA, PKC and PLC pathways. It is also noteworthy that COPD patients exhibit reduced threshold to capsaicin induced cough compared to non-disease controls, indicating increased TRPV1 sensitivity in COPD patients (Doherty et al. 2000). It has also been reported that TRPV1 agonist capsaicin induces cytokine release and cell death (in a dose-dependent fashion) in human epithelial cell lines BEAS-2B and A549 (Reilly et al. 2003). Furthermore, a recent study identified TRPV1 as an important mediator of cellular toxicity in the lungs to inhaled particulate matter, which may also be an important risk factor for COPD (Deering-Rice et al. 2012).

A search of the literature yielded few studies examining the role of TRPV1 in *in vivo* smoke exposure models, although it has been previously reported that neonatal treatment of rats with capsaicin to ablate TRPV1 abolished increased vascular permeability induced by cigarette smoke (Lundberg et al. 1983). Therefore the significant decrease of neutrophilia in TRPV1-/- mice compared to wild-types acutely exposed to cigarette smoke is a novel finding. An interesting feature of this experiment was the finding that TRPV1 ablation inhibited IL-1β levels in BALF compared to wild-types, but KC levels were similarly increased in both wild-type and TRPV1-/- animals. This pattern was recapitulated in TRPV4-/- animals, where again neutrophilia and IL-1β levels were decreased in the BALF, but KC levels remained increased. Taken alone these findings suggest that IL-1β and not KC is important in the recruitment of neutrophils to airways in response to acute cigarette smoke. However TRPM8-/- mice exhibited approximately 85% reduction in neutrophilia, significantly reduced KC levels and only a small reduction in IL-1β levels. Together these results suggest two distinct mechanisms for cigarette smoke induced neutrophilia, one involving TRPV1/4 and IL-1β, and another involving TRPM8 and KC, both of which contribute to the overall cellular burden in the airways.

It is also of note that ATP release was reduced in TRPV1-/- and TRPV4-/- animals but not in TRPM8-/- animals. TRPV4 activation has been previously reported to cause ATP release from airway epithelial cells via pannexin channels in response to osmotic stress (Seminario-Vidal et al. 2011), and ATP is increased in BALF from COPD patients (Mortaz et al. 2009). ATP activates $P2X_7$ receptors leading to inflammasome activation of caspase-1 and consequently cleavage of pro-IL-1β and pro-IL-18 to their respective mature forms. What is more, inflammation in the 3-day smoke exposure model was shown to be sensitive to $P2X_7$ ablation and antagonism (Eltom et al. 2011). It is therefore likely that TRPV1 and TRPV4 are involved upstream of $P2X_7$, mediating ATP release after exposure to cigarette smoke, which activates the $P2X_7$ – inflammasome - caspase-1 axis, resulting in the cleavage of pro-IL-1β into mature IL-1β, which contributes to the recruitment of neutrophils into the airway lumen.

The mechanism of TRPM8 induced inflammation is much harder to determine, as very few publications have dealt with this relationship. TRPM8 is activated by cold temperatures (8-22°C) as well as menthol with invokes cold sensations. Exposure to cold air has many effects

on the respiratory system, including bronchoconstriction, increased mucus secretion and decreased mucociliary clearance, and prolonged exposure can lead to inflammatory cell infiltration and increased cytokine transcription (Davis & Freed 2001; Davis et al. 2005). It has also been recently identified that a functional TRPM8 variant is expressed in human epithelial cells, activation of which promotes Ca^{2+} release from the endoplasmic reticulum and increased transcription of numerous inflammatory cytokines including IL-8 (Sabnis et al. 2008a; Sabnis et al. 2008b). IL-8 activates the CXCR2 receptor in humans, the equivalent receptor of which in mice is activated by KC, and therefore KC is often considered as the functional homologue of IL-8 in humans. The mechanism by which TRPM8 activation induces the transcription of various cytokines has not been elucidated, although activation of TRPM8 receptors is known to induce strong influxes of Ca^{2+} into the cell, and it is therefore conceivable that this influx leads to activation of PKC pathways which are known to control NF-κB mediated transcription of pro-inflammatory genes (Page et al. 2003).

In many ways the results obtained with the genetic ablation of TRPM2 were the most surprising of this chapter. TRPM2 is a prime candidate for a role in cigarette smoke induced inflammation as it is known to be activated by oxidative stress, a key inflammatory insult associated with cigarette smoke, and it is widely expressed on inflammatory cells including neutrophils. Activation of TRPM2 *in vitro* has also been shown to induce IL-8 release from monocytes (Yamamoto, Shimizu, and Mori 2009). Furthermore TRPM2-/- mice have been shown to exhibit significantly decreased neutrophilia in a model of DSS-induced colitis (Araki et al. 2006). These data suggest a pro-inflammatory role for TRPM2 in response to oxidative stimuli, prompting the suggestion that inhibition of TRPM2 could be beneficial in COPD (Banner et al. 2011). A more recently published paper however, found no differences in neutrophilia or cytokine levels in BALF between TRPM2-/- mice and wild-types after exposure to cigarette smoke for 3 days, in a protocol similar to one used in this chapter (Hardaker et al. 2012). However Hardaker *et al* did not report cell counts in the lung tissue, which is where significant increases in neutrophil and macrophage levels were observed in Figure 4.4 of this chapter. Neither these data nor the Hardaker *et al* study support the hypothesis that inhibition of TRPM2 would provide any benefit in COPD, and in fact it may actually potentiate the inflammation.

4.4.2 Summary

The data in this chapter support the theory that there are at least 2 distinct, albeit interacting, inflammatory pathways contributing to cellular infiltration in the 3-day smoke exposure model of airway inflammation. Knocking out TRPM8 inhibited neutrophilia and KC levels whereas knocking out TRPV1 or TRPV4 inhibited neutrophilia, ATP levels and IL-1β levels. TRPM2 may play a protective role against CS-induced inflammation, as mice deficient for this channel exhibited higher levels of neutrophilia and cytokine release. TRPA1 and TRPC6 had no discernible role in this model.

What is not discernible from the data in this chapter, is whether TRP channels are involved specifically in cigarette smoke mediated inflammatory pathways, or whether they have a general role in the innate inflammatory responses of the lung. Innate inflammatory responses are part of the lungs first line of defence to invading pathogens such as bacteria and viruses. Bacteria and viruses are thought to be responsible for the majority of exacerbations experienced by COPD patients, and exacerbations are the prime cause for hospitalisation and mortality in the disease. For this reason it would be therapeutically beneficial to target the COPD relevant inflammatory pathways, without compromising the innate immune system and in so doing risking exacerbations. Models of bacterial infection in mice using aerosolised lipopolysaccharide (LPS) exposure have been well-characterised by many research groups, and will provide the ideal system to compare the role of TRP channels in innate defence like inflammatory responses.

Chapter 5

LPS Exposure Model

5.1 Rationale

Chapter 4 examined the role of TRP channels in the development of inflammation using acute cigarette smoke exposure as the agent of induction. Genetic deletion of various TRP channels was shown to affect the levels of key inflammatory markers, thought to be important in the development of COPD. However, it is not clear whether these effects are specific to CS-mediated inflammatory pathways, or whether they are a result of the respective TRP channels being part of a normal innate inflammatory response. In order to answer this question, an alternative model of airway inflammation will be employed, namely, acute lipopolysaccharide (LPS) exposure which is known to activate an innate inflammatory response.

The innate inflammatory response to bacteria is well characterised, and is essential to host defence. LPS is an integral component of the cell membrane of gram-negative bacteria, which can directly activate Toll-Like Receptor 4 (TLR4), causing the production of pro-inflammatory cytokines via myeloid differentiation factor 88 (MyD88) and NF-κB. Aerosolised LPS challenge induces acute airway neutrophil recruitment, as seen with acute CS exposure, however the LPS induced inflammatory mechanism does not reflect the dysregulated and self-propagating inflammation in COPD.

Inflammation induced by LPS is effectively inhibited by glucocorticoid treatment and resolves rapidly (Birrell et al. 2005), and furthermore, repeated LPS administration attenuates neutrophilic infiltration (Brass et al. 2008). This is in contrast to cigarette smoke induced inflammation and that observed in COPD, where the inflammation is progressive, glucocorticoid-resistant and slowly resolving. The acute inflammatory response to CS in mice has also been shown to be independent of NF-κB, which is a key pro-inflammatory mediator of the LPS response (Rastrick et al. 2013).

The major aim of this chapter is to investigate whether the selected TRP channels are involved in the innate inflammatory response in the airways, as opposed to a specific CS-mediated inflammatory pathway.

5.2 Methods

5.2.1 Animals

Animals were bred as described in section 2.1. Wild-type and knock-out mice were age matched for each study and were 10 – 12 weeks old. Mice were housed in individually ventilated cages (IVCs) with littermates. TRPA1-/- and +/+ littermate controls were identified by genotyping of tail-tips a minimum of one week prior to the study. All other colonies were genotyped after the experiments.

5.2.2 LPS Exposure Model

Animals were exposed to either aerosolised 1 mg/ml LPS in saline, or control (sterile saline) for 30 minutes. Wild-type and knock-out mice were challenged concurrently, to ensure identical exposure conditions. Mice were returned to their IVCs after exposure, with water and food available *ad libitum*. For further details of the LPS exposure system, please refer to section 2.3.2.

6 hours after the challenge, the animals were terminally anaesthetised by inter-peritoneal injection of sodium pentobarbitone (200 mg/kg). BALF and lung tissue were subsequently collected as described in sections 2.4.1 and 2.4.2 and processed to establish total and differential cell counts. Cytokines were also measured in the BALF as detailed in section 2.4.4. Collection and processing of samples was performed in an identical fashion to the acute smoke model.

5.2.2.1 Characterisation of the LPS Exposure Model

The LPS exposure model was previously characterised by Respiratory Pharmacology, and is published in Eltom *et al.* 2011. As with the acute smoke exposure model, a sub-maximal dose of 1 mg/ml LPS in saline was established after a dose-response, by measuring BALF neutrophilia. A time-course study was subsequently performed to identify the relevant time-points for key inflammatory mediators, especially cellular recruitment in the BALF and

lung tissue, as well as cytokine release. LPS induced inflammation was found to share many phenotypic similarities with 3-day smoke induced inflammation in mice, including neutrophilia, KC release and IL-1β release (Figure 5.1).

There are however a number of differences as well, notably that neutrophil levels are far higher after LPS than they are after smoke, and these higher levels also peak at 24 hours after exposure rather than 48 hours. It is also worth noting that while in the acute smoke model neutrophils were the only cell type significantly increased in the BALF, increased levels of eosinophils (peaking at 2 hours), macrophages and lymphocytes (24 – 96 hours) are observed after LPS exposure. The increases in these cell-types after LPS exposure can also be measured in the lung tissue (Figure 5.2), whereas changes are limited to BALF after the acute smoke model.

The cytokine levels similarly peak at earlier time-points after LPS exposure compared to after acute smoke exposure (Figures 5.1 and 4.1). Levels of KC and IL-1β both peak at or before 2 hours (the earliest time point measured), compared to 24 hours in the acute smoke model. A number of other cytokines are also significantly increased after LPS, including IL-6, TNF-α and IL-18.

From these data the 6 hour time-point was chosen to examine the role of TRP channels, as this is a sub-maximal time-point for neutrophilia, IL-1β and KC levels, which were the main end-points measured in the acute smoke model.

5.2.3 Statistical analysis

Data are expressed as mean ± S.E.M in all graphs. All experiments were performed with an *n* of at least 6 (please refer to individual figure legends). Statistical significance of differences between KO and wild-type groups was determined by a Mann-Whitney U test for non-parametric data. P values < 0.05 were taken as significant, and are denoted with a * on each graph.

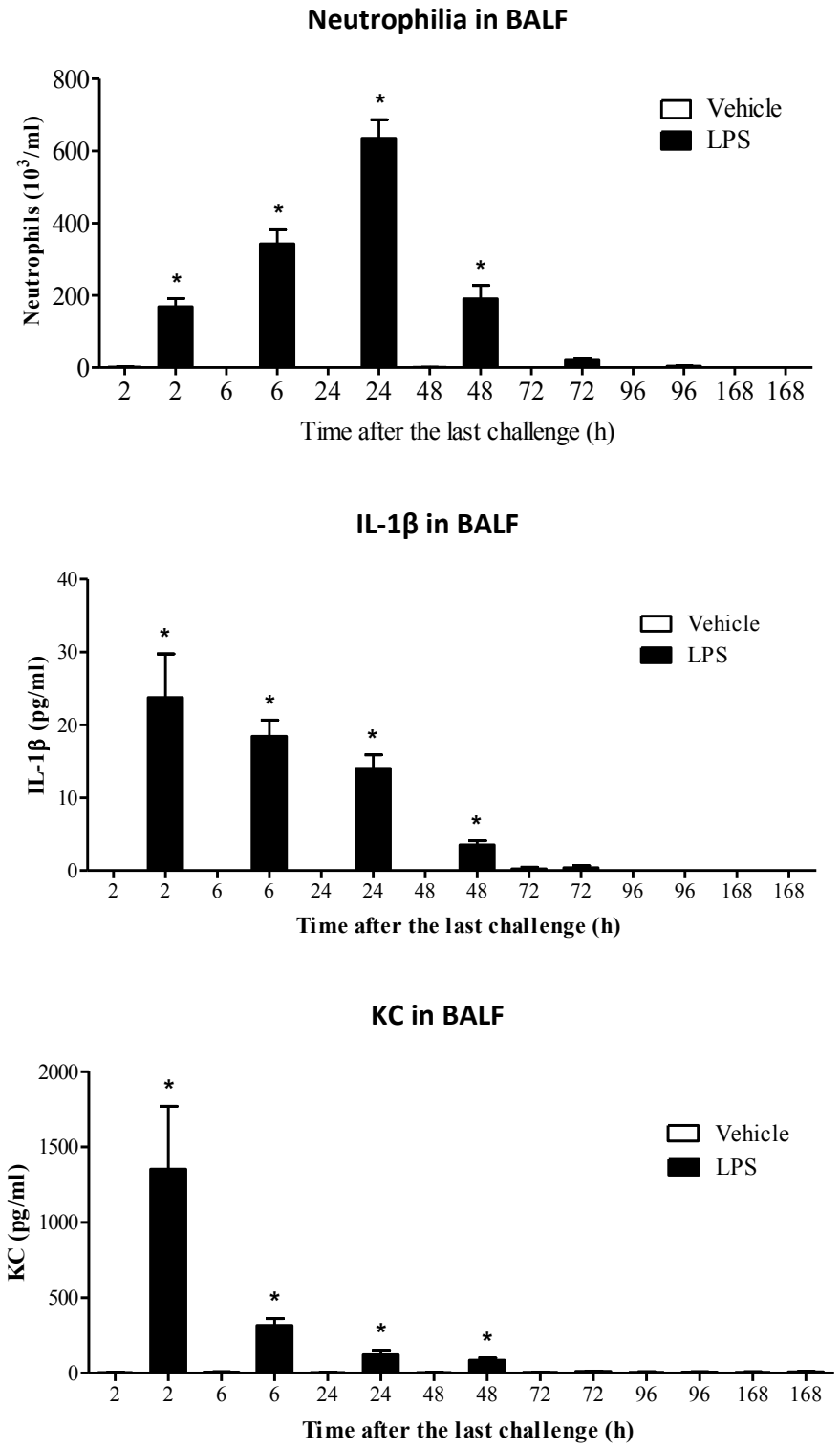

Figure 5.1 Temporal characterisation of inflammation in BALF after LPS exposure model. *Wild-type (C57BL/6) mice were exposed to LPS (grey bars), or Vehicle (Saline, white bars), for 30 minutes. BALF samples were collected at 2, 6, 24, 48, 72, 96 and 168 hours after the final exposure for analysis of inflammatory markers, including neutrophilia and cytokine release. Data are represented as mean ± SEM for n=6 animals in each group. Statistical significance was determined using Mann-Whitney U test. * = P<0.05, denoting a significant difference between the smoke exposed group and the time-matched air exposed control.*

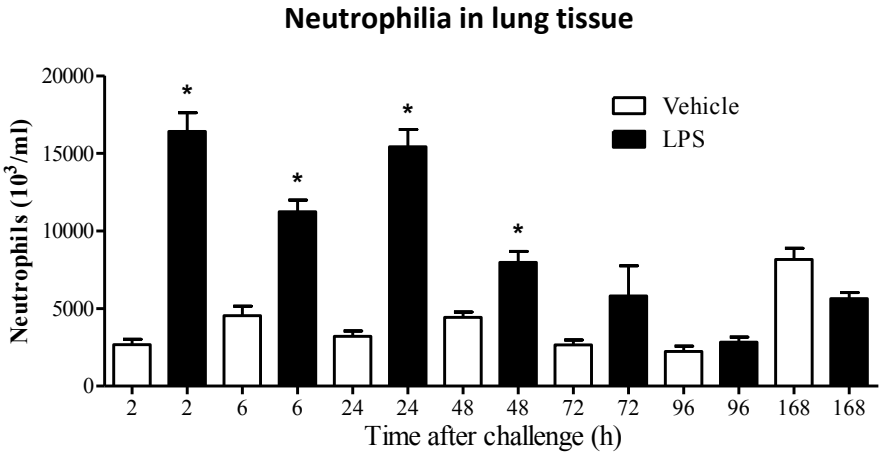

Figure 5.2 Temporal characterisation of inflammation in tissue after LPS exposure model. *Wild-type (C57BL/6) mice were exposed to LPS (grey bars), or Vehicle (Saline, white bars), for 30 minutes. BALF samples were collected at 2, 6, 24, 48, 72, 96 and 168 hours after the final exposure for analysis of neutrophilia. Data are represented as mean ± SEM for n=6 animals in each group. * = P<0.05, denoting a significant difference between the smoke exposed group and the time-matched air exposed control.*

5.3 Results

Knock-out and wild-type control animals were exposed to LPS concurrently, before being returned to their own cages. Six hours later the animals were terminally anaesthetised before collection of tail-tips for genotyping, then BALF and lung tissue for analysis of inflammation. The main end-points measured in this model were neutrophil recruitment in the BALF, as well as cytokines including IL-1β and KC. These end-points are common to both the acute smoke exposure model and inflammatory markers measured in COPD patients.

In all of the following experiments, LPS exposed wild-type mice exhibited significantly increased levels of neutrophilia and cytokine release in BALF when examined at the 6 hour time-point. Cellular recruitment was also measured in the lung tissue for the TRPA1, TRPM2 and TRPV1 knock-out mice in conjunction with the relevant wild-types. All cellular data from BALF and lung tissue is presented in tables in the appendix. Below the BALF neutrophil and cytokine data is presented for each of the channels, as these are the end-points which are in common with the acute CS model, and for which the 6 hour time-point was chosen. Lung tissue cellular infiltration data is also presented for the TRPM2 experiment, as this was the only channel where a significant effect was observed.

As in the previous chapter, all strains of knock-out mice were viable, fertile and largely indistinguishable from their wild-type controls. Tail-tips were collected from all animals for DNA extraction and genotyping, to confirm their genetic integrity.

5.3.1 TRPA1 and TRPC6

It was shown in the previous chapter that genetic deletion of either TRPA1 or TRPC6 had no effect on inflammation induced by 3-days of smoke exposure in mice. These results are recapitulated using the LPS model. There were no significant differences in the levels of any of the inflammatory mediators measured between either TRPC6-/-, or, TRPA1-/- mice and their respective wild-type controls. These results are represented in Figures 5.3 and 5.4, where levels of BALF neutrophilia, IL-6, IL-1β and KC are shown. In addition there were no significant changes in the numbers of neutrophils measured in the lung tissue between wild-types and TRPA1 knock-outs. These data suggest that neither TRPA1 nor TRPC6 play a role in LPS-induced inflammatory responses in the airways.

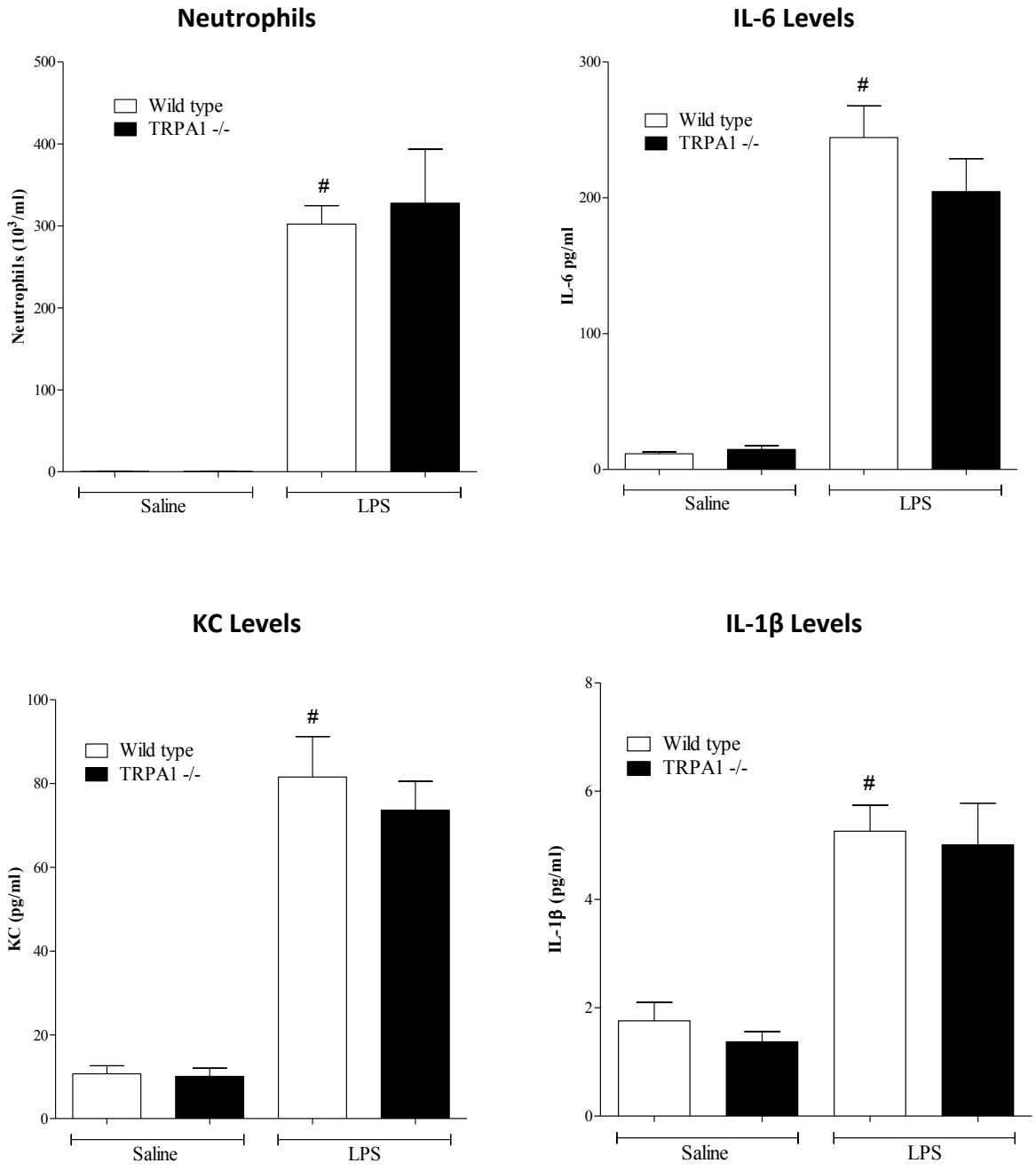

Figure 5.3 The role of TRPA1 on markers of inflammation measured in BALF after LPS exposure. *Wild-type and TRPA1-/- mice were exposed to LPS, or Vehicle (Saline), for 30minutes. BALF samples were collected 6 hours after the final exposure for analysis of neutrophilia and cytokine levels. Data are represented as mean ± SEM for n=8 animals in each group. Statistical significance was determined using a Mann-Whitney U test. # = P<0.05, denoting a significant difference between the smoke exposed and air exposed wild-types.*

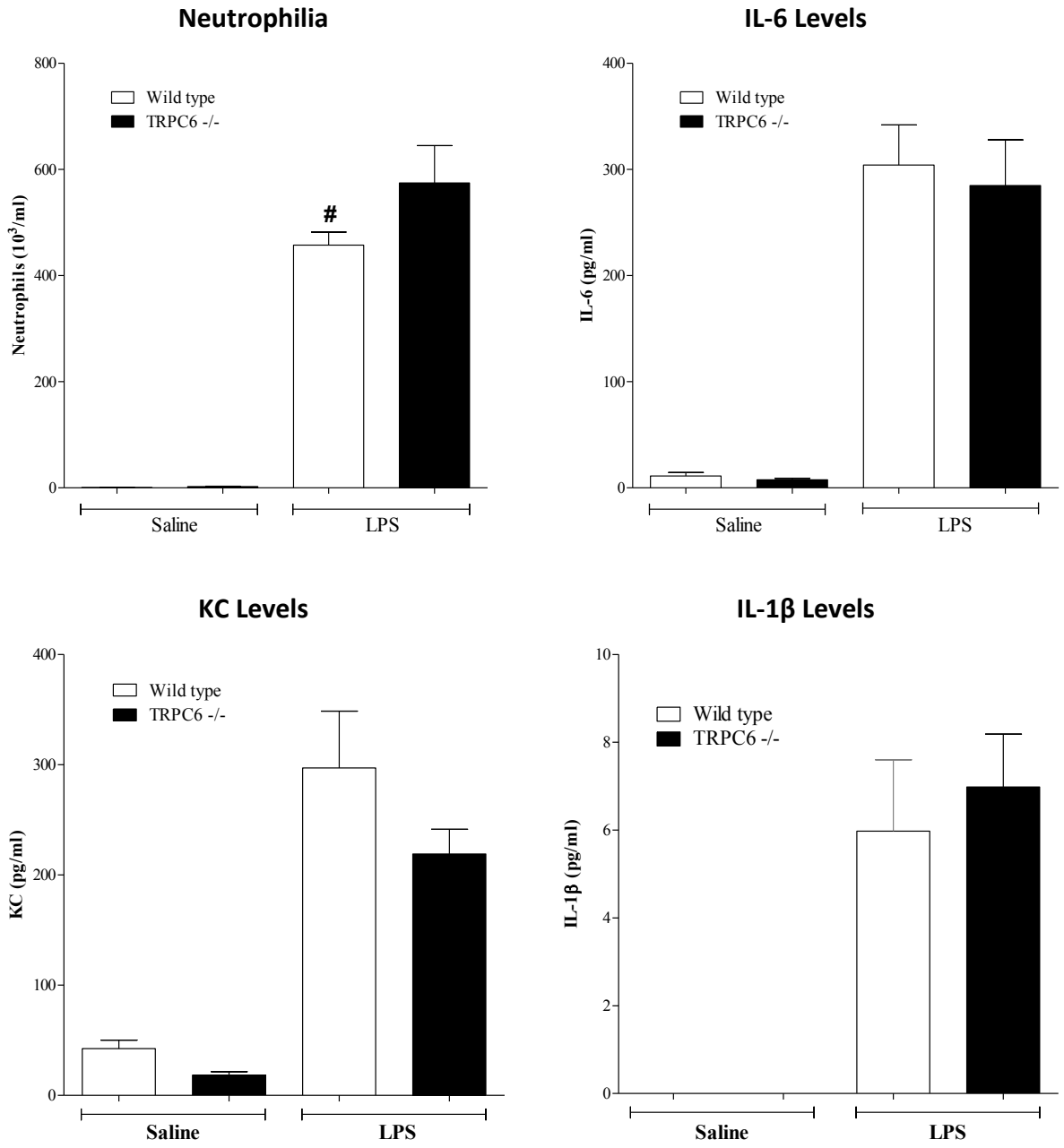

Figure 5.4 The role of TRPC6 on markers of inflammation measured in BALF after LPS exposure. *Wild-type and TRPC6-/- mice were exposed to LPS, or Vehicle (Saline), for 30 minutes. BALF samples were collected 6 hours after the final exposure for analysis of neutrophilia and cytokine levels. Data are represented as mean ± SEM for n=8 animals in each group. Statistical significance was determined using a Mann-Whitney U test. # = P<0.05, denoting a significant difference between the smoke exposed and air exposed wild-types.*

5.3.2 TRPV1, TRPV4 and TRPM8

The data in chapter 4 implicated a role for TRPV1, TRPV4 and TRPM8 in cigarette smoke induced airway inflammation. Genetic deletion of any of these channels resulted in significantly decreased levels of airway neutrophilia after acute smoke exposure, however, the mediator profile of TRPV1 and V4 was different to that of TRPM8.

After LPS challenge there was no significant difference in the levels of neutrophilia or cytokine release in the BALF between TRPV1-/- or TRPV4-/- mice and the wild-type (Figures 5.5 and 5.6).

TRPV1-/- and TRPV4-/- mice exhibited reduced levels of ATP and IL-1β in the BALF after acute smoke exposure (compared to wild-types), suggesting they may be involved in the $P2X_7$-Inflammasome-caspase-1 pathway. However ATP release and caspase-1 activation are not increased in wild-type mice after LPS activation. The fact, therefore, that genetic ablation of TRPV1 or TRPV4 had no effect on LPS-induced inflammation adds credence to the theory that these two channels are activated by cigarette smoke driven inflammatory pathways and not by innate immune inflammatory pathways.

TRPM8-/- mice also exhibited significantly decreased neutrophilia compared to wild-types after acute smoke exposure, however they had normal levels of ATP and IL-1β release. They did however have significantly decreased levels of KC, prompting the suggestion that TRPM8 is involved in a separate inflammatory pathway to TRPV1 and V4. Release of KC is also a major feature of the LPS exposure model, however genetic ablation of TRPM8 did not reduce KC release in this model. Similarly there was no significant effect on the levels of other cytokines, including IL-6, IL-1β or TNF-α, and there was no difference in neutrophil recruitment (Figure 5.7). These data suggest that TRPM8 is involved in cigarette smoke induced release of KC and neutrophil recruitment, but not in LPS induced inflammatory pathways.

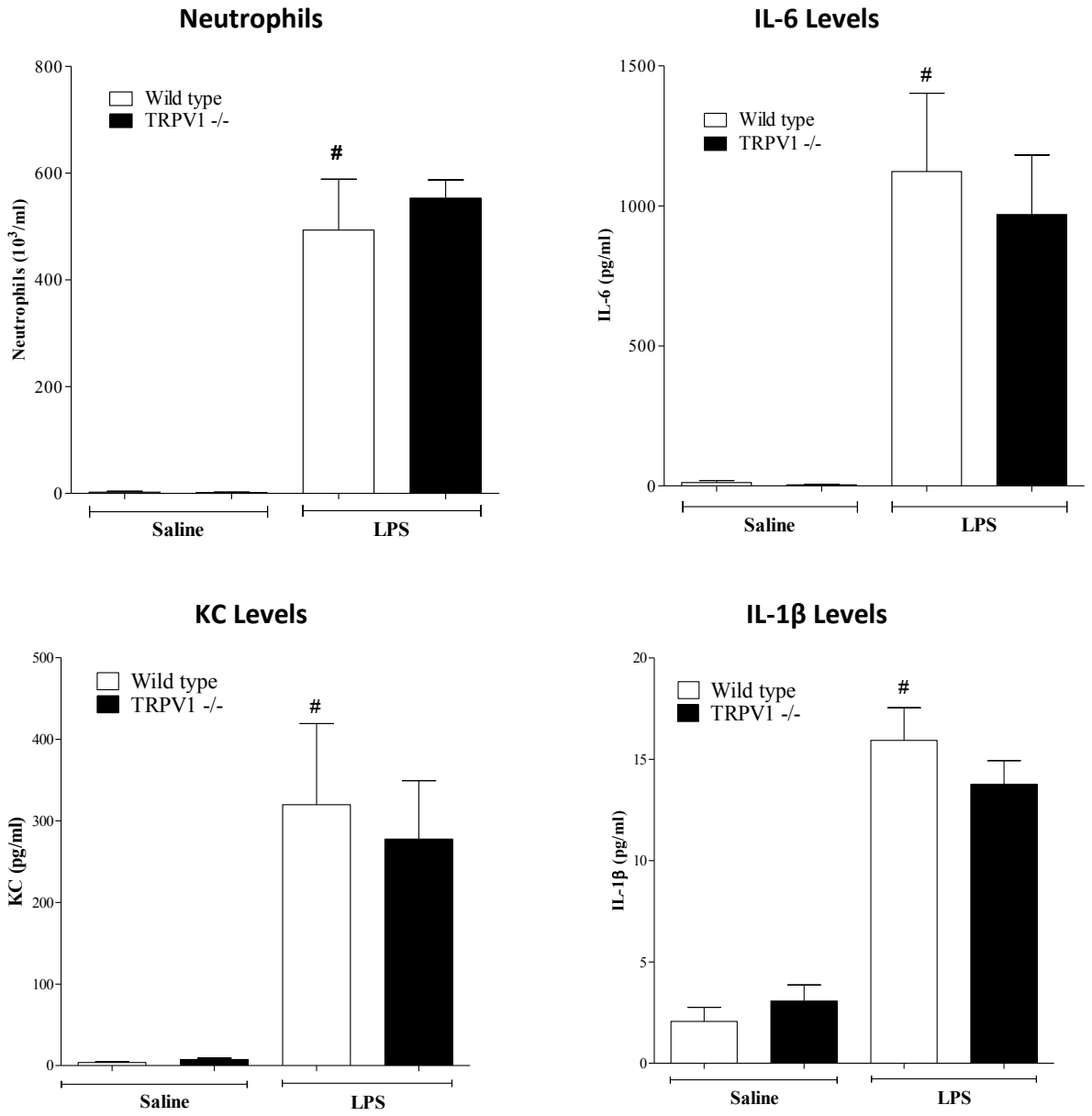

Figure 5.5 The role of TRPV1 on markers of inflammation measured in BALF after LPS exposure. *Wild-type and TRPV1-/- mice were exposed to LPS, or Vehicle (Saline), for 30 minutes. BALF samples were collected 6 hours after the final exposure for analysis of neutrophilia and cytokine levels. Data are represented as mean ± SEM for n=8 animals in each group. Statistical significance was determined using a Mann-Whitney U test. # = P<0.05, denoting a significant difference between the smoke exposed and air exposed wild-types.*

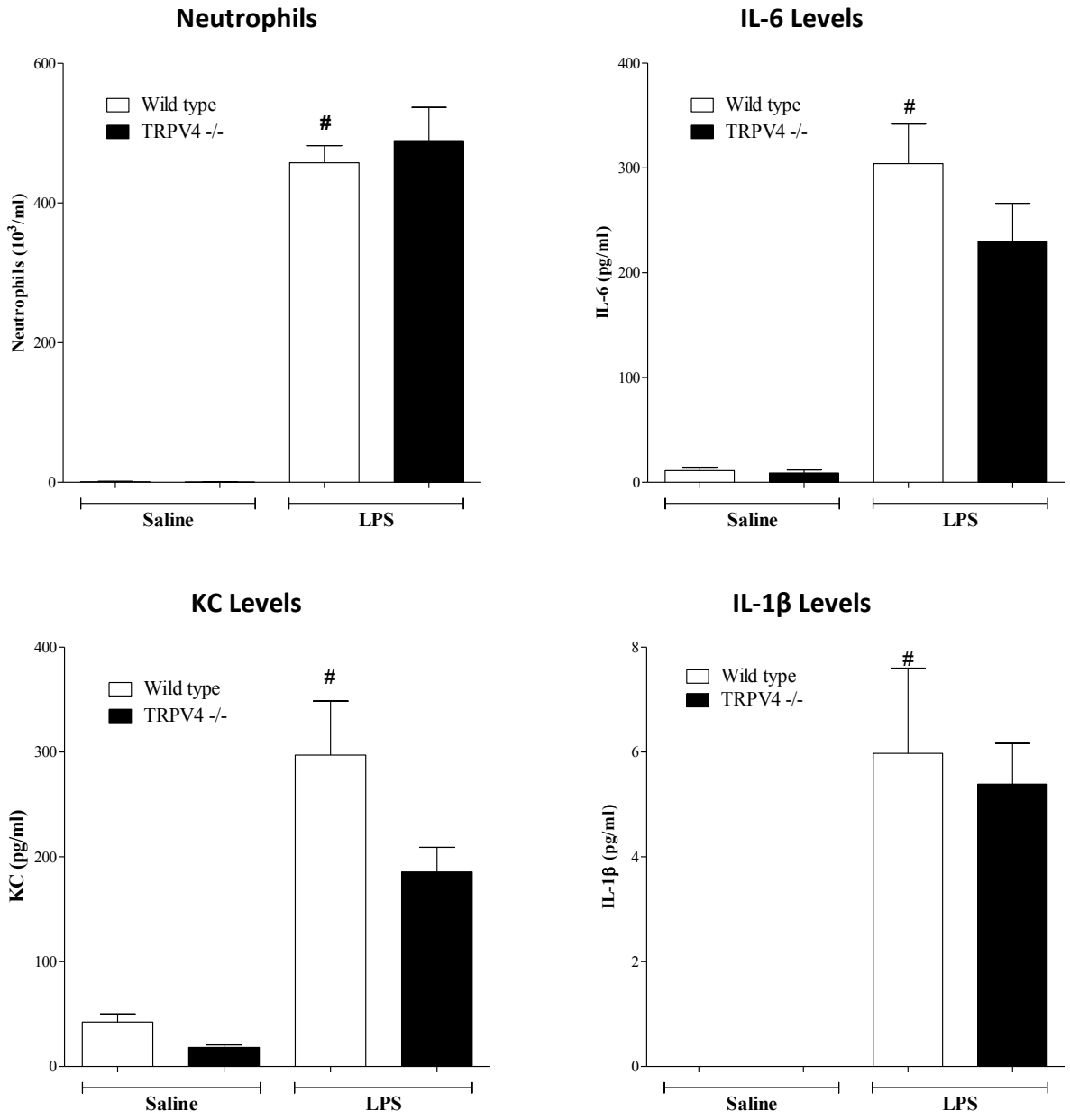

Figure 5.6 The role of TRPV4 on markers of inflammation measured in BALF after LPS exposure. *Wild-type and TRPV4-/- mice were exposed to LPS, or Vehicle (Saline), for 30 minutes. BALF samples were collected 6 hours after the final exposure for analysis of neutrophilia and cytokine levels. Data are represented as mean ± SEM for n=6 animals in each group. Statistical significance was determined using a Mann-Whitney U test. # = P<0.05, denoting a significant difference between the smoke exposed and air exposed wild-types.*

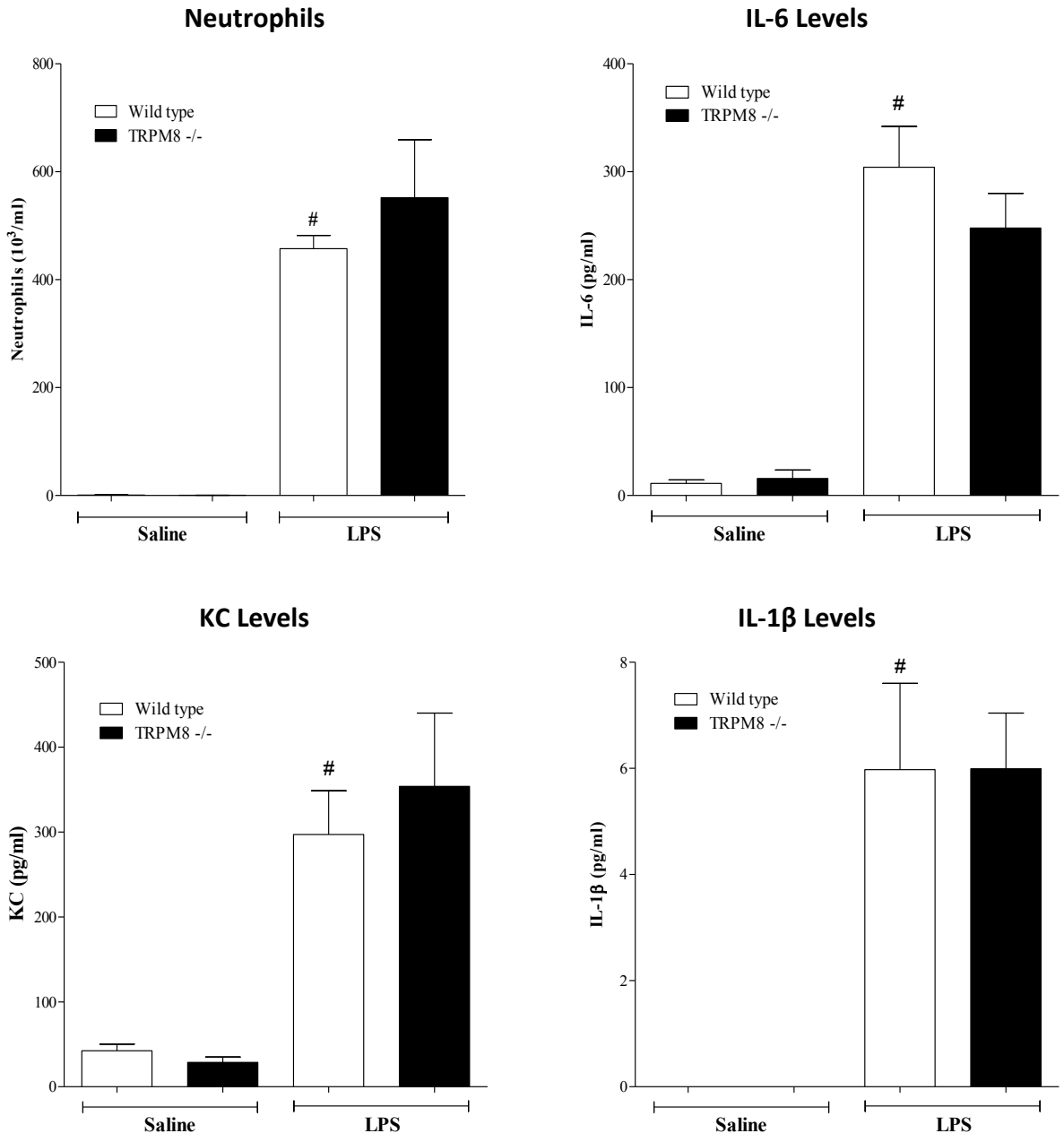

Figure 5.7 The role of TRPM8 on markers of inflammation measured in BALF after LPS exposure. *Wild-type and TRPM8-/- mice were exposed to LPS, or Vehicle (Saline), for one 30 minutes. BALF samples were collected 6 hours after the final exposure for analysis of neutrophilia and cytokine levels. Data are represented as mean ± SEM for n=6 animals in each group. Statistical significance was determined using a Mann-Whitney U test. # = P<0.05, denoting a significant difference between the smoke exposed and air exposed wild-types.*

5.3.3 TRPM2

In chapter 4 it was shown that TRPM2 deficient mice exhibited higher levels of lung neutrophil and macrophage infiltration after acute smoke exposure. This result suggested that TRPM2 may provide a protective role against cigarette smoke driven airway inflammation.

After LPS exposure TRPM2-/- mice exhibited double the number of neutrophils in BALF compared to the wild-types, which was a statistically significant change, as shown in Figure 5.8. This figure also shows there were significant increases of inflammatory cytokines: IL-6 (>200% increase), IL-1β (>30% increase), and KC (>500% increase). The other inflammatory cytokines measured were TNF-α, MIP-1α and MIP-2, and all were similarly significantly increased in TRPM2-/- mice compared to wild-types. These data suggest that lack of TRPM2 exacerbates the inflammatory reaction in the airways to LPS stimulus, and therefore it is reasonable to suggest that TRPM2 may be involved in negative regulation of the innate inflammatory response, at least in the lungs.

After acute smoke exposure, the major site of cellular recruitment in TRPM2-/- mice was the lung tissue as opposed to the BALF. It was also noted that in TRPM2-/- mice the phenotype of this model had changed from purely neutrophil infiltration to neutrophil and macrophage infiltration. Wild-types exhibited significantly increased lung tissue neutrophil infiltration after LPS exposure, and this was further and significantly increased in TRPM2-/- mice at the 6 hour time-point (Figure 5.9). Wild-types also show significantly decreased numbers of macrophages in the tissue after 6 hours, however the number of macrophages increases slightly (although non-significantly) in TRPM2 mice. This is again evidence of a slight phenotype change in the nature of the inflammation seen in TRPM2 deficient mice.

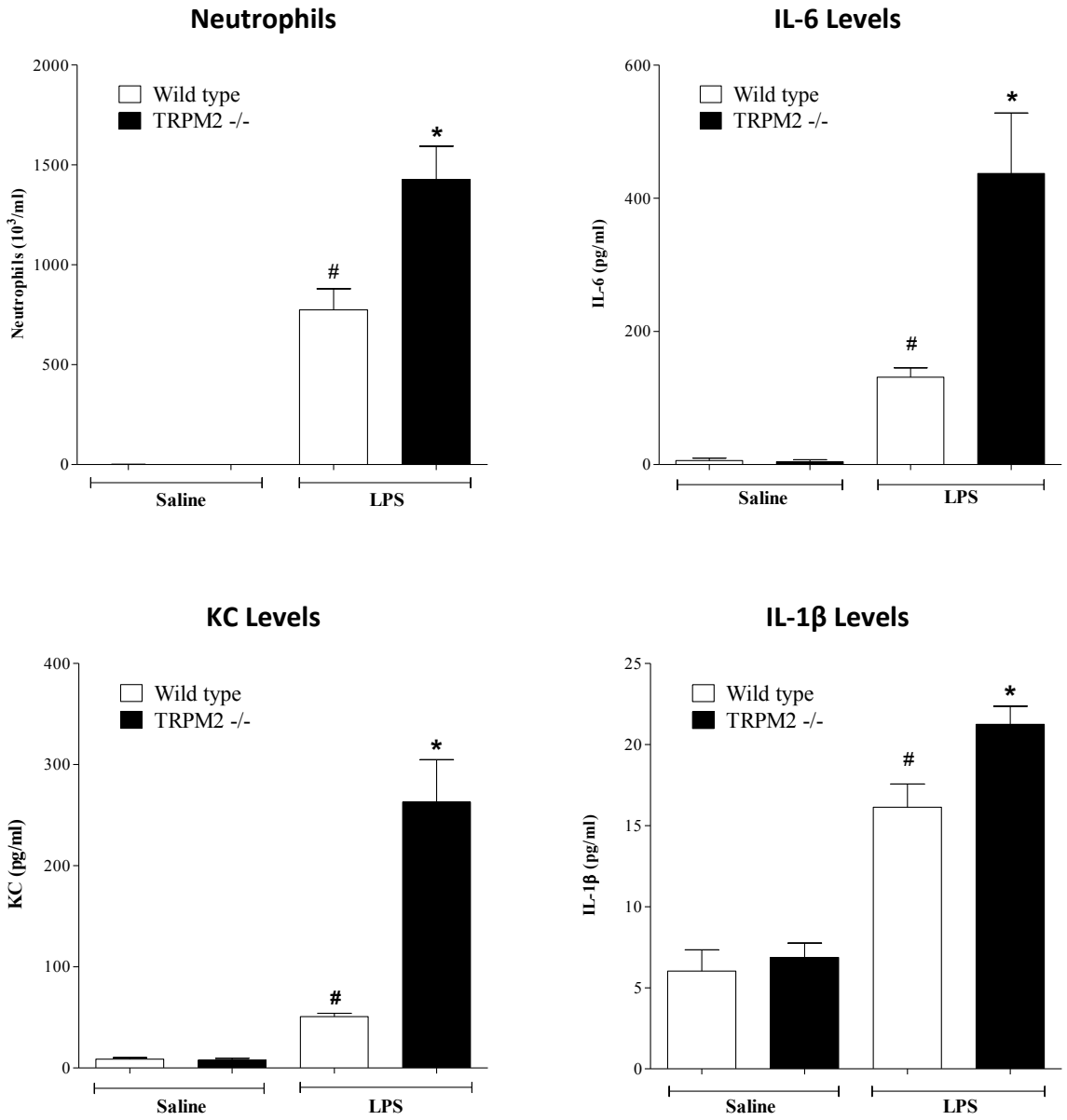

Figure 5.8 The role of TRPM2 on markers of inflammation measured in BALF after LPS exposure. *Wild-type and TRPM2-/- mice were exposed to LPS, or Vehicle (Saline), for 30 minutes. BALF samples were collected 6 hours after the final exposure for analysis of neutrophilia and cytokine levels. Data are represented as mean ± SEM for n=8 animals in each group. Statistical significance was determined using a Mann-Whitney U test. # = P<0.05, denoting a significant difference between the smoke exposed and air exposed wild-types; * = P<0.05, denoting a significant difference between the smoke exposed wild-types and knock-outs.*

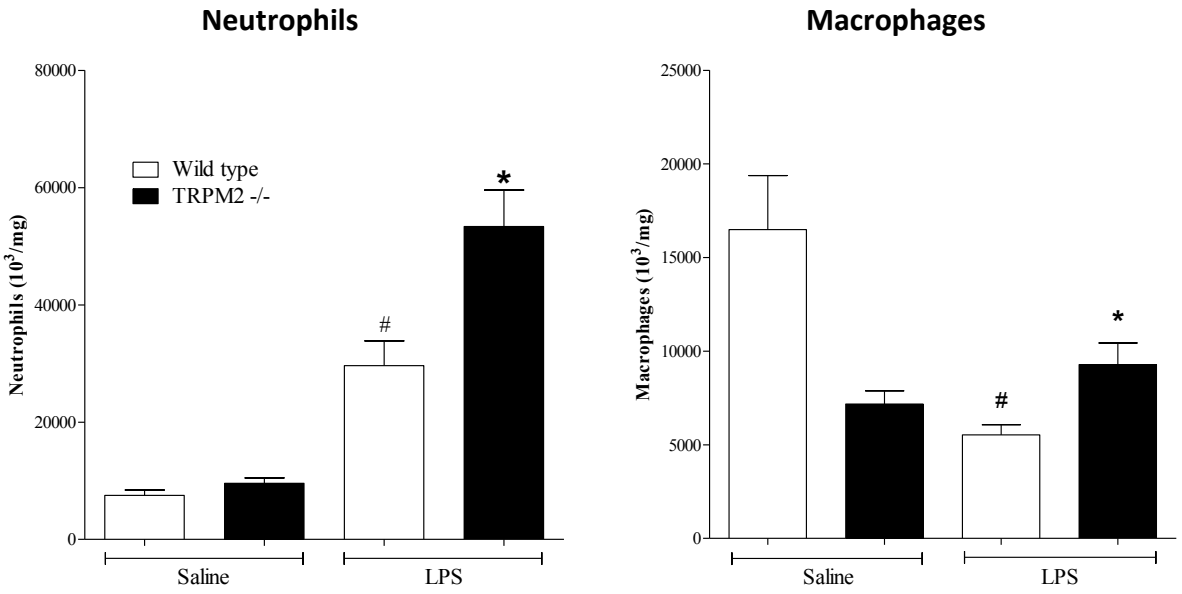

Figure 5.9 The role of TRPM2 on inflammatory cell recruitment measured in Lung Tissue after LPS exposure. *Wild-type and TRPM2-/- mice were exposed to LPS, or Vehicle (Saline), for 30 minutes. Lung tissue was collected 6 hours after the final exposure for analysis of cellular recruitment. Data are represented as mean ± SEM for n=8 animals in each group. Statistical significance was determined using a Mann-Whitney U test. # = P<0.05, denoting a significant difference between the smoke exposed and air exposed wild-types; * = P<0.05, denoting a significant difference between the smoke exposed wild-types and knock-outs.*

5.4 Discussion

The aim of this chapter was to assess whether specific TRP channels were involved in the innate inflammatory response to bacterial endotoxin, modelled by acute LPS exposure in mice. LPS consists of a lipid covalently joined to a polysaccharide, and is found on the outer membrane of gram-negative bacteria. Challenging mice with exposure to aerosolised LPS initiates an innate inflammatory response mediated by TLR4 receptors, causing increased transcription and production of pro-inflammatory cytokines, including a number of neutrophil attracting chemokines such as KC. This leads to acute neutrophilia, peaking at around 24 hours and resolving by 72 hours after challenge (Figure 5.1). LPS exposure therefore stimulates an inflammatory phenotype with many similarities to acute smoke exposure in mice but via an innate defence pathway, which unlike the acute smoke exposure model and human COPD is sensitive to glucocorticoid treatment.

5.4.1 Discussion of Results

Genetic deletion of TRPC6 did not affect cellular recruitment or cytokine release in either the acute smoke model or the LPS model. This finding is juxtaposed to very recently published data where TRPC6-/- mice, also bred on a C57BL/6 background were completely protected from LPS induced airway inflammation (1 mg/ml) and sepsis (40 mg/kg) (Tauseef et al. 2012). The authors of this paper present data showing that LPS exposure caused TLR4-dependent DAG generation in endothelial cells from wild-type and TRPC6-/- mice, but subsequent increases in cytosolic Ca^{2+} were elicited only by wild-type cells and not by TRPC6-/- cells. It is further shown that TRPC6-/- mice are protected against LPS induced increases in oedema, leukocyte sequestration and myeloperoxidase (MPO) activation. It should be noted that Tauseef *et al* took measurements at the 3.5 hour time-point, whereas the data in this chapter was collected at the 6 hour time-point, and that cell counts were performed by manual counting of histologically fixed lung slides as opposed to use of an automated cell counter on BALF samples. Furthermore Tauseef *et al* did not measure cytokines in the BALF, however these variations in protocol do not justify the irreconcilably different data sets observed between the present study and Tauseef *et al*.

As with TRPC6, no evidence was found for a TRPA1 dependent mechanism in LPS induced lung inflammation. There are currently no other studies in the literature which have reported this experiment. TRPA1-/- mice were also not protected against acute smoke driven neutrophilia and cytokine release. These data suggest that TRPA1 is not a viable target for therapeutic targeting of airway inflammation associated with COPD. It has been reported, however, that TRPA1-/- mice are protected against allergen-induced leukocyte infiltration in the airways, a result recapitulated in wild-type mice treated with TRPA1 antagonist HC-030031 (Caceres et al. 2009), suggesting it may be involved in the airway inflammation associated with asthma.

Mice deficient for either TRPM8, TRPV1 or TRPV4 all benefitted from significantly reduced neutrophilia after smoke exposure, however the cytokine pathways affected seem to vary between TRPM8 and the two Vanilloid channels. The data after acute smoke exposure supported the theory that TRPV1 and TRPV4 are activated by cigarette smoke upstream of ATP release, which in turn mediates activation of the $P2X_7$-Inflammasome-caspase-1 dependent cleavage of pro-IL-1β. Previous examination of the LPS time-course study confirmed that there is no significant increase in ATP levels or caspase-1 activation at any of the time-points following LPS exposure. Furthermore, it has been published that $P2X_7$ inhibitor A-438079 is ineffective at decreasing LPS-induced neutrophilia in mice (Eltom et al. 2011). This indicates that the $P2X_7$-inflammasome-caspase-1 axis, shown to be important in the acute smoke model is not involved in the inflammatory response to LPS. Despite this there is a significant increase in IL-1β levels. This is likely due to the fact that IL-1β mRNA expression is significantly increased at 2 and 6 hours after LPS exposure, whereas there are no transcriptional changes in the smoke model (Eltom et al. 2011). This highlights the difference between the mechanisms of inflammatory regulation in the LPS and the acute smoke models. This mechanistic difference provides a reasonable explanation for the fact that TRPV1 and TRPV4 appear to mediate the inflammatory response to acute smoke but not to LPS exposure. Furthermore this makes these two channels attractive targets for therapeutic intervention against the inflammation which drives COPD. From the data in chapters 4 and 5 it is reasonable to hypothesise that antagonism of TRPV1 or TRPV4 may help attenuate the inflammation caused by cigarette smoke which drives the majority of COPD cases, without compromising the patient's innate immune defence and in so doing

risking increased exacerbations. It should be noted however that a TRPV1 agonist was reported to attenuate airway neutrophilia and TNF-α release in LPS challenged mice (Tsuji et al. 2010), although no evidence was found in the present studies to suggest that TRPV1 may provide a protective role against inflammation, indeed there were no increases in any of the inflammatory markers measured in TRPV1-/- compared to wild-types before or after LPS challenge (Figure 5.5). It is also important to mention that ablation of TRPV1 was found to enhance Th2-biased immune responses in mice sensitised intra-nasally with OVA or HDM (Mori et al. 2011). Further to this, it has been reported that loss of TRPV1 function due to a genetic variant is associated with lower risk of active childhood asthma (Cantero-Recasens et al. 2010). This research is important to bear in mind with the subset of COPD patients who exhibit concurrent asthmatic phenotypes. There are no reported studies where the role of TRPV4 or TRPM8 in the response to LPS or murine models of asthma has been examined in the available literature.

Genetic deletion of TRPM2 was the only channel which yielded significantly increased markers of inflammation compared to the wild-type after LPS exposure. This result correlated with the increased inflammation seen in TRPM2 knockouts after acute smoke exposure in chapter 4. As previously discussed, TRPM2 has been often considered as mediating pro-inflammatory processes to oxidative stress (Banner et al. 2011). TRPM2 is known to be activated by ROS and LPS (Wehage et al. 2002), and for this reason it was hypothesised that genetic ablation of TRPM2 would attenuate the inflammatory reaction to LPS. Further to this it has been reported that down-regulation of TRPM2 using small hairpin RNA (shRNA) decreased the production of inflammatory cytokines including IL-6, IL-8, IL-10 and TNF-α, from human monocytes, in response to LPS challenge (Wehrhahn et al. 2010). In contrast to this data, it has been reported that TRPM2-/- mice exhibited normal levels of neutrophil, macrophage, and inflammatory cytokines after LPS exposure at 3 and 24 hour time-points in BALF (Hardaker et al. 2012). However another group have published data which concurs with the findings in this chapter. Di *et al* found that TRPM2-/- mice exhibited increased cytokine release and increased MPO activity after LPS challenge compared to wild-types (Di et al. 2012). Furthermore it was observed in this publication that ROS production was increased in polymorphonuclear (PMN) cells and bone marrow derived macrophages (BMDMs) from TRPM2-/- mice, a result which was confirmed by blocking

TRPM2 channel function using DPQ (3,4-dihydro-5-[4-(1-piperidinyl)butoxyl]-1(2H)-isoquinolinone) in wild-type macrophages. The authors went on to suggest that TRPM2 controlled ROS production by modulating membrane depolarisation and consequently NADPH oxidase activity. NADPH oxidase is a membrane bound enzyme which catalyses the production of superoxide anion radical O_2^- (Schrenzel et al. 1998). The data in this chapter compliment the findings of Di *et al*, and suggest that TRPM2 is likely to function as a negative feedback mechanism for the production of ROS in phagocytes. The consequence therefore of removing or inhibiting TRPM2, is dys-regulated ROS production in response to inflammatory stimuli, resulting in exacerbated inflammatory responses.

5.4.2 Summary

The data in this chapter indicate no role for TRPA1 or TRPC6 in endotoxin induced airway inflammation. This result conforms with the data in the previous chapter, which showed that neither of these channels plays a role in acute smoke driven inflammation either. Taking these studies together it must be surmised that neither channel is important in the modulation of airway inflammation in these murine models. Consequently, there is no evidence here to support either TRPA1 or TRPC6 as a possible therapeutic target in COPD.

The data obtained with TRPM2-/- mice, support the theory that this channel has a protective role against airway inflammation, and this is consistent with the data obtained after acute smoke exposure. It is likely, therefore, that antagonistic blockade of TRPM2 would exacerbate COPD, there is no data available however to indicate whether TRPM2 function is altered in this disease.

TRPM8, TRPV1 and TRPV4 knock-out mice all showed attenuated inflammation after acute smoke exposure, but reacted normally to LPS challenge. From these observations it is reasonable to suggest that targeting these channels may reduce cigarette smoke induced inflammation, without compromising the innate immune response to bacteria. The next step to further test this theory is to examine the role of these channels in a more chronic model of cigarette smoke induced inflammation, which more closely resembles the slowly resolving inflammation which characterises COPD.

Chapter 6

Sub-Chronic Cigarette Smoke Exposure Model

6.1 Rationale

Chapter 4 examined the role of TRP channels A1, C6, M2, M8, V1 and V4 in the initiation of the inflammatory response using an acute murine model of cigarette smoke (CS) exposure. Genetic ablation of channel A1 or C6 was found to have no effect on the markers of airway inflammation measured, namely, the levels of neutrophilia and cytokine release in the BALF. TRPM2 knock-out mice were found to experience exacerbated levels of inflammation compared to the wild-type. It was, however, observed that TRPV1 knock-out, TRPV4 knock-out and TRPM8 knock-out mice exhibited reduced levels of CS-induced neutrophilia, a key hall-mark of the inflammatory phenotype observed in COPD. This effect was not recapitulated after aerosolised LPS exposure, where TRPV1, TRPV4 and TRPM8 knock-outs were found to exhibit normal inflammatory responses to this innate stimulus.

When taken together, these data may indicate that TRPV1, TRPV4 and TRPM8 are specifically involved in certain CS-mediated inflammatory pathways. This makes these channels good candidates for a role in the inflammatory mechanisms thought to drive COPD and, therefore, potential therapeutic targets for the disease. In particular, TRPV1 and TRPV4 were indicatively involved in an inflammatory pathway dependent on the P2X7-caspase-1 axis. Caspase-1 activity is increased in lung tissue from smokers and COPD patients compared to non-smokers (Eltom et al. 2011), and furthermore, TRPV1 and TRPV4 expression was shown in chapter 3 to be significantly up-regulated at the transcriptional level in COPD patients compared to non-smokers. These observations advocate a possible role for TRPV1 and TRPV4 in the pathogenesis of COPD, potentially through the development of inflammation.

The major aim of this chapter is, therefore, to test the hypothesis that TRPV1 and TRPV4 are involved in the generation of a COPD-like inflammatory phenotype.

To test this hypothesis, a longer cigarette smoke exposure in wild-type mice will be characterised to identify at which point the inflammatory phenotype changes, and whether the modified inflammatory phenotype resembled that of the human disease more closely. Once a longer smoke exposure time-course has been characterised where the endpoints more closely reflect the nature of the underlying inflammation in COPD, experimental parameters can be selected to test whether TRPV1 and TRPV4 are involved in the

development of the disease-like phenotype. This information will provide an indication of whether targeting these channels is a feasible strategy for treating the inflammatory mechanisms which drive the progression of COPD.

6.2 Methods

6.2.1 Animals

Animals were bred as described in section 2.1. Identical conditions were employed to obtain both wild-type and knock-out mice, as were detailed in the methods sections of chapters 4 and 5. Animals were a minimum of 10 weeks old and were age matched between different experimental groups. All mice were housed in IVCs and were allowed to acclimatise in a room adjacent to the smoke exposure room for one week before the commencement of the experiments.

6.2.2 Establishing a length of cigarette smoke exposure for sub-chronic model

In order to establish how many consecutive days of CS exposure were required to elicit a change in the inflammatory phenotype from a purely neutrophilic response to a simultaneously macrophage and lymphocyte driven response, wild-type mice were separated into groups of 8 and exposed to CS for one hour, twice a day, and for between 3 and 28 consecutive days. Time-matched controls were exposed to room air instead of CS. The time-points chosen for examination were 3, 7, 10, 14, 17, 21, 24 and 28 days of consecutive smoke exposures. All time-point groups began their exposures together on the same day. 24 hours after the respective number of exposures, the mice were terminally anaesthetised as described in section 2.4. The 24 hour time-point was chosen for this experiment as it was previously shown in the characterisation of the acute smoke exposure model to elicit a statistically significant sub-maximal inflammatory response (Figure 4.1).

BALF neutrophil levels were increased after 3 days of CS exposures, peaking after 10 days, and subsequently remained at a similar level through to the 28 day time-point. Macrophage infiltration to the airway lumen, as evidenced by increased macrophage levels in the BALF,

begins after 10 days of CS exposure (Figure 6.1). Macrophage recruitment peaks after 14 days and, as with neutrophil recruitment, remains elevated at every subsequent time-point up to 28 days exposure. Similarly to the macrophage response, lymphocyte recruitment begins after 10 days and peaks at 14 days of CS exposure.

Lung tissue samples were also collected for total and differential cell counts, as described in more detail in section 2.4.2. After 3 days CS exposure (acute model), there were no observed increases in the number of lung tissue leukocytes, recapitulating the findings of the previous acute CS model characterisation in chapter 4. The data in figure 6.2 shows that macrophage recruitment in the lung tissue begins after 7 days of CS exposure, one time-point earlier than in the BALF (Figure 6.2). The macrophage levels were statistically increased compared to the time-matched control group at every time-point subsequently, with the exception of 21-days. Neutrophil levels were significantly increased only at the 7-day and 24-day time-points. These increases, however, were extremely modest compared to the increases observed in the macrophage data. Tissue lymphocyte levels are significantly increased from 10 days of CS exposure, and remain generally higher than the air exposed control groups from then on. From the lung tissue cellular recruitment data, it can be concluded that macrophage recruitment is the main feature of 10-28 day smoke exposure regimens.

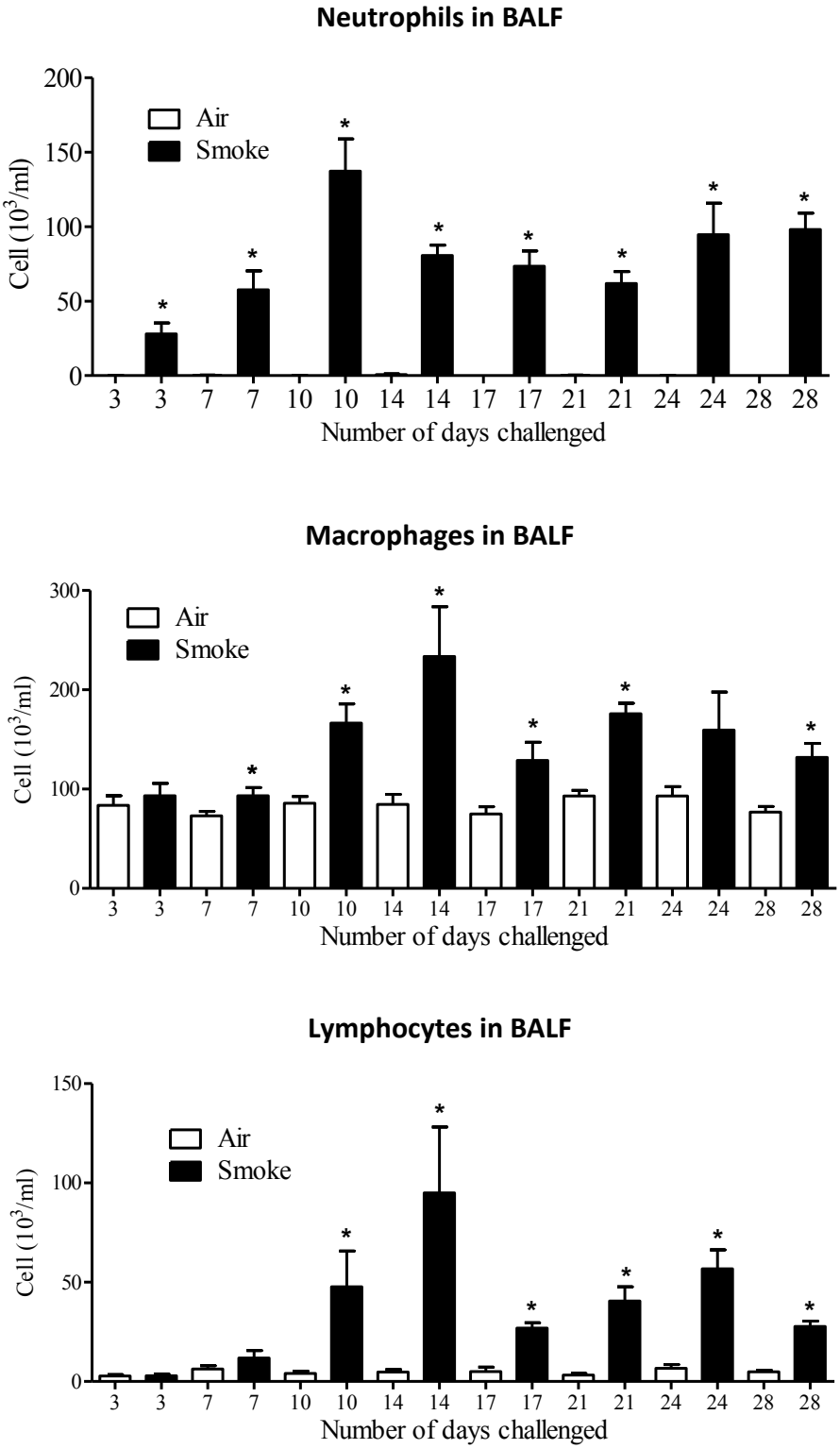

Figure 6.1 The effect of cigarette smoke exposure on cellular recruitment in BALF over time. *Wild-type (C57BL/6) mice were exposed to cigarette smoke (grey bars), or control (room air, white bars), for one hour twice daily for 3, 7, 10, 14, 17, 21, 24 or 28 consecutive days. BALF samples were collected at 24 hours after the final exposure for analysis of cellular recruitment. Data are represented as mean ± SEM for n=6 animals in each group. Statistical significance was determined using Mann-Whitney U test. * = P<0.05, denoting a significant difference between the smoke exposed group and the time-matched air exposed control.*

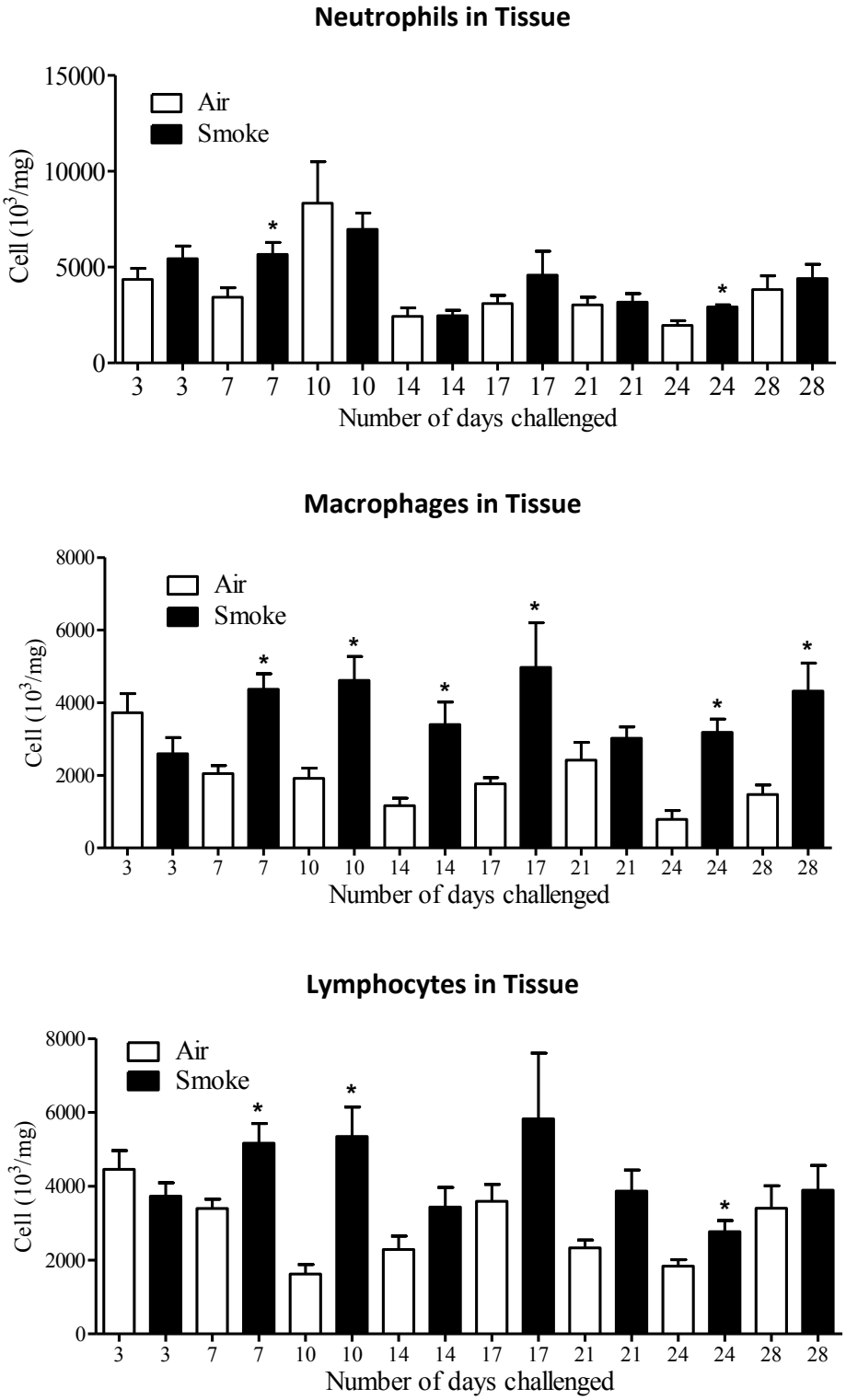

Figure 6.2 The effect of cigarette smoke exposure on cellular recruitment in lung tissue over time. *Wild-type (C57BL/6) mice were exposed to cigarette smoke (grey bars), or control (room air, white bars), for one hour twice daily for 3, 7, 10, 14, 17, 21, 24 or 28 consecutive days. Lung tissue samples were collected at 24 hours after the final exposure for analysis of cellular recruitment. Data are represented as mean ± SEM for n=6 animals in each group. Statistical significance was determined using Mann-Whitney U test. * = P<0.05, denoting a significant difference between the smoke exposed group and the time-matched air exposed control.*

6.2.3 Temporal analysis of 14-day smoke exposure model

From the data in figures 6.1 and 6.2 it was identified that the CS-induced inflammatory phenotype changed from exclusively neutrophil infiltration to neutrophil, macrophage and lymphocyte infiltration after a minimum of 10 days exposure, and that this phenotype persisted until the final exposure, which was at 28 days of exposure. After 14 days, the macrophage phenotype is fully developed. From these data, 14 consecutive days of smoke exposure was selected as the CS exposure length for examining the contribution of TRPV1 and TRPV4 to the more advanced inflammatory phenotype.

A time-course study was subsequently performed after 14 days CS exposure, to characterise the model, as was performed for the acute smoke exposure and LPS exposure models in chapters 4 and 5 respectively. Wild-type mice were exposed to 14 consecutive days of CS, and culled at time-points between 2 hours and 168 hours after the final exposure.

Neutrophil, macrophage and lymphocyte levels in the BALF were increased at all time-points after 14-days of CS exposure (Figure 6.3). This demonstrates that the inflammation is now poorly resolving compared to the acute smoke exposure model, where cellular infiltration was predominantly resolved by 72 hours after the last exposure (Figure 4.1). This phenotype more closely resembles the inflammation observed in COPD, where neutrophil, macrophage and lymphocyte levels remain elevated indefinitely, even once patients have ceased smoking.

In the lung tissue, macrophage levels are elevated above those of the time-matched air-exposed controls at the 24, 48 and 72 hour time-points. Lymphocyte levels were not statistically increased at any time-point in this analysis, although there was a small increase at the 24 hour time-point (Figure 6.4). This indicates that the main site of lymphocyte infiltration in the 14-day exposure model is in the BALF rather than the tissue.

In view of the time-course data collected for the 14-day CS exposure model, as well as for consistency with the acute smoke model, the 24 hour time-point was selected to examine the effect of genetic ablation of TRPV1 and TRPV4 in the sub-chronic smoke exposure model.

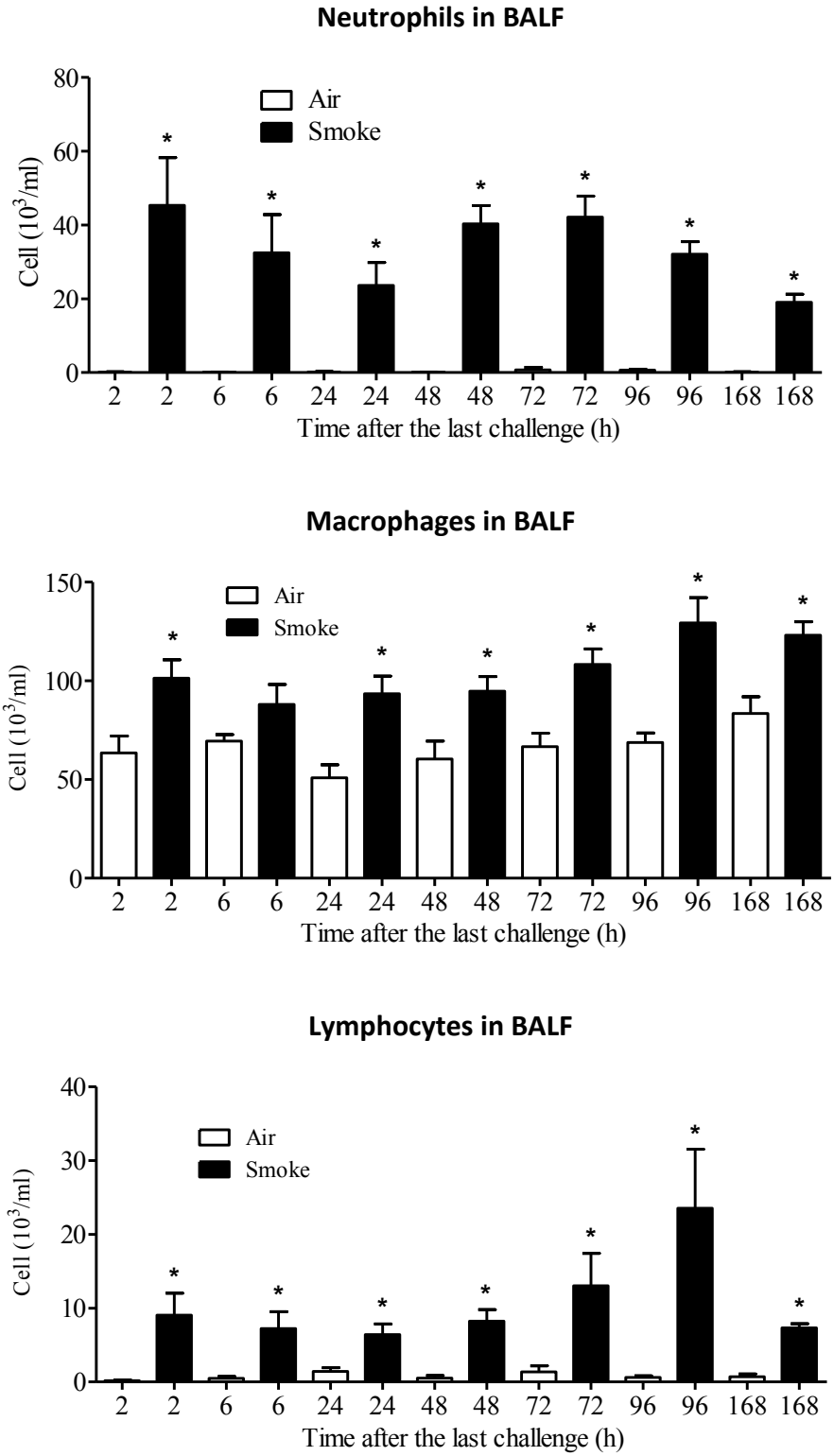

Figure 6.3 Temporal characterisation of cellular recruitment in BALF after 14-day CS exposure. *Wild-type (C57BL/6) mice were exposed to cigarette smoke (grey bars), or control (room air, white bars), for one hour twice daily for 14 consecutive days. BALF samples were collected at 2, 6, 24, 48, 72, 96 and 168 hours after the final exposure for analysis of cellular recruitment. Data are represented as mean ± SEM for n=6 animals in each group. Statistical significance was determined using Mann-Whitney U test. * = P<0.05, denoting a significant difference between the smoke exposed group and the time-matched air exposed control.*

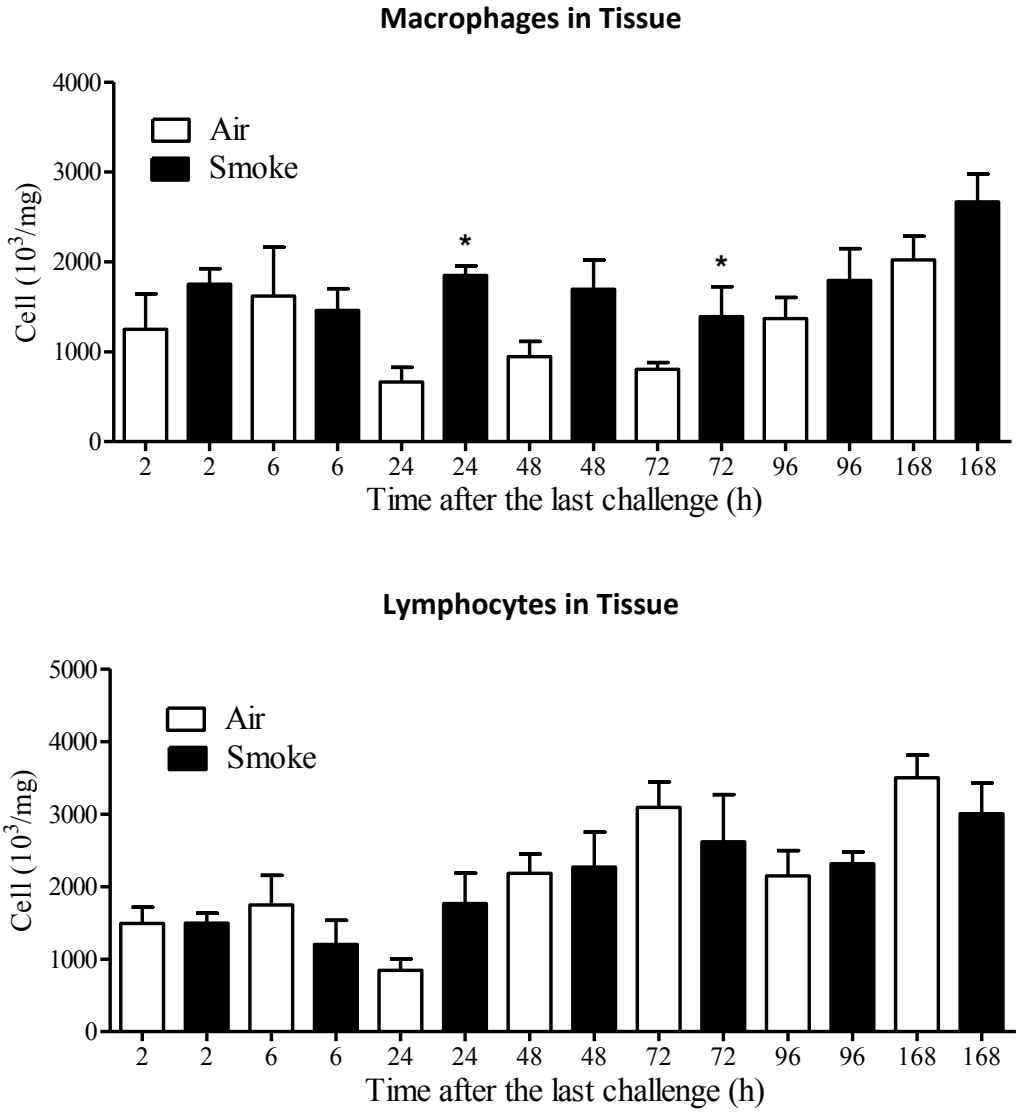

Figure 6.4 Temporal characterisation of macrophage and lymphocyte recruitment in lung tissue after 14-day CS exposure. *Wild-type (C57BL/6) mice were exposed to cigarette smoke (grey bars), or control (room air, white bars), for one hour twice daily for 14 consecutive days. BALF samples were collected at 2, 6, 24, 48, 72, 96 and 168 hours after the final exposure for analysis of cellular recruitment. Data are represented as mean ± SEM for n=6 animals in each group. Statistical significance was determined using Mann-Whitney U test. * = P<0.05, denoting a significant difference between the smoke exposed group and the time-matched air exposed control.*

6.2.4 Investigating the role of TRPV1 and TRPV4 in sub-chronic smoke exposure

Having determined a length of exposure of 14-days and a time-point of 24 hours after the last exposure for the sub-chronic CS exposure model, groups of age-matched wild-type, TRPV1-/- and TRPV4-/- mice were bred. These mice were sub-divided into CS-exposure and air-exposure (control) groups as previously described in chapters 4 and 5.

6.2.5 Statistical Analysis

Data are expressed as mean ± S.E.M in all graphs. All experiments were performed with an *n* of at least 6 (please refer to individual figure legends). Statistical significance of differences between KO and wild-type groups was determined by a Mann-Whitney U test for non-parametric data. P values < 0.05 were taken as significant, and are denoted with a * on each graph.

6.3 Results

It has been previously established in chapter 4 that TRPV1 and TRPV4 are involved in the inflammatory response of C57BL/6 (wild-type) mice to CS, evidently mediated by the release of ATP and the subsequent cleavage of pro-IL-1β into its mature form, IL-1β. In order to probe the role of these two channels in a more chronic CS-induced inflammatory phenotype, the development of inflammation in wild-type mice was tracked for 28 days. It was observed that after 14 days of CS exposure the inflammatory phenotype had changed from that of the acute (3 day) exposure model. In the acute exposure model, the main endpoints were increased levels of neutrophils and cytokines in the BALF. After 14 days exposure, neutrophil and cytokine levels were still increased in the BALF but, macrophage levels were also increased in the BALF and the lung tissue. Furthermore the cellular infiltration into the airway lumen after 14 days CS exposure remained unresolved after 168 hours (7 days). These observations more closely resemble the phenotype of inflammation in COPD compared to the acute smoke exposure model, providing an improved platform to test the role of TRPV1 and TRPV4 in the generation of the disease phenotypes.

TRPV1-/- and TRPV4-/- mice were therefore exposed to CS for 14 consecutive days, concurrently with wild-type mice, in order to examine the role of these channels in the development of this COPD-like inflammatory phenotype. The numbers of inflammatory cells and the levels of inflammatory mediators were subsequently measured in the BALF and lung tissue, 24 hours after the final exposure.

6.3.1 Cellular Infiltration

The concentrations of neutrophils, macrophages and lymphocytes in BALF samples of wild-type mice were significantly increased after 14 days of CS exposure, compared to the air exposed controls. As in the acute smoke model, mice lacking either TRPV1 or TRPV4 exhibited decreased neutrophil numbers in the BALF compared to the wild-type animals, however only the difference between TRPV1 and wild-types reached statistical significance using the Mann-Whitney U test (Figure 6.5). Genetic ablation of either channel, however,

had no effect on the increase in macrophage levels or lymphocyte levels (Figure 6.5). There were no statistically significant differences in eosinophil levels between any of the groups.

Differential cell counts in the lung tissue revealed a significant increase in macrophage levels in the smoke exposed wild-type mice, compared to the air exposed controls. Genetic ablation of either TRPV1 or TRPV4 resulted in a statistically significant 50% reduction of this increase in macrophage levels (Figure 6.6). There was also a small but non-significant increase in neutrophil levels in the smoke exposed wild-type group. Conversely in the TRPV1-/- and the TRPV4-/- mice there was, in fact, a decrease in lung tissue neutrophil levels after 14 days CS exposure. It is important to note however that there were no statistically significant differences between the neutrophil levels in any of the groups, so the drawing of any conclusions from these trends should be exercised with caution. There was no statistical increase in lymphocytes or eosinophils in 14-day CS exposed wild-type mice compared to air exposed controls. Lymphocyte and eosinophil levels were not increased in TRPV1-/- or TRPV4-/- mice compared to wild-types, demonstrating that no other cell type was compensating for the reduction in neutrophil and macrophage levels after CS exposure.

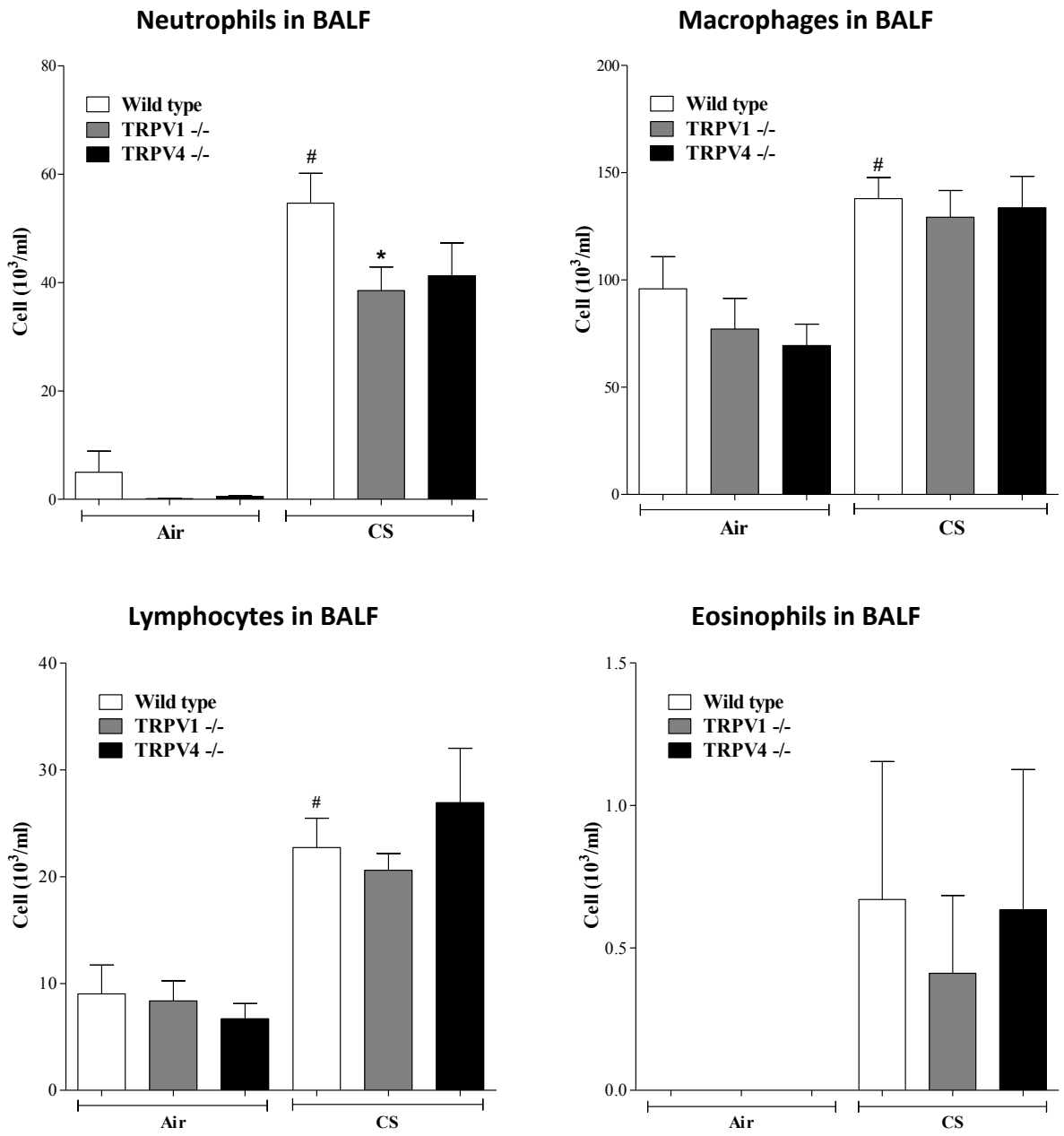

Figure 6.5 The role of TRPV1 and TRPV4 on leukocyte recruitment measured in BALF after 14-day smoke model. *Wild-type, TRPV1-/- and TRPV4-/- mice were exposed to cigarette smoke, or control (room air), for one hour twice daily for 14 consecutive days. BALF samples were collected 24 hours after the final exposure for analysis of cellular recruitment. Data are represented as mean ± SEM for n=8 animals in each group. Statistical significance was determined using a Mann-Whitney U test. # = P<0.05, denoting a significant difference between the smoke exposed and air exposed wild-types; * = P<0.05, denoting a significant difference between the smoke exposed wild-types and knock-outs.*

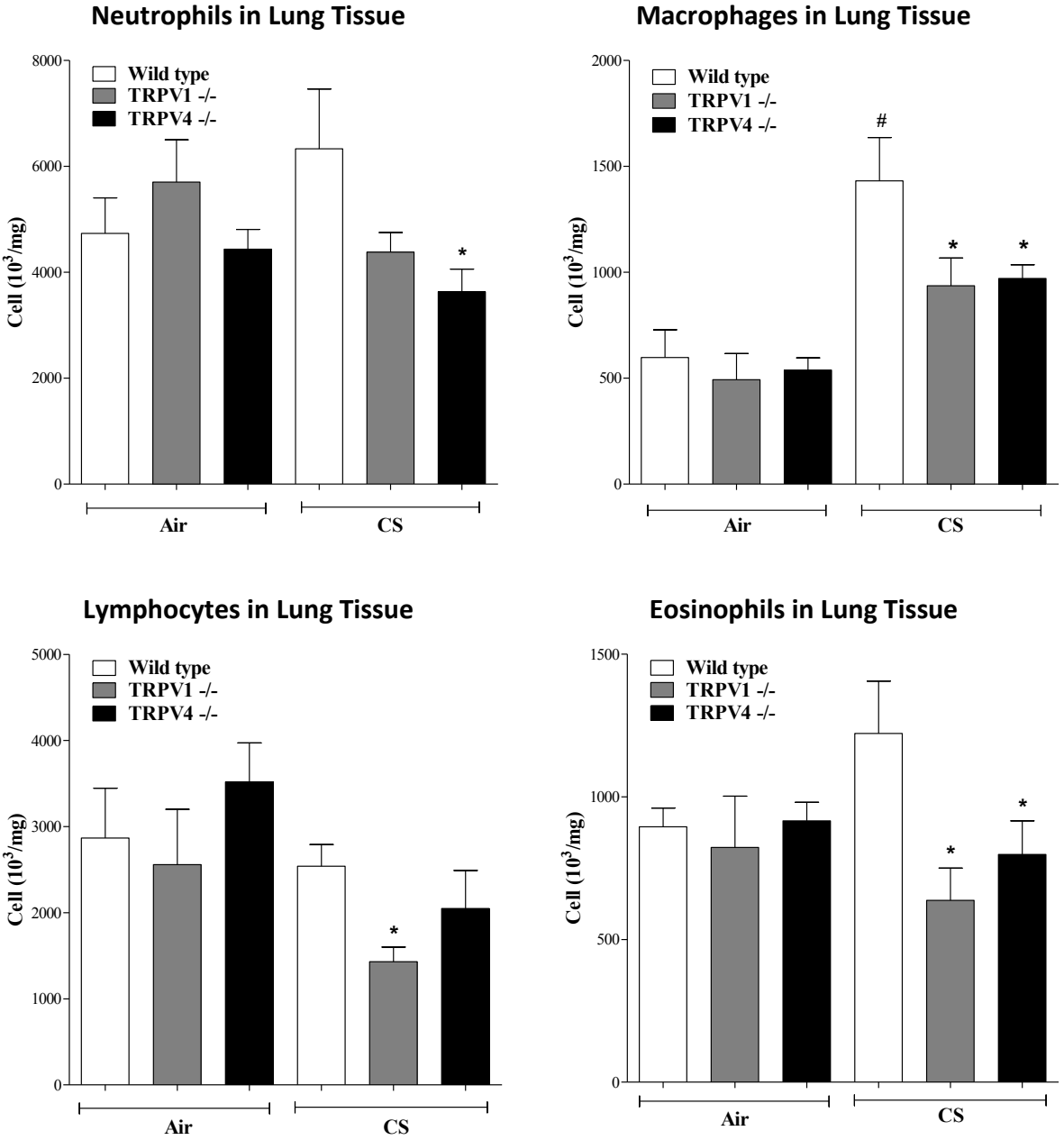

Figure 6.6 The role of TRPV1 and TRPV4 on leukocyte recruitment measured in lung tissue after 14-day smoke model. *Wild-type, TRPV1-/- and TRPV4-/- mice were exposed to cigarette smoke, or control (room air), for one hour twice daily for 14 consecutive days. Lung tissue samples were collected 24 hours after the final exposure for analysis of cellular recruitment. Data are represented as mean ± SEM for n=8 animals in each group. Statistical significance was determined using a Mann-Whitney U test. # = P<0.05, denoting a significant difference between the smoke exposed and air exposed wild-types; * = P<0.05, denoting a significant difference between the smoke exposed wild-types and knock-outs.*

6.3.2 Mediators

Genetic ablation of either TRPV1 or TRPV4 reduces the levels of inflammatory cell recruitment in both the acute and sub-chronic smoke exposure models. In chapter 4 it was observed that the CS exposed TRPV1-/- and TRPV4-/- mice also exhibited reduced levels of ATP and IL-1β compared to wild-types, but normal levels of KC. As previously discussed in chapter 4, these data indicated the possible involvement of the P2X7-inflammasome-caspase-1 axis in the TRPV1 and TRPV4 dependent CS-induced inflammatory pathway. In order to further test this theory, a number of inflammatory mediators were measured in the lung tissue and BALF samples from the wild-type, TRPV1-/- and TRPV4-/- mice after 14-days CS exposure. The inflammatory mediators measured included the levels of ATP, caspase-1 activation, and inflammatory cytokines including IL-1β and KC.

Caspase-1 activation was significantly increased in CS-exposed wild-type mice compared to air exposed controls. Caspase-1 activation was similarly increased in CS exposed TRPV1-/- and CS-exposed TRPV4-/- mice compared to their respective air exposed controls. There was no reduction in the levels of CS-induced caspase-1 activation in the knock-out strains compared to those of the CS-exposed wild-type group. It is therefore not surprising that there was similarly no reduction in the CS-induced IL-1β levels in either of the knock-out strains compared to the wild-types. This result is in contrast to the data collected in the acute smoke exposure model, where IL-1β levels were significantly reduced by genetic ablation of either TRPV1 or TRPV4. The ATP levels were below the detection limit of the assay in the BALF samples collected from this study.

Interestingly the levels of KC release in response to CS exposure were increased slightly in the TRPV4-/- mice and increased significantly in the TRPV1-/- mice compared to the wild-type group.

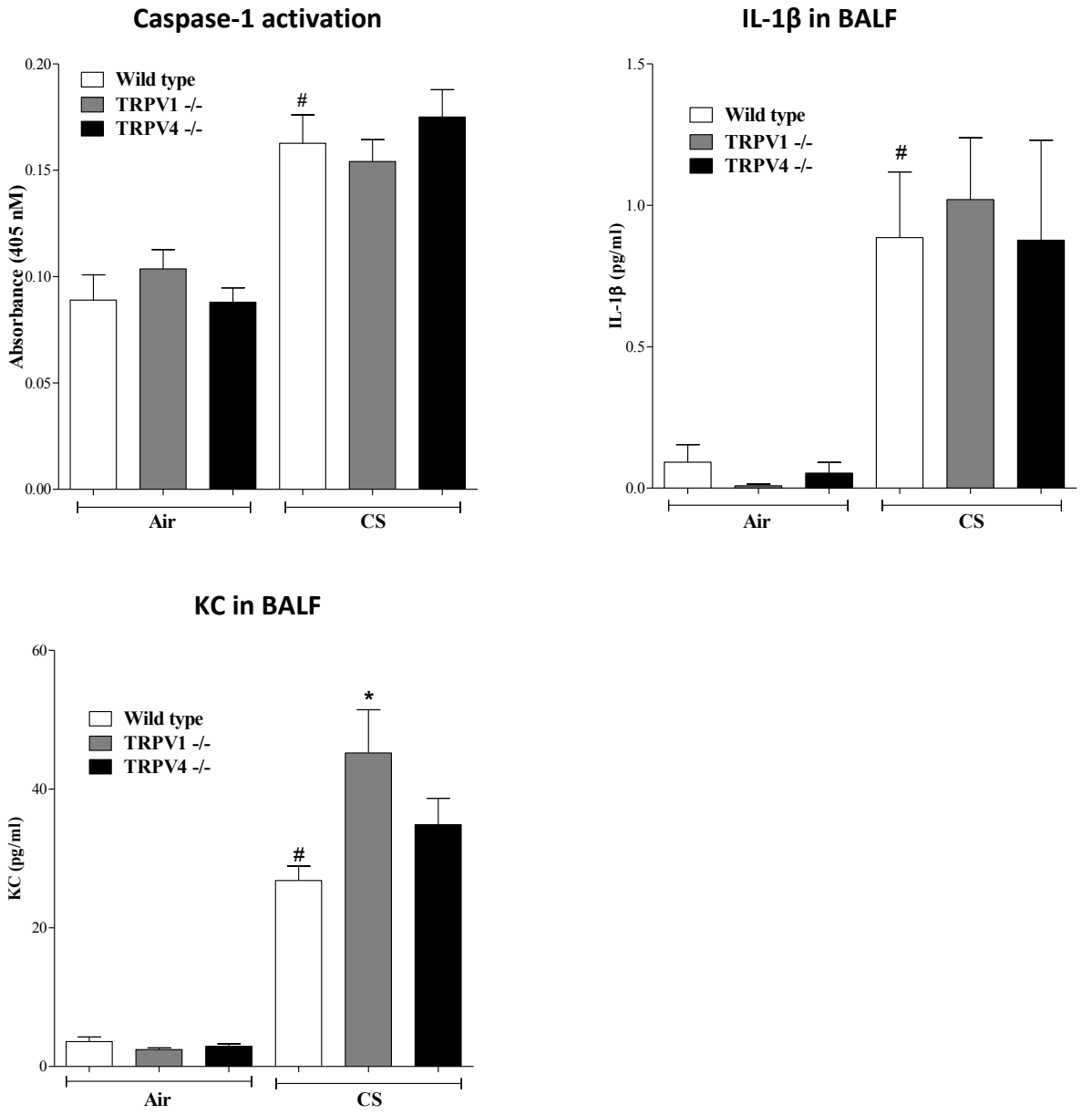

Figure 6.7 The effect of TRPV1 and TRPV4 on inflammatory mediators after 14-day smoke model. *Wild-type, TRPV1-/- and TRPV4-/- mice were exposed to cigarette smoke, or control (room air), for one hour twice daily for 14 consecutive days. BALF and lung tissue samples were collected 24 hours after the final exposure for analysis of Caspase-1 activation and cytokine release. Data are represented as mean ± SEM for n=8 animals in each group. Statistical significance was determined using a Mann-Whitney U test. # = P<0.05, denoting a significant difference between the smoke exposed and air exposed wild-types; * = P<0.05, denoting a significant difference between the smoke exposed wild-types and knock-outs.*

6.4 Discussion

The aim of this chapter was to characterise a longer CS-exposure model, which more closely recapitulates the inflammatory phenotype of COPD patients. Once characterised this model was used to test the role of TRPV1 and TRPV4, channels identified in chapters 4 and 5 as playing a role in the acute inflammatory response to CS, but not to the innate immune response to LPS.

Wild-type mice exposed to CS for 14 days (sub-chronic), exhibited a distinctly different inflammatory phenotype compared to mice exposed to CS for 3 days (acute). The main differences included the progression from an exclusively neutrophilic airway infiltrate in the acute model, to a concurrent infiltration of macrophages in both the lung tissue and the airways in the sub-chronic model. This progression recapitulates the findings of other previously reported studies from various research groups (including: D'hulst et al. 2005; Stevenson et al., 2007; and reviewed in: Stevenson & Birrell, 2011). The cellular infiltrate in the sub-chronic model, more closely resembles that of COPD patients. In addition the inflammation in the sub-chronic model is also comparatively slow to resolve, indeed, it was not resolved by the longest time-point examined: 168 hours (1 week) after the final exposure. This is an important feature as the inflammation in COPD patients similarly does not resolve after they stop smoking (Rabe et al. 2007). Furthermore it has been demonstrated that C57BL/6 mice exposed to repeated CS-challenges in this way, do go on to develop emphysematous lung changes and that the inflammation does exhibit glucocorticoid resistance (Marwick et al. 2004). These features make the sub-chronic smoke model an appropriate tool to test the role of specific TRP channels in the pathophysiology of COPD.

In the previous results chapters, it was demonstrated that genetic deletion of either TRPV1 or TRPV4 significantly reduced the infiltration of neutrophils induced by acute CS exposure, but did not affect the neutrophilic response to LPS, which is a model of innate inflammation. The inflammatory mediator profile of CS-exposed TRPV1-/- or TRPV4-/- mice indicated the importance of ATP and IL-1β in the induction of neutrophil infiltration, suggesting the importance of the $P2X_7$ – inflammasome - caspase-1 axis to this model.

After acute smoke exposure, genetic ablation of either TRPV1 or TRPV4 resulted in a more than 50% reduction in BALF neutrophil recruitment. Genetic ablation of either TRPV1 or TRPV4 also reduced neutrophil recruitment after sub-chronic CS exposure; the effect, however, of around 20% reduction, was noticeably less profound. Furthermore the levels of IL-1β, which were significantly decreased in the acute model, were no longer affected by TRPV1 or TRPV4 ablation after sub-chronic exposure. The levels of caspase-1 activation were similarly not affected by ablation of TRPV1 or TRPV4. It can therefore be surmised that the reduced effectiveness of TRPV1 or TRPV4 ablation on reducing neutrophilia after sub-chronic CS exposure is most likely related to the levels of caspase-1 activation returning to that of the wild-type after prolonged CS exposure. Unfortunately, the ATP levels were below detection in the BALF samples from this study. This makes it difficult to determine whether ATP dependent activation of $P2X_7$ is responsible for the caspase-1 activation levels returning to normal in the genetically modified mice, or whether an alternative compensatory pathway is involved. It is, however, noteworthy that particularly in the TRPV1-/- mice, the levels of KC release are significantly increased compared to the wild-type. In the acute exposure model, there was no change in the KC levels in the TRPV1-/- or TRPV4-/- mice compared to the wild-type. This may indicate that compensatory or, possibly, alternative inflammatory mechanisms are of more importance in the longer exposure model, and that these mechanisms operate independently of TRPV1 and TRPV4 activation.

It has been demonstrated that genetic ablation of $P2X_7$ in C57BL/6 mice does significantly reduce the levels of caspase-1 activation, IL-1β and neutrophil recruitment after 14-day CS exposure (Eltom et al. 2011). This data would support the hypothesis that the $P2X_7$-inflammasome-caspase-1 axis activation inducing the maturation of IL-1β is still a key mechanism in the regulation of inflammation in the sub-chronic CS exposure model. It would be logical to suggest, therefore, that the reduced inhibition of inflammation in TRPV1-/- and TRPV4-/- mice after prolonged CS exposure occurs due to alternative pathways of $P2X_7$ activation, rather than a mechanism independent of $P2X_7$.

A possible explanation for increased P2X7-inflammasome-caspase-1 activation in the TRPV1-/- and TRPV4-/- mice after prolonged CS exposure, is the overlap in functionality of TRPV1 and TRPV4. As described in chapter 4, deletion of either channel resulted in approximately a 50% reduction in neutrophilia, and similar reductions in the levels of ATP and IL-1β,

indicating they are involved in the same inflammatory mechanistic response to CS. This raises the possibility that TRPV1 and TRPV4 are both partly responsible the ATP release in response to CS exposure. It is therefore possible that when only one of these two channels is deleted, the function of the other is still enough to initiate an escalating inflammatory response. It may therefore be reasonable to interpret the results in this chapter as indicative of a delay in the inflammatory response caused by genetic deletion of either TRPV1 or TRPV4. In TRPV1-/- mice, the presence of functional TRPV4 results in lower levels of ATP release and subsequent $P2X_7$ – inflammasome – caspase-1 activation, and thus lower levels of IL-1β release. These levels of IL-1β release are, however, enough to cause neutrophil recruitment, but this neutrophil recruitment takes longer (or rather, more CS exposures) to reach the same levels as the wild-type where both channels are functionally present. It is conceivable that in this scenario, TRPV1-/- mice exposed to sub-chronic CS could exhibit normal levels of caspase-1 activation and, consequently, IL-1β release, but that they reach this level later than the wild-type, explaining the slightly lower levels of neutrophilia. Similarly the situation could work *vice versa* for the TRPV4-/- mice.

What is clear from the data in this chapter is that genetic deletion of either TRPV1 or TRPV4 is not sufficient to prevent the inflammation induced by repeated CS exposure on a chronic or sub-chronic basis, from developing from a purely neutrophillic phenotype, to a more advanced COPD-resembling phenotype, including macrophage infiltration. If the theory, that, the ineffectiveness of deletion of either of these channels in the sub-chronic model is due to the overlap of functionality between the two channels, is in fact correct, there is the possibility that ablation or inhibition of both of the channels may successfully inhibit the inflammatory response to longer term CS exposure. One way to address this possibility would be the breeding of “double knock-outs”, i.e. a TRPV1-/-V4-/- colony. There are, however, no studies in the literature reporting the successful breeding or use of a colony of such mice, so it is unclear whether the young of such a colony would remain fertile and viable for study.

In summary, the data in this chapter demonstrate that TRPV1-/- and TRPV4-/- do play a role in the development of inflammation after sub-chronic exposure to CS, as evidenced by the significantly reduced levels of BALF neutrophils and tissue macrophages compared to the wild-type. Whilst these aspects of the cellular infiltration are inhibited, the degree of

inhibition is considerably reduced compared to the acute smoke model. More importantly the inflammatory mediators which were reduced by ablation of TRPV1 or TRPV4, namely IL-1β and caspase-1 are no longer inhibited in the sub-chronic model. This may be due to the overlap of function between the two channels, or a compensatory mechanism which could explain the increased levels of KC compared to the wild-types, or a combination of both. Therefore it can be surmised that deletion of either TRPV1 or TRPV4 alone is not sufficient to inhibit the progression of the inflammation in chronically CS-exposed C57BL/6 mice.

Chapter 7

Conclusions

7.1 Summary and general discussion

The aim of this thesis was to examine the hypothesis that members of the TRP cation channel family are involved in the pathogenesis of COPD and, furthermore, to evaluate the potential of various TRP channels as therapeutic targets for this disease. Establishing new therapeutic targets for COPD sufferers is of crucial global importance, as this disease is currently the 4th most common cause of death worldwide, and the prevalence is on the increase (WHO; Lopez et al., 2006). COPD therefore represents a critically important social and economic burden. The currently available therapies, however, are ineffective at arresting the progression of the disease, providing at best only modest symptomatic improvements.

The term 'COPD' encompasses three separate airway pathologies, namely, chronic bronchitis, small airways disease, and emphysema. Patients may present with one, two or all of these pathologies and in varying degrees of severity, and all contribute to the progressively worsening obstruction of airflow (Vestbo et al. 2012). The uniting feature of the three pathologies is chronic, non-resolving inflammation, which is thought to drive disease progression and functional changes (Hogg 2004). The main therapies currently used for the treatment of COPD are bronchodilators for symptomatic relief, and inhaled corticosteroids to attenuate the inflammation. Inhaled corticosteroids, however, are ineffective at attenuating the inflammation in the vast majority of COPD patients (Barnes 2010). For this reason, finding new therapies, which are effective at attenuating the inflammation in COPD, is of crucial importance to improving disease management. In order to find new anti-inflammatory therapies for COPD, the inflammatory response must first be characterised and understood, so that viable and effective drug-targets can be elucidated. As a contribution to this goal, this thesis aimed to evaluate the role of specific members of the TRP channel family in the pathogenesis of COPD.

The TRP channels are a superfamily of cation channels which are responsible for modulating a wide range of cellular functions. Specific members of this family were selected based on their expression on relevant cell types, particularly airway and inflammatory cells, and their involvement in inflammatory processes both in the airways and other organs. Based on these criteria, TRP channels A1, C6, M2, M8, V1 and V4 were selected for investigation in

the pathogenesis of COPD. It was decided to use murine models of airway inflammation to study these channels.

Murine models of inflammation provide an excellent tool for examining the role of specific proteins in disease-like systems, in particular because there is a wide range of genetically modified mouse strains available. Cigarette smoke (CS) driven models have become the preferred preclinical system to model the inflammatory pathways and mechanisms which characterise COPD (Stevenson and Birrell 2011). CS is thought to be responsible for around 90% of the cases of COPD globally, making it the ideal stimulus to recapitulate some of the important features of COPD-like airway inflammation.

Cigarette smoke exposure models of airway inflammation in rodents appear to have two main phases: a transient acute phase where the main feature is neutrophilia in the BALF, which normally peaks during the first week of exposure; followed by a more chronic phase characterised by recruitment of neutrophils, macrophages and lymphocytes to the lung, which typically starts after 2-4 weeks of exposure (Eltom et al. 2011; Stevenson et al. 2007). These changes recapitulate many of the features of COPD, including those thought to be important to disease development and progression. For example, COPD patients have significantly increased levels of neutrophils and IL-1β in their BALF, which are important features of both the acute and sub-chronic phases of CS-induced lung inflammation in the murine models used in this thesis. It is also encouraging to note that the inflammation seen in more chronic murine models is resistant to glucocorticoid treatment (Marwick et al. 2004, Marwick et al.2009). It has therefore been concluded that small rodent models can mimic the progressive, low-grade, slowly resolving and steroid-insensitive inflammation associated with COPD (Stevenson and Birrell 2011).

The acute CS exposure model (3-days exposure), which was characterised by BALF neutrophilia and cytokine release (in particular: KC and IL-1β), was used in chapter 4 to examine the role of each channel in the initiation of inflammation as a response to CS. Mouse colonies genetically deficient for each of the TRP channels, were established and exposed to the acute CS model alongside wild-type controls. The animals were all male, age-matched, fed on identical diets and housed together. Any differences in inflammatory responses between the wild-type colony and the knock-out colony were, therefore,

considered to be a direct result of the modified gene, and an indication of its role in the disease model. The major aim of this chapter was to test the hypothesis that the six selected TRP channels were involved in the acute response to the disease relevant stimulus, CS. The major findings of this chapter were that mice lacking TRPV1, TRPV4 or TRPM8 exhibited reduced markers of inflammation compared to wild-types, whilst those lacking TRPA1 or TRPC6 exhibited no change, and those lacking TRPM2 exhibited exacerbated inflammation. The hypothesis was, therefore, fulfilled with respect to TRPV1, TRPV4, TRPM8 and TRPM2, but not with respect to TRPA1 and TRPC6.

Having identified that certain TRP channels were important in the acute response to CS in chapter 4, the following chapter sought to identify whether any of the six selected TRP channels were involved in the innate airway inflammatory response. In order to test this, knock-out mice were exposed to a previously characterised model of LPS induced airway inflammation. As with the acute CS-exposure model, LPS exposure induces acute airway neutrophilia and cytokine release which can be measured in the BALF. LPS is thought to induce inflammation predominantly through TLR-4 dependent activation of NF-κB. This inflammatory pathway is extremely sensitive to inhibition by glucocorticoids (Birrell et al. 2005) and, moreover, repeated LPS challenge leads to attenuation of the neutrophilic response, i.e. immunological tolerance (Brass et al. 2008). Therefore it does not reflect the progressive steroid-insensitive inflammation which is central to the pathogenesis of COPD (Stevenson and Birrell 2011). By profiling the TRP knock-out mouse colonies in both the LPS model and the acute smoke model in parallel, it was thus possible to differentiate whether a given TRP channel was involved in innate airway inflammatory responses, or whether the channel's involvement was specific to CS-driven (disease relevant) inflammatory processes. The major findings of chapter 5, were that TRPA1, C6, M8, V1 and V4 had no effect on the airway inflammatory response to LPS, whilst mice lacking TRPM2 exhibited exacerbated inflammation.

After identifying that TRPV1, TRPV4 and TRPM8 were important to the inflammatory response to acute CS, but not to that of LPS, it was hypothesised that these channels may be important in the progression of the CS-mediated inflammatory response from the acute neutrophilic phenotype, to a more COPD-like phenotype seen in the sub-chronic CS model. Furthermore, it had been earlier observed, in chapter 3, that transcriptional expression of

TRPV1 and TRPV4 was increased in lung parenchymal samples from COPD patients compared to non-smoker controls. TRPV1 and TRPV4 were, therefore, tested in the sub-chronic CS model alongside wild-type controls.

The data obtained, in all three of these *in vivo* models of airway inflammation, are discussed below, in the context of other relevant published findings for each of these channels.

7.1.1 TRPA1 and TRPC6

TRPA1 was a prime candidate for involvement in CS-induced airway inflammation, as it is reportedly expressed in airway epithelial cells, which were found to release IL-8 in response to the TRPA1 agonist acrolein (Nassini et al. 2012). Acrolein and crotonaldehyde, both potent activators of TRPA1, are also found in cigarette smoke, providing a feasible mechanism of airway inflammation initiation. Furthermore, activation of TRPA1 in human lung cells by wood smoke particulate, an increasingly prevalent cause of COPD, has also been recently reported (Shapiro et al. 2013). The data in this thesis, however, provide no evidence that TRPA1 performs a role in the development of CS-induced airway inflammation. Genetic deletion of TRPA1 in C57BL/6 mice had no effect on inflammation induced by either acute CS or LPS. It is of particular note that TRPA1-/- mice exhibited no reduction in acute CS induced KC release compared to the wild-type. This is in contrast to published data, where BALF was taken from naïve TRPA1-/- and wild-type mice, then treated with CS *in vitro*. BALF from TRPA1-/- mice exhibited significantly reduced KC release after CS treatment compared to BALF from the wild-type (Nassini et al. 2012). In this paper however, it is not clear whether cellular analysis was performed on the BALF before and/or after CSE treatment. It is therefore possible that the BALF from the naïve TRPA1-/- mice contained fewer inflammatory leukocytes from which to release KC compared to the wild-type BALF, or even that there was a different leukocyte profile between the colonies. Because the experiment was performed *in vitro*, it is also not possible to assess whether the KC release induced was associated with cellular recruitment. It was, however, also observed that specific TRPA1 antagonist HC-030031 reduced the BALF KC-release from wild-type mice after intra-tracheal instillation of CSE. One possible explanation for this discrepancy with the results obtained in chapter 4, is the fact that the origin of the TRPA1-/- mice used in the

Nassini paper is different to the origin of those used in this thesis, and furthermore the genetic background is also different. It is also notable that BALF KC levels after intra-tracheal CSE instillation are not reported in TRPA1-/- mice, raising the possibility that the effects that Nassini *et al* observed with the knock-outs may have been unique to the *in vitro* experiments.

Similarly with TRPC6, the inflammatory markers measured after exposure to acute CS or to LPS were the same in the knock-outs as in the wild-types. TRPC6 has previously been reported to play a fundamental role in the migration of human neutrophils (Damann *et al.* 2009) and, furthermore, has been shown to be up-regulated in the macrophages collected from COPD patients compared to non-disease controls (Finney-Hayward et al. 2010). These two studies would advocate a role for TRPC6 in the pathological processes of COPD. The murine data in this thesis, however, found no role for TRPC6 in the migration of neutrophils into the airways after either CS or LPS challenge. The discrepancy between these data sets may highlight a difference in the biochemical physiology of C57BL/6 mice compared to humans. Alternatively it is possible that the neutrophils used in the Damann study behaved differently to those in an *in vivo* system, due to the experiment being performed *in vitro*.

7.1.2 TRPM2

Oxidant – antioxidant imbalance is a key feature of COPD (Schaberg et al. 1992), and is thought to be a significant factor contributing to airway inflammation and lung tissue damage. Indeed, oxidative stress activates the redox sensitive transcription factor NF-κB, to modulate the expression of inflammatory cytokines (Dröge 2002). The identity of the protein(s) responsible for transducing Reactive Oxygen Species (ROS) signals into inflammatory signals was an elusive mystery for many years, until the identification of TRPM2 (formerly TRPC7) in 1998 (Nagamine et al. 1998). Two independent research groups published the observation that H_2O_2 activates TRPM2 to facilitate Ca^{2+} permeability in 2002. Wehage *et al.* reported that H_2O_2 induced Ca^{2+} entry by direct oxidation of TRPM2 proteins (Wehage et al. 2002); whereas Hara *et al.* observed that TRPM2 is activated by H_2O_2 via the action of β-nicotinamide adenine dinucleotide (NAD^+) (Hara et al. 2002). CD38 in the plasma membrane mediates the hydrolysis of NAD^+ to form nicotinamide and adenosine

diphosphate ribose (ADPR) (Lund 2006), the latter being a potent intracellular ligand for TRPM2. It remains unclear, however, whether this affects intracellular ADPR levels, as the enzymatic activity of CD38 is thought to occur extracellularly (Takahashi et al., 2011).

The evidence of a role for TRPM2 in the ROS-mediated pathogenesis of COPD was strengthened by the observation that Ca^{2+} influx through H_2O_2-activated TRPM2 channels can elicit cytokine (particularly IL-8) transcription by Pyk2 phosphorylation resulting in Ras-dependent Erk activation, inducing nuclear translocation of RelA (Takahashi et al. 2011).

These observations are in agreement with *in vivo* data obtained, showing that TRPM2-/- mice were protected from inflammation in a model of DSS-induced colitis (Araki et al. 2006). Furthermore, it has been reported that TRPM2 is required for LPS-induced cytokine production in human monocytes (Wehrhahn et al. 2010).

In view of the above evidence, it was hypothesised that TRPM2-/- mice would be protected against inflammation after acute CS exposure. In stark contrast to this hypothesis, it was observed in chapter 4 that genetic deletion of TRPM2 resulted in significantly exacerbated inflammation including higher levels of cytokine production and neutrophil recruitment. In chapter 5 it was further shown that this response was recapitulated in the LPS exposure model. These results suggest that the pro-inflammatory effect of TRPM2 deletion was not specific to CS, but was a general effect on airway inflammation. It should be noted, however, that TRPM2-/- mice exhibited no difference from the wild-type in an OVA-induced model of allergic airway inflammation (Sumoza-Toledo *et al*. 2013).

In support of the observations in this thesis, Di *et al.* reported that TRPM2-/- mice exhibited significantly increased inflammation after LPS exposure (Di et al. 2012), conforming with the data in chapter 5, and contradicting the original hypothesis that TRPM2 activation by ROS positively mediates cytokine production. It has been further demonstrated that TRPM2-/- polymorphonuclear leukocytes (PMNs) and bone-marrow derived macrophages (BMDMs) stimulated with LPS exhibited more ROS production than TRPM2+/+ cells (Di et al. 2012). This ROS production was shown to be NADPH oxidase dependent using the inhibitor diphenyleneiodonium (DPI). Furthermore, elevated ROS production in TRPM2-/- macrophages was reduced to the levels of TRPM2+/+ macrophages by controlling the

membrane potential with extracellular K^+ (Di et al. 2012). This suggests that TRPM2 controls the NADPH oxidase production of ROS by modulating the membrane potential.

The data in this thesis supports the work of Di *et al.* and extends the findings to a COPD relevant murine model of CS induced airway inflammation. It is possible, therefore, that TRPM2 may function as a negative feedback mechanism for the regulation of NADPH oxidase dependent ROS production in situations of CS- and LPS- induced airway inflammation, as represented in *Figure 7.1*. Antagonism of TRPM2 in COPD patients would therefore not be a viable therapeutic option, based on this data, however further experiments are required to assess whether this murine data translates to human biochemistry.

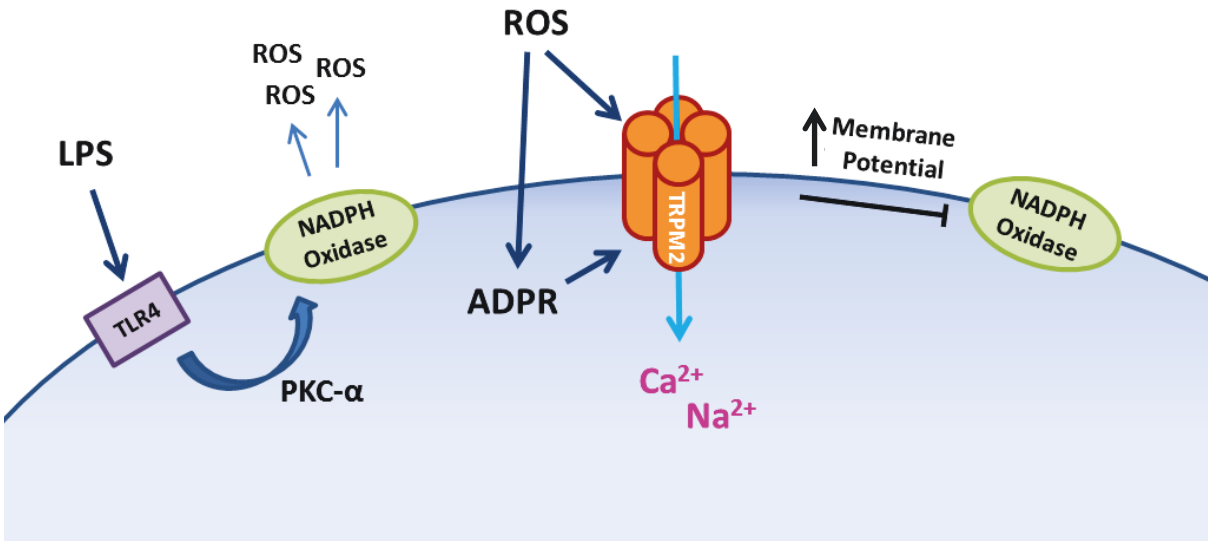

Figure 7.1 TRPM2 as a negative feedback regulator for oxidative stress. *Reactive Oxygen Species (ROS) are released from the membrane-bound enzyme NADPH Oxidase as a response to LPS through TLR4-mediated activation of PKC-α. Alternatively, cells may be directly exposed to ROS from exogenous sources such as cigarette smoke. ROS is thought to activate TRPM2 both directly, and through ADPR. TRPM2 activation causes the cation channel to open, allowing Ca^{2+} (and Na^{2+}) to travel across the cell membrane. This movement of cations results in membrane depolarisation, which deactivates NADPH Oxidase and, thus, negatively regulates the production of ROS.*

It is also notable that there were no observable differences in TRPM2 expression in the lung parenchyma collected from human non-smokers, smokers and emphysema patients, as shown in *Figure 3.5 (chapter 3)*. Similarly, it has been reported that there is no change in TRPM2 transcriptional expression in macrophages isolated from the lungs of COPD patients, smokers and controls (Finney-Hayward et al. 2010). These data indicate that a deficiency in TRPM2 expression is not responsible for the development of COPD in response to CS exposure. In the murine models of inflammation, TRPM2 expression was significantly increased, particularly at acute time-points. It is possible that in COPD, the up-regulation of TRPM2 as a response to CS exposure is lost, impairing the lungs ability to respond to ROS burden.

The role of TRPM2 in the control of oxidant-antioxidant balance merits further research in the understanding of the pathogenesis of COPD, and the identification of novel therapies for the disease. Oxidant-antioxidant imbalance has been implicated for a role in the pathogenesis of COPD, and the generation of the abnormal, poorly-resolving inflammatory response (Schaberg et al. 1992, Babior 2000). It is possible, that stimulation of TRPM2 receptors may help to negatively regulate the production of endogenous reactive oxygen species, thus helping to redress the oxidant-antioxidant imbalance in COPD patients. Furthermore, oxidative stress is thought to be a key mechanism contributing to the inefficacy of glucocorticoids against inflammation in COPD (Barnes 2013). Oxidative stress is thought to inactivate HDAC-2, a key protein in the mechanism of glucocorticoid dependent regulation of inflammation, both through the generation of peroxynitrites and through the activation of PI3K (Barnes 2013). It is therefore possible, that stimulation of TRPM2 in COPD patients to reduce the production of reactive oxygen species, could reverse the steroid-insensitivity of the disease, improving the efficacy of glucocorticoid treatment.

7.1.3 TRPV1, TRPV4 and TRPM8

The inflammatory responses of TRPV1-/-, TRPV4-/- and TRPM8-/- mice were significantly decreased after acute CS exposure compared to the wild-type. The inflammatory responses of these knock-out mice to LPS, however, remained the same as in the wild-type. These results indicate that TRPV1, TRPV4 and TRPM8 are specifically involved in the propagation of the inflammatory response to acute CS in this murine model. The further observation was made that TRPM8 was involved in a separate inflammatory mechanism to TRPV1 and TRPV4. The inhibition of airway neutrophilia in TRPM8-/- was attributable to a decrease in the neutrophil chemotactic KC, whilst the elevation in the levels of ATP and IL-1β remained unaffected. Conversely, TRPV1-/- mice and TRPV4-/- mice exhibited a 50% reduction in ATP, IL-1β and neutrophil levels compared to wild-types, but the elevation in KC levels remained unaffected. These data suggest that at least two independent inflammatory mechanisms are responsible for neutrophil recruitment in the acute CS exposure model. Indeed, it seems likely there are a number of interacting inflammatory cascades which all contribute to the inflammatory phenotype of COPD, and a number of cytokines have been identified as having key roles (Barnes 2009). Many of these cytokines and their receptors have been identified as potential therapeutic targets, notably including TNF-α, IL-1β, CXCR2. A blocking antibody for TNF-α, *infliximab*, failed to show any clinical benefit in COPD patients at doses where it is effective in rheumatoid arthritis (Rennard et al. 2007). There is also an IL-1β antibody, *canakinumab*, which is currently in clinical trials for the treatment of COPD (Dhimolea 2010). The results of this trial will help to further elucidate the role of IL-1β in the pathogenesis of COPD, indicating whether upstream targets of IL-1β (such as $P2X_7$ and possibly TRPV1/TRPV4) may be of therapeutic benefit in COPD. Small molecule antagonists of CXCR2 are also currently in clinical development for COPD patients (Chapman et al. 2009). However, given the overlap in functionalities between many cytokines and cytokine receptors, together with the complex nature of the cytokine network in COPD (Barnes 2009), it seems unlikely that any therapy targeting only one specific cytokine/receptor will provide a significant clinical benefit to patients. One alternative to targeting individual cytokines, is to target the upstream mediators of cytokine release. This strategy could potentially modulate multiple inflammatory cytokines simultaneously and, thus, prove more

effective at attenuating the chronic inflammation in COPD patients. One such mediator, identified in this thesis, is ATP, which appears to be an upstream regulator of IL-1β release.

The level of extracellular ATP was found to be increased in COPD patients, correlating positively with BALF neutrophilia and negatively with lung function (Lommatzsch et al. 2010). Indeed, ATP has been suggested as an important inflammatory mediator in the airways through the activation of purinergic receptors (Bours et al. 2006; Mohsenin and Blackburn 2006), as well as an important mediator in the regulation of mucus clearance, through its action on purinoceptors in the apical membrane (Button et al. 2013).

Interestingly, the reduction in ATP levels in CS exposed TRPV1-/- and TRPV4-/- mice indicates the possible involvement of purinergic receptors in the transduction of the CS-induced inflammatory response. This hypothesis fits with published data showing that genetic deletion of $P2X_7$ receptors in C57BL/6 mice also reduces neutrophilia and IL-1β levels in acute and sub-chronic models of CS exposure (Eltom et al. 2011; Lucattelli et al. 2011), and selective pharmacological inhibition of $P2X_7$ receptors prevented the development of emphysema in a more chronic murine model (Lucattelli et al. 2011). It has been suggested that the $P2X_7$-inflammasome-caspase-1 axis is responsible for the cleavage of pro-IL-1β into the mature form in these murine CS exposure models. The fact that CS-exposed TRPV1-/- and TRPV4-/- mice exhibited reduction in ATP release in combination with reduced IL-1β levels and neutrophilia, suggests that these two ion channels may play a role in the ATP release mediated activation of the $P2X_7$-inflammasome-caspase-1 mechanism of airway inflammation induction.

In view of this evidence, the role of TRPV1 and TRPV4 were subsequently examined in a sub-chronic (14-day) model of CS-induced inflammation, to test their involvement in the development of a more COPD-like inflammatory phenotype. The inflammatory phenotype in the sub-chronic model progresses from the phenotype of the acute model to include macrophage and lymphocyte infiltration in the BALF in addition to neutrophils, as well as significantly increased leukocyte infiltration in the lung tissue. The inflammation in the sub-chronic model is also more slowly resolving than in the acute model. The phenotype of the sub-chronic model therefore more closely resembles the inflammation thought to drive COPD.

When tested in the sub-chronic CS model, genetic ablation of TRPV1 or TRPV4 provided only modest reductions in BALF neutrophilia and lung tissue macrophage recruitment. Macrophage recruitment in the BALF, IL-1β levels and caspase-1 activation were unaffected by genetic ablation of either TRPV1 or TRPV4. Furthermore, KC levels were increased in both knock-out strains compared to the wild-type. Whilst these data still support the theory that both TRPV1 and TRPV4 play a role in the development of COPD-like inflammation in this sub-chronic CS murine model, the data also suggest it is unlikely that targeting either channel independently would be sufficient to provide a therapeutic benefit in a disease state. It is possible that due to the evident overlap in functionality between TRPV1 and TRPV4, ablation of one channel is compensated for by the other channel in the sub-chronic model. This TRPV1/4-ATP-IL-1β axis, however, is evidently of significant importance to the initiation and progression of CS mediated inflammation and, furthermore, translational data indicates that the pathway may be important to the pathogenesis of COPD, as increased levels of $P2X_7$ and caspase-1 activation has been observed in CS-exposed wild-type mice and in COPD patients (Lommatszch et al. 2010, Eltom et al. 2011). In addition to this, it was observed in chapter 3 of this thesis that, transcriptional expression of TRPV1 and TRPV4 is increased lung tissue from COPD patients, indicating that these channels may be important for CS-mediated release of extracellular ATP in COPD patients. These observations may also provide some insight into COPD susceptibility. Although many people smoke, not all smokers will develop COPD (Hurst et al. 2010), indicating that genetics play a role in disease susceptibility. The human tissue data in this thesis may indicate that individuals with higher expression levels of TRPV1 and/or TRPV4 may be more susceptible to developing the disease. Indeed, two separate genome-wide association studies have identified SNPs of TRPV4 which are associated with COPD.

An interesting feature, of this TRPV1/4-ATP-IL-1β inflammatory pathway, is that eATP dependent activation of the $P2X_7$-inflammasome-caspase-1 axis facilitates the production of IL-1β (and IL-18) from its pro-form, without the need for up-regulation at the transcriptional level. The pro-forms of IL-1β and IL-18 are constitutively high in the cytosol and previous data has indicated that CS does not affect the transcriptional regulation of IL-1β, despite significantly increasing levels of the mature protein in murine models (Eltom et al. 2011). This may partly explain why the inflammatory response in COPD patients is normally

insensitive to glucocorticoids. Glucocorticoids are thought to exert their anti-inflammatory effects, predominantly, through modulating transcriptional regulation of inflammatory cytokines and chemokines (Barnes 2013). Targeting of this pathway may, therefore, provide a therapy which can succeed where glucocorticoids have failed.

It is also noteworthy that, TRPV1-/- and TRPV4-/- mice exhibited a normal innate immune response to aerosolised LPS. This observation suggests that potential COPD therapies which target this pathway, would not compromise the innate immune response in patients. This could be an important feature of a successful COPD therapy, as the vast majority of exacerbations are thought to be caused by bacterial (and/or viral) infections (Papi et al. 2006). Avoiding exacerbations is of critical importance in COPD, as frequency of exacerbations correlates strongly to disease progression (Soler-Cataluña et al. 2005), and in the UK alone, COPD exacerbations account for over 90,000 hospital admissions a year, which is approximately 10% of all UK hospital admissions (Price et al.2006).

Further elucidation and translation of this inflammatory pathway is, therefore, highly warranted, in the search for new, effective therapies to treat COPD patients, and tackle the ever-increasing social and economic burden of this devastating disease.

7.2 Future Directions

The aim of this thesis was to investigate the potential role of various TRP channels in the pathogenesis of COPD, with the intention of elucidating new therapeutic targets. The murine *in vivo* data presented implicates a prominent role for TRPV1, TRPV4 and TRPM8 in the initiation of inflammation as a response to CS, the main risk factor for development of COPD. Further work, however, is required to qualify these targets in human COPD patients. The data in this thesis have also raised a number of other interesting questions which I believe justify further study. The following sections detail the future directions, in which I believe, the research in this thesis should be extended.

7.2.1 Human tissue

The expression of TRP channels, measured in human lung samples in chapter 3, is possibly the most disease-relevant data obtained in this thesis, as it enabled the direct comparison of COPD patients with 'healthy' smokers and non-smoker controls. However, the conclusions which can be drawn from this data are limited, both by the relatively low *n* number in the patient subsets, and by the limited patient background information available.

Future work in this area, should aim to elucidate whether there is any correlation between TRP channel expression in COPD patients and markers of pulmonary function (i.e. FEV_1), GOLD status, frequency of exacerbations, current medications and smoking history. It would also be of interest to investigate whether there is a correlation between the number of pack-years and TRP channel expression in the 'healthy' smokers. It would also be of interest to identify whether increased expression of TRPV1 and TRPV4 is associated more strongly to one of the individual pathologies of COPD (i.e. chronic bronchitis, small airways and emphysema). Obtaining this data may help to identify patient sub-sets where therapies targeting these channels are more or less likely to be effective.

In addition to this, further research should focus on identifying the cell-types where differences in TRP channel expression manifest. In the present work, whole lung parenchyma was used for the expression studies. Parenchyma is comprised of a number of cell types, including, epithelial, endothelial, fibroblasts, leukocytes and others. Isolating the

cell types where TRP channel expression is increased (or decreased) may help to further elucidate the inflammatory mechanisms which are thought to drive this disease.

7.2.2 *In vitro* models of CS-induced inflammation

Models of *in vitro* CS exposure allow direct activation of human cells by the most important risk factor for COPD, allowing the observation of precise cellular mechanisms involved in disease generation. Furthermore, equivalent mouse and human cell types can be examined under the same exposure conditions to provide translational information for *in vivo* murine models to human systems.

The most commonly employed *in vitro* model for COPD research involves the treatment of cell populations with cigarette smoke extract (CSE) (Krimmer and Oliver 2011). CSE is generated by bubbling the smoke from cigarettes through a volume of cell media, in order to dissolve soluble compounds from the CS. The constituents of CSE are thought to correlate well to those during *in vivo* CS inhalation, as compounds inhaled *in vivo* must have solubility in order to pass through the aqueous mucus layer in the lungs. Indeed, data has been published showing that genetic changes observed in the bronchial epithelial cells of heavy smokers *in vivo* correlate with changes to cells exposed to CSE *in vitro* (Pierrou et al. 2007).

In vitro models of CSE exposure would, therefore, provide an excellent platform for the further investigation of the relationship between TRP channels and CS-induced ATP signalling, in disease relevant cell-types, such as airway epithelial cells and leukocytes. Furthermore, translational data could be obtained by running parallel experiments in human and mouse cells. This data may provide information on the clinical relevance of the *in vivo* models used in this thesis.

7.2.2.1 The role of TRPV1 and TRPV4 in CS-induced ATP release *in vitro*

Levels of ATP are increased in the BALF retrieved from COPD patients (Mortaz et al. 2009). TRPV4 activation can induce ATP release from airway epithelial cells via pannexin channels

(Seminario-Vidal et al. 2011), and the data in chapter 4 indicate the importance of this pathway in the generation of CS-induced inflammation in the murine model.

During the course of these studies I also found that ATP release is a key feature of CSE treated cell-lines. Murine epithelial (LA-4), murine monocyte (J774), human epithelial (A549) and human monocyte (THP-1) cell lines were cultured in their respective mediums, as described in section 2.5.1. When exposed to increasing concentrations of CSM as described in section 2.5.2, dose-dependent ATP release was measured at 30 minutes after exposure, as shown in figure 7.2.1 below. There were no significant differences in cell viability between treatment groups after 24 hours, as measured by MTT assay, indicating that cell death was not responsible for the ATP release.

These data indicate that ATP release is a common feature of epithelial cells and monocytes, in both mouse and human systems, in response to CSE. This observation adds credibility to the translational value of the *in vivo* data in chapter 4, where ATP release was shown to correlate with other inflammatory markers of inflammation in CS-exposed mice, indicating that ATP release may be a key mediator in the initiation of the inflammatory response to CS in humans.

This data provides the ideal platform to investigate the relationship between TRPV1 and TRPV4 channels and CS-induced ATP release. Furthermore, I investigated the expression of TRPV1 and TRPV4 in murine and human cell types. The murine cell panel contained LA-4, J774 and primary airway fibroblasts. TRPV4 expression was detected in all 3 cell types, and TRPV1 expression was detected in LA-4 and fibroblast cell types. The human cell panel contained A549, THP-1, U-937 (human monocyte line), HASM (primary human airway smooth muscle) and primary human macrophages. TRPV1 expression was detected in all human cell types, and TRPV4 expression was measured in both primary cell types but not in the cell lines.

To establish the role of TRPV1 and TRPV4 in CSE induced ATP release, these murine and human cell types could be characterised with dose-response and time-course experiments using CSE, as was performed in the *in vivo* CS exposure models. Pharmacological tools could then be used to probe the relationship between the ion channels and ATP release. Specifically TRPV1 antagonist JNJ-17203212 (Swanson et al. 2005), and TRPV4 antagonist

HC-067047 (Martin et al. 2012) could be used in an attempt to inhibit CSE-induced ATP release. Furthermore, specific TRPV1 agonist capsaicin and TRPV4 agonist GSK1016790A (Thorneloe et al. 2008) could be used to attempt to induce ATP release independently.

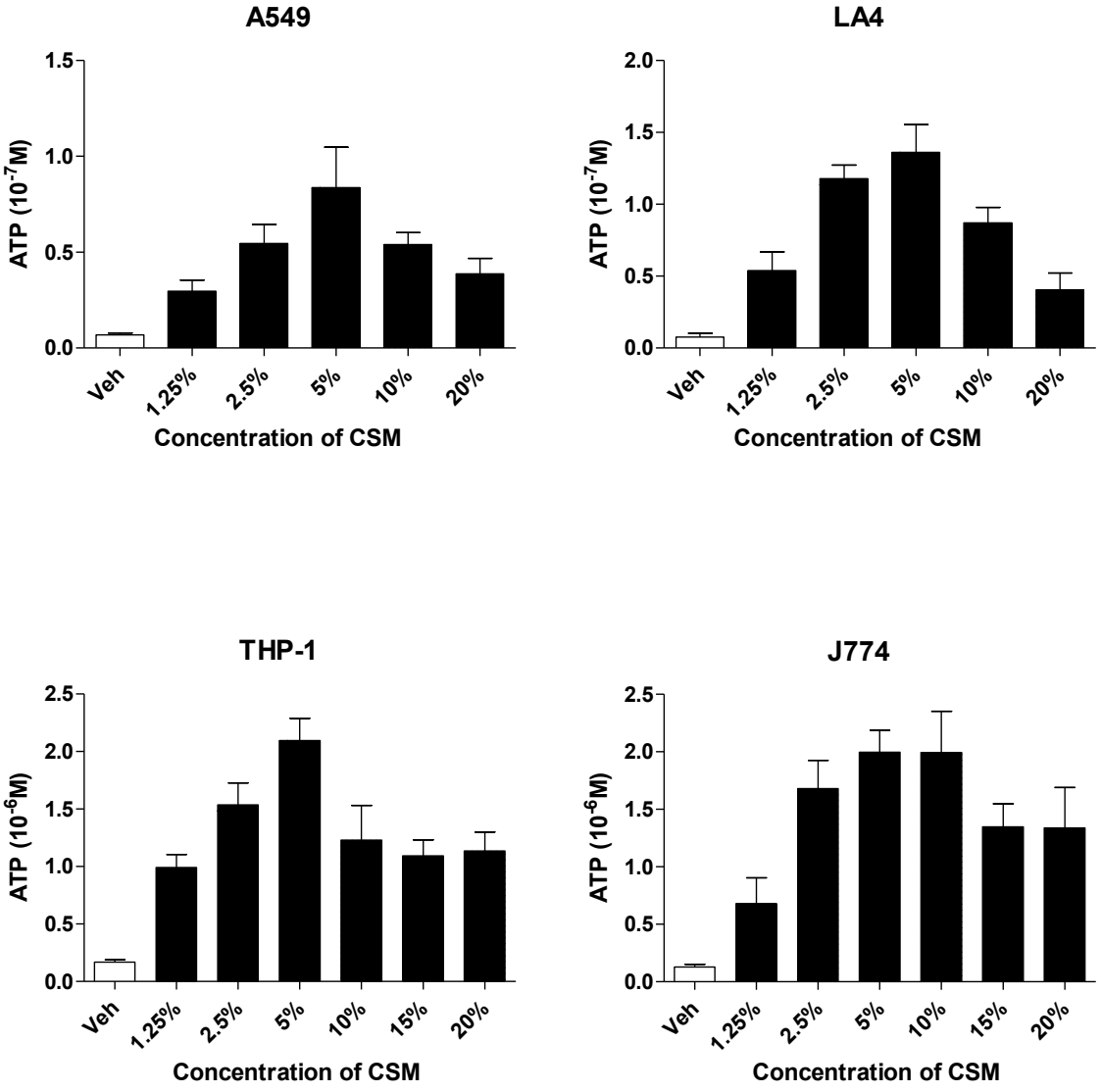

Figure 7.2 The release of ATP from murine and human cell lines in response to increasing concentrations of CSE. *A549, LA-4, THP-1 and J-774 cell lines were cultured in their respective media. Cells were plated in 24-well plates and growth arrested before treatment with 1.25, 2.5, 5, 10, 15 or 20% CSE, or vehicle (normal growth arrest media), 3 wells per plate per treatment. 30 minutes after treatment, the cell supernatants were collected and the concentration of ATP was measured. The data are represented as mean ± SEM for n=3 separate experiments.*

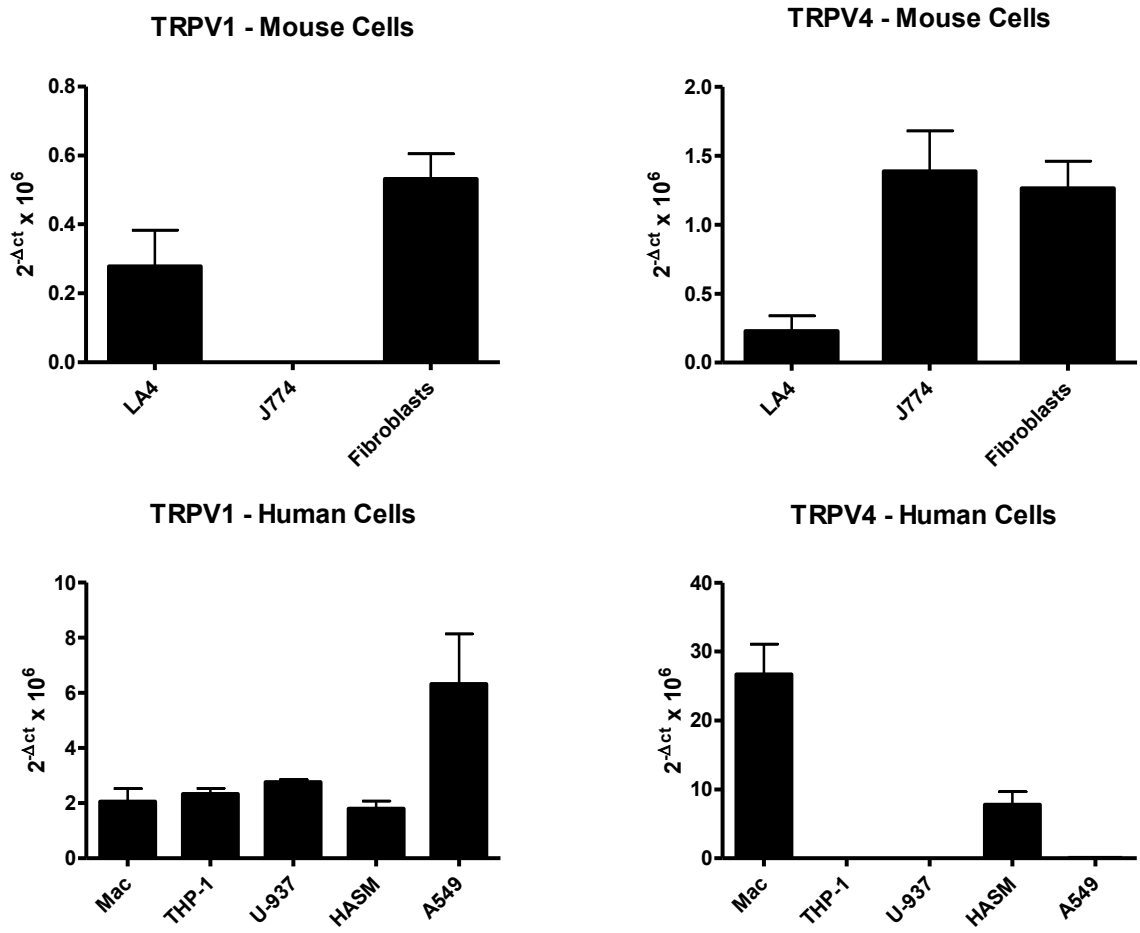

Figure 7.3 The expression of TRPV1 and TRPV4 channels in human and mouse cell types. *Human cell lines THP-1, U-937, A549 and primary HASM and Macrophages were cultured in their respective media. Murine cell lines LA-4, J-774 and primary airway fibroblasts were cultured in their respective media. RNA was isolated from separate populations from each cell type then reverse transcribed to cDNA. TaqMan reagents and primers were used to measure the expression of TRPV1 and TRPV4 relative to 18s in each sample. Data are expressed as mean ± SEM. For cell lines n=4 separately cultured cell populations; for primary cells n=6 patients/animals.*

7.2.3 Pharmacological modulation of TRP channels *in vivo*

The pharmacological tools mentioned in the above section (7.2.1), which modulate TRPV1 and TRPV4, could further be employed to probe the *in vivo* murine smoke and LPS models. Knock-out mice are an incredibly useful tool to identify the role of individual proteins, however genetically modifying an organism from birth has the potential to affect the development of the organism in unforeseen ways. Although the mice from genetically modified strains in this thesis were shown to be fertile and viable, it is possible that subtle biochemical changes were effected which were not observed. These subtle changes may have in turn affected the response of these animals to the given stimuli via mechanisms independent of the ablated gene itself. Pharmacological modulation of the TRP channels in experiments parallel to the studies performed with the knock-outs would act to confirm the validity of the data.

In order to employ a pharmacological tool for an *in vivo* model, the compound must first be validated using pharmacodynamic markers, to establish effective dose concentrations and time-points. The pharmacokinetics and pharmacodynamics of JNJ-17023212 have been previously reported in rats (Swanson et al. 2005) and guinea-pigs (Bhattacharya et al., 2007; Grace et al., 2012), but never in mice. Ideally the pharmacodynamic markers measured for the purposes of this research should involve airway functions, as the distribution of compounds may differ between organs.

Once the pharmacodynamic activity of the compounds is established, a dosing regimen can be devised to ensure adequate pharmacological blockade of the relevant receptor is achieved during each of the model exposures. Littermates dosed with vehicle only should be used as the negative control, and the relevant knock-outs could be used as a positive control for the acute CS model.

7.2.4 Are TRPV1 and TRPV4 solely responsible for the CS-mediated release of ATP?

The data in chapter 4 indicated that genetic deletion of either TRPV1 or TRPV4 reduced acute CS-induced ATP release by 50%. It is possible therefore, but by no means certain, that these are the only two channels involved in this response. In order to test this hypothesis, dual pharmacological antagonism of the two channels could be induced using JNJ-17203212 and HC-067047 in combination, before testing mice in the acute smoke model and human and murine cells in an *in vitro* CSE driven model. Alternatively, or in compliment, a 'double knock-out' colony of mice could be bred by crossing mice from the TRPV1-/- and TRPV4-/- colonies. Such a colony of TRPV1-/- X TRPV4-/- mice have not been reported in the literature to date, so it is unclear whether such a colony would produce viable, fertile offspring. If double knock-out mice could be produced, then primary cell types, including airway epithelial cells and macrophages could be isolated from the animals and cultured, to provide parallel *in vitro* data.

If it were to be found, using the above tools, that ATP is still elicited by CS or CSE despite pharmacological inhibition and/or genetic disruption of TRPV1 and TRPV4, then the next question to address would be whether the ATP is being released via other members of the TRP channel family, or whether a different class of receptor is involved. A non-selective TRP channel blocker such as ruthenium red (Raemdonck et al. 2012), would be a potential tool for testing this hypothesis, as it is known to inhibit all members of the TRPV family. Ruthenium red is however also known to interact with a number of non-TRP mammalian ion channels, including ryanodine receptors and P/Q type voltage gated calcium channels (Bulmer et al. 2005).

7.2.5 Pharmacological modulation of TRP channels in pre-established inflammation

Elucidating the mechanisms which initiate CS-induced inflammation is of critical importance to the discovery of novel therapeutic targets for COPD. It must be remembered however that COPD is a chronic disease where inflammation is long-established and self-propagating. Any potential therapy would therefore need to reduce pre-established and sustained inflammation. A number of chronic murine smoke exposure models have been shown to

establish COPD-like functional changes, such as emphysema, after 6 months of exposures (Churg et al. 2004; Shapiro et al. 2003). It would be of interest to test whether the inhibition of specific TRP receptors in mice where chronic inflammation has already been induced, affects the severity of inflammation or the progression of emphysema. Initially a chronic murine smoke exposure model would have to be characterised to establish changes in inflammatory phenotypes, the onset and progression of emphysematous changes, and the rate of decline of lung function. From this characterisation, the time-points could be determined for the initiation of TRP inhibition, i.e. before the onset of emphysema and/or once emphysema is established.

7.2.6 Chronic CS-exposure model

In this thesis, acute (3-day) and sub-chronic (14-day) murine CS exposure models were utilised to determine the role of TRP channels in the initiation of the inflammation. The inflammatory phenotype was shown to change from exclusively neutrophilic-driven in the acute model, to neutrophil, lymphocyte and macrophage driven in the sub-chronic model. Multiple research groups have shown that in chronic models (>6 months) of CS exposure, functional lung changes occur which recapitulate the features of COPD, such as emphysema, reduced airway compliance and the development of B-cell follicles (D'hulst et al., 2005; Seimetz et al., 2011; van der Strate et al., 2006). Testing TRP knock-outs and TRP antagonists in a chronic murine model could provide crucial information about the role of TRP channels in CS-induced the lung functional changes associated with the severity and prognosis of COPD.

It should also be noted that other murine models of emphysema have been characterised in the literature, most notably elastase (Birrell et al. 2005). This would be another potential model for examining the role of TRP channels in the development of emphysema. However, one major drawback is that the inflammation exhibited after elastase instillation into the lungs resolves rapidly. This observation indicates that the elastase model may not truly recapitulate the mechanisms of emphysema development seen in COPD.

7.2.7 TRPM2 regulation in human macrophages from COPD patients

As discussed in section 7.1.3, there is strong evidence that TRPM2 functions as a negative feedback mechanism for the production of ROS in murine macrophages (Di et al. 2012). In addition to this evidence, the data in this thesis showed that TRPM2-deficient mice exhibited increased severity of airway inflammation as a response to CS and LPS. These findings raise the possibility that TRPM2 may be involved in the regulation of inflammatory responses found in COPD patients, where ROS are thought to play a role in driving the progression of the disease.

TRPM2 was found to be significantly up-regulated in wild-type mice lung tissue after acute CS, sub-chronic CS and LPS challenge, presumably due to the increased ROS production from sequestered leukocytes. It is possible that the oxidant/anti-oxidant imbalance observed in COPD patients may be a result of a deficiency in TRPM2, a deficiency in the up-regulation response to inflammatory insult, or possibly as a result of TRPM2 dysfunction.

To test these possibilities, macrophages could be isolated from human patients (non-smoker, smoker, and COPD), cultured, and then challenged with CSE before measuring the levels of ROS production and the expression of TRPM2. The function of TRPM2 channels in this model could be further elucidated using pharmacological tools. There are, however, no pharmacological antagonists currently available which specifically target TRPM2. Channel function can, however, be modulated by blocking ADPR, although this will have a number of other non-TRPM2 cellular effects, making the exact role of TRPM2 in human cells difficult to ascertain.

7.2.8 How does TRPM8 regulate KC after murine CS exposure?

One of the most interesting observations in this thesis was the ability of TRPM8 to modulate the CS-mediated release of KC in the acute murine smoke exposure model. This highlighted a CS-activated inflammatory pathway which was independent of the TRPV1/4 – ATP – IL-1β pathway. Furthermore CS-exposed TRPM8-/- mice exhibited almost completely attenuated BALF neutrophil recruitment, as shown in figure 4.5, indicating the importance of KC to the inflammatory response. Furthermore the LPS-induced inflammatory response was

unaffected by TRPM8 deficiency. These observations justify the investigation of TRPM8 in sub-chronic and chronic murine exposure models, to assess the relative contribution of this pathway in the progression of the CS-mediated inflammatory phenotype.

Characterising the pathway by which TRPM8 mediates inflammatory cytokines would be of particular interest. To date, little research has been published which examines this mechanism, although a functional TRPM8 variant has been identified in human lung epithelial cells which mediates inflammatory cytokine transcription (Sabnis, Reilly, et al. 2008). The mechanism by which this functional TRPM8 variant exerts these effects, however, has not been elucidated. This may be due to a lack of pharmacological tools with TRPM8 specificity. Once better tools become available, I believe this will be an important future avenue to further the work in this thesis.

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Appendix – Tabulated cellular recruitment data from *in vivo* murine experiments

BALF		Cells 10^3/ml			
Genotype	**Treatment**	**Lymphocytes**	**Eosinophils**	**Macrophages**	**Neutrophils**
WT	Air	0.96 ± 0.15	0.54 ± 0.28	22.74 ± 2.68	2.12 ± 0.79
	Smoke	3.04 ± 0.87	0.61 ± 0.43	51.76 ± 3.63 #	35.10 ± 14.99 #
TRPA1-/-	Air	1.17 ±0.37	0.03 ± 0.03	13.15 ± 2.01	0.15 ± 0.11
	Smoke	2.24 ± 0.41	0.18 ± 0.18	40.03 ± 3.39	28.55 ± 8.63
Lung tissue		Cells 10^3/mg			
WT	Air	368 ± 76	123 ± 18	85 ± 11	495 ± 92
	Smoke	289 ± 30	104 ± 14	96 ± 14	304 ± 37
TRPA1-/-	Air	258 ± 23	137 ± 23	83 ± 12	364 ± 33
	Smoke	215 ± 25	105 ± 14	67 ± 7	277 ± 34

Table 8.1 – The role of TRPA1 channels in Acute Smoke Model induced inflammatory cell recruitment. *Wild-type and TRPA1-/- mice were exposed to room air or CS for 3 consecutive days. BALF and lung tissue were collected 24 hours after the last exposure for 4-way differential cell counts. Data are represented as Mean ± SEM for n=8 observations. Statistical significance was determined using the Mann-Whitney U-test. # = P<0.05, denoting a significant difference between the smoke exposed and air exposed wild-types; * = P<0.05, denoting a significant difference between the smoke exposed wild-types and knock-outs*

BALF		Cells 10^3/ml			
Genotype	**Treatment**	**Lymphocytes**	**Eosinophils**	**Macrophages**	**Neutrophils**
WT	Air	5.92 ± 1.02	0.05 ± 0.05	75.48 ± 9.92	0.55 ± 0.36
	Smoke	11.78 ± 1.04	0.49 ± 0.33	117.76 ± 14.56	132.0 ± 18.26 #
TRPC6-/-	Air	22.86 ± 4.02	0.39 ± 0.28	84.72 ± 6.79	1.03 ± 0.45
	Smoke	15.3 ± 3.70	0.26 ± 0.26	230.38 ± 38.73	163.15 ± 36.02

Table 8.2 – The role of TRPC6 channels in Acute Smoke Model induced inflammatory cell recruitment. *Wild-type and TRPC6-/- mice were exposed to room air or CS for 3 consecutive days. BALF was collected 24 hours after the last exposure for 4-way differential cell counts. Data are represented as Mean ± SEM for n=8 observations. Statistical significance was determined using the Mann-Whitney U-test. # = P<0.05, denoting a significant difference between the smoke exposed and air exposed wild-types; * = P<0.05, denoting a significant difference between the smoke exposed wild-types and knock-outs.*

BALF		Cells 10^3/ml			
Genotype	**Treatment**	**Lymphocytes**	**Eosinophils**	**Macrophages**	**Neutrophils**
WT	Air	1.25 ± 0.44	0.2 ± 0.14	72.19 ± 7.49	1.48 ± 1.23
	Smoke	2.11 ± 0.64	0.2 ± 0.2	83.4 ± 8.07	61.46 ± 8.91 #
TRPM2-/-	Air	0.49 ± 0.16	0.11 ± 0.11	63.15 ± 5.20	0 ± 0
	Smoke	1.19 ± 0.15	0.43 ± 0.33	93.34 ± 8.60	23.8 ±9.78
Lung tissue		**Cells 10^3/mg**			
WT	Air	8602 ± 2064	3994 ± 817	4390 ± 920	10785 ± 2265
	Smoke	3508 ± 899	1972 ± 270	3696 ± 825	9578 ± 1965
TRPM2-/-	Air	4896 ± 1088	1757 ± 450	3900 ± 657	7259 ± 1321
	Smoke	6714 ± 1631	2533 ± 441	6553 ± 833 *	17288 ± 3555 *

Table 8.3 – The role of TRPM2 channels in Acute Smoke Model induced inflammatory cell recruitment. *Wild-type and TRPM2-/- mice were exposed to room air or CS for 3 consecutive days. BALF and lung tissue were collected 24 hours after the last exposure for 4-way differential cell counts. Data are represented as Mean ± SEM for n=8 observations. Statistical significance was determined using the Mann-Whitney U-test. # = P<0.05, denoting a significant difference between the smoke exposed and air exposed wild-types; * = P<0.05, denoting a significant difference between the smoke exposed wild-types and knock-outs.*

BALF		Cells 10^3/ml			
Genotype	**Treatment**	**Lymphocytes**	**Eosinophils**	**Macrophages**	**Neutrophils**
WT	Air	1.25 ± 0.44	0.2 ± 0.14	72.19 ± 7.49	1.48 ± 1.23
	Smoke	2.11 ± 0.64	0.2 ± 0.2	83.4 ± 8.07	61.46 ± 8.91 #
TRPM8-/-	Air	0.47 ± 0.21	3.21 ± 3.21	49.9 ± 3.71	0.7 ± 0.56
	Smoke	2.21 ± 1.16	0.75 ± 0.75	102.93 ± 11.21	7.85 ± 7.19 *
Lung tissue		**Cells 10^3/mg**			
WT	Air	8602 ± 2064	3994 ± 817	4390 ± 920	10785 ± 2265
	Smoke	3508 ± 899	1972 ± 270	3696 ± 825	9578 ± 1965
TRPM8-/-	Air	6530 ± 1104	3556 ± 720	4021 ± 915	9493 ± 2522
	Smoke	3136 ± 734	2010 ± 542	3302 ± 588	8380 ± 1532

Table 8.4 – The role of TRPM8 channels in Acute Smoke Model induced inflammatory cell recruitment. *Wild-type and TRPM8-/- mice were exposed to room air or CS for 3 consecutive days. BALF and lung tissue were collected 24 hours after the last exposure for 4-way differential cell counts. Data are represented as Mean ± SEM for n=8 observations. Statistical significance was determined using the Mann-Whitney U-test. # = P<0.05, denoting a significant difference between the smoke exposed and air exposed wild-types; * = P<0.05, denoting a significant difference between the smoke exposed wild-types and knock-outs.*

BALF		Cells 10^3/ml			
Genotype	**Treatment**	**Lymphocytes**	**Eosinophils**	**Macrophages**	**Neutrophils**
WT	Air	10.77 ± 3.76	0.84 ± 0.84	31.78 ± 6.37	0.36 ± 0.26
	Smoke	5.74 ± 2.28	1.35 ± 0.65	76.05 ± 5.56 #	25.61 ± 5.06 #
TRPV1-/-	Air	6.32 ± 1.77	0.23 ± 0.23	53.96 ± 8.16	4.49 ± 4.06
	Smoke	2.21 ± 0.58	0.16 ± 0.16	108.04 ± 11.72	8.34 ± 4.08 *
Lung tissue		**Cells 10^3/mg**			
WT	Air	2391 ± 287	1876 ± 353	1404 ± 251	3762 ± 354
	Smoke	2373 ± 275	1643 ± 158	953 ± 152	4077 ± 575
TRPV1-/-	Air	3083 ± 364	2830 ± 265	1208 ± 207	5477 ± 466
	Smoke	2661 ± 227	2430 ± 169	747 ± 109	4651 ± 410

Table 8.5 – The role of TRPV1 channels in Acute Smoke Model induced inflammatory cell recruitment. *Wild-type and TRPV1-/- mice were exposed to room air or CS for 3 consecutive days. BALF and lung tissue were collected 24 hours after the last exposure for 4-way differential cell counts. Data are represented as Mean ± SEM for n=8 observations. Statistical significance was determined using the Mann-Whitney U-test. # = P<0.05, denoting a significant difference between the smoke exposed and air exposed wild-types; * = P<0.05, denoting a significant difference between the smoke exposed wild-types and knock-outs.*

BALF		Cells 10^3/ml			
Genotype	**Treatment**	**Lymphocytes**	**Eosinophils**	**Macrophages**	**Neutrophils**
WT	Air	1.25 ± 0.44	0.2 ± 0.14	72.19 ± 7.49	1.48 ± 1.23
	Smoke	2.11 ± 0.64	0.2 ± 0.2	83.4 ± 8.07	61.46 ± 8.91 #
TRPV4-/-	Air	1.21 ± 0.19	0 ± 0	52.48 ± 6.19	0.06 ± 0.06
	Smoke	1.98 ± 0.56	0.9 ± 0.9	76.2 ± 5.22	16.74 ± 7.75 *
Lung tissue		**Cells 10^3/mg**			
WT	Air	8602 ± 2064	3994 ± 817	4390 ± 920	10785 ± 2265
	Smoke	3508 ± 899	1972 ± 270	3696 ± 825	9578 ± 1965
TRPV4-/-	Air	10026 ± 1473	4878 ± 703	5022 ± 517	12135 ± 1103
	Smoke	3241 ± 492	2020 ± 353	3191 ± 245	8545 ± 1134

Table 8.6 – The role of TRPV4 channels in Acute Smoke Model induced inflammatory cell recruitment. *Wild-type and TRPV4-/- mice were exposed to room air or CS for 3 consecutive days. BALF and lung tissue were collected 24 hours after the last exposure for 4-way differential cell counts. Data are represented as Mean ± SEM for n=8 observations. Statistical significance was determined using the Mann-Whitney U-test. # = P<0.05, denoting a significant difference between the smoke exposed and air exposed wild-types; * = P<0.05, denoting a significant difference between the smoke exposed wild-types and knock-outs.*

BALF		Cells 10^3/ml		
Genotype	**Treatment**	**Eosinophils**	**Lympho-mononuclear**	**Neutrophils**
WT	Saline	0.52 ± 0.29	119.9 ± 16.63	0.58 ± 0.33
	LPS	2.44 ± 1.19	49.64 ± 5.83	302.36 ± 22.45 #
TRPA1-/-	Saline	0 ± 0	97.08 ± 16.35	0.43 ± 0.35
	LPS	0 ± 0	46.97 ± 10.1	295.03 ± 67.45
Lung tissue		**Cells 10^3/mg**		
WT	Saline	1317 ± 116	5191 ± 610	3819 ± 435
	LPS	772 ± 167	1924 ± 307	16267 ± 1411 #
TRPA1-/-	Saline	1651 ± 271	1197 ± 170	3711 ± 329
	LPS	828 ± 218	1444 ± 245	13279 ± 1625

Table 8.7 – The role of TRPA1 channels in LPS induced inflammatory cell recruitment. *Wild-type and TRPA1-/- mice were exposed to saline or LPS for 30 minutes. BALF and lung tissue were collected 24 hours after the last exposure for 3-way differential cell counts. Data are represented as Mean ± SEM for n=8 observations. Statistical significance was determined using the Mann-Whitney U-test. # = P<0.05, denoting a significant difference between the LPS exposed and saline exposed wild-types; * = P<0.05, denoting a significant difference between the LPS exposed wild-types and knock-outs.*

BALF		Cells 10^3/ml		
Genotype	**Treatment**	**Eosinophils**	**Lympho-mononuclear**	**Neutrophils**
WT	Saline	0 ± 0	76.7 ± 8.68	0.8 ± 0.43
	LPS	3.35 ± 2.0	40.38 ± 6.06	457.53 ± 24.61 #
TRPC6-/-	Saline	0 ± 0	72.6 ± 4.47	2.4 ± 0.63
	LPS	1.65 ± 1.08	48.84 ± 10.0	574.51 ± 70.4

Table 8.8 – The role of TRPC6 channels in LPS induced inflammatory cell recruitment. *Wild-type and TRPC6-/- mice were exposed to saline or LPS for 30 minutes. BALF was collected 24 hours after the last exposure for 3-way differential cell counts. Data are represented as Mean ± SEM for n=8 observations. Statistical significance was determined using the Mann-Whitney U-test. # = P<0.05, denoting a significant difference between the LPS exposed and saline exposed wild-types; * = P<0.05, denoting a significant difference between the LPS exposed wild-types and knock-outs.*

BALF		Cells 10^3/ml		
Genotype	**Treatment**	**Eosinophils**	**Lympho-mononuclear**	**Neutrophils**
WT	Saline	0 ± 0	76.7 ± 8.68	0.8 ± 0.43
	LPS	3.35 ± 2.0	40.38 ± 6.06	457.53 ± 24.61 #
TRPM2-/-	Saline	0.75 ± 0.75	60.52 ± 13.53	15.1 ± 14.8
	LPS	0.48 ± 0.48	51.61 ± 4.91	845.41 ± 232.9 *
Lung tissue		**Cells 10^3/mg**		
WT	Saline	1313 ± 212	16491 ± 2891	7502 ± 928
	LPS	945 ± 274	5525 ± 549 #	29651 ± 4223 #
TRPM2-/-	Saline	891 ± 134	7178 ± 699	9568 ± 915
	LPS	1254 ± 319	9284 ± 1159 *	53376 ± 6203 *

Table 8.9 – The role of TRPM2 channels in LPS induced inflammatory cell recruitment. *Wild-type and TRPM2-/- mice were exposed to saline or LPS for 30 minutes. BALF was collected 24 hours after the last exposure for 3-way differential cell counts. Data are represented as Mean ± SEM for n=8 observations. Statistical significance was determined using the Mann-Whitney U-test. # = P<0.05, denoting a significant difference between the LPS exposed and saline exposed wild-types; * = P<0.05, denoting a significant difference between the LPS exposed wild-types and knock-outs.*

BALF		Cells 10^3/ml		
Genotype	**Treatment**	**Eosinophils**	**Lympho-mononuclear**	**Neutrophils**
WT	Saline	0 ± 0	76.7 ± 8.68	0.8 ± 0.43
	LPS	3.35 ± 2.0	40.38 ± 6.06	457.53 ± 24.61 #
TRPM8-/-	Saline	0 ± 0	63.08 ± 12.61	13.58 ± 13.12
	LPS	2.46 ± 1.48	43.69 ± 5.49	670.09 ± 148.75

Table 8.10 – The role of TRPM8 channels in LPS induced inflammatory cell recruitment. *Wild-type and TRPM8-/- mice were exposed to saline or LPS for 30 minutes. BALF was collected 24 hours after the last exposure for 3-way differential cell counts. Data are represented as Mean ± SEM for n=8 observations. Statistical significance was determined using the Mann-Whitney U-test. # = P<0.05, denoting a significant difference between the LPS exposed and saline exposed wild-types; * = P<0.05, denoting a significant difference between the LPS exposed wild-types and knock-outs.*

BALF		Cells 10^3/ml		
Genotype	**Treatment**	**Eosinophils**	**Lympho-mononuclear**	**Neutrophils**
WT	Saline	0.14 ± 0.13	76.78 ± 18.28	2.61 ± 1.71
	LPS	4.01 ± 1.20	85.93 ± 27.71	462.28 ± 95.52 #
TRPV1-/-	Saline	0 ± 0	129.87 ± 18.39	1.31 ± 0.43
	LPS	0 ± 0	73.89 ± 8.22	558.33 ± 35.89
Lung tissue		**Cells 10^3/mg**		
WT	Saline	1089 ± 188	2509 ± 216	2675 ± 473
	LPS	626 ± 180	3540 ± 457	17217 ± 1662 #
TRPV1-/-	Saline	1035 ± 105	2666 ± 189	8549 ± 1719
	LPS	837 ± 280	4788 ± 574	20352 ± 1662

Table 8.11 – The role of TRPV1 channels in LPS induced inflammatory cell recruitment. *Wild-type and TRPV1-/- mice were exposed to saline or LPS for 30 minutes. BALF and lung tissue were collected 24 hours after the last exposure for 3-way differential cell counts. Data are represented as Mean ± SEM for n=8 observations. Statistical significance was determined using the Mann-Whitney U-test. # = P<0.05, denoting a significant difference between the LPS exposed and saline exposed wild-types; * = P<0.05, denoting a significant difference between the LPS exposed wild-types and knock-outs.*

BALF		Cells 10^3/ml		
Genotype	**Treatment**	**Eosinophils**	**Lympho-mononuclear**	**Neutrophils**
WT	Saline	0 ± 0	76.7 ± 8.68	0.8 ± 0.43
	LPS	3.35 ± 2.0	40.38 ± 6.06	457.53 ± 24.61 #
TRPV4-/-	Saline	0 ± 0	67.81 ± 5.08	0.76 ± 0.24
	LPS	0.95 ± 0.95	37.41 ± 10.04	489.14 ± 47.57

Table 8.12 – The role of TRPV4 channels in LPS induced inflammatory cell recruitment. *Wild-type and TRPM8-/- mice were exposed to saline or LPS for 30 minutes. BALF was collected 24 hours after the last exposure for 3-way differential cell counts. Data are represented as Mean ± SEM for n=8 observations. Statistical significance was determined using the Mann-Whitney U-test. # = P<0.05, denoting a significant difference between the LPS exposed and saline exposed wild-types; * = P<0.05, denoting a significant difference between the LPS exposed wild-types and knock-outs.*