GENETIC AND EPIGENETIC CHARACTERIZATION OF THE SPHINGOSINE-1-PHOSPHATE SIGNALLING SYSTEM IN MACROPHAGES IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE

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LIST OF ABBREVIATIONS

AM	Alveolar macrophage	MCP-1	macrophage chemotactic protein
BAL	Bronchoalveolar lavage	MMP	matrix metalloproteinases
BSP	bisulfite specific PCR	mRNA	Messenger ribonucleic acid
CS	cigarette smoke	NO	nitric oxide
CTGF	Connective tissue growth factor	NTHi	non-typeable H. influenzae.
DNA	Deoxyribonucleic acid	mRNA	Messenger ribonucleic acid
EDTA	Ethylene diamine tetra-acetic acid	PE	Phycoerythrin
EGFR	Epithelial growth factor receptors	PMA	Phorbol 12-myristate 13-acetate
Facs tube	Polystyrene tube (5 ml)	ROS	Reactive oxygen species
FCS	Fetal calf serum	RPMI	Roswell Park Memorial Institute Culture Media
FEV-1	Forced expiratory volume in one second	S1P	Sphingosine 1-phosphate
FVC	Forced vital capacity	S1PR	Sphingosine 1-phosphate receptor
GOLD	Global Strategy for Chronic Obstructive Lung disease	SPHK	Sphingosine kinases
GRO-a	growth related oncogene-alpha	SGPL1	Sphingosine 1-phosphate lyase 1
HDAC	histone deacetylase	SD	Standard deviation
IHC	lmmunohistochemistry	TFBS	Transcription factor binding sites
IL-	Interleukin	TQ	thymoquinone
IP-10	interferon-gamma inducible protein	µg	Microgram
I-TAC	interferon-inducible T-cell alpha-chemoattractant	µI	Microlitre
LPS	Lipopolysaccharide	Wash buffer	0.5% BSA in lsoton II
LTB4	leukotriene B4		
Mab	Monoclonal antibody		

DECLARATION

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LIST OF PUBLICATIONS/MANUSCRIPTS AND ABSTRACTS

Publications/manuscripts related to this research

Hodge G, **Barnawi J**, Jurisevic C, Moffat D, Holmes M, Reynolds P.N, Jersmann H, Hodge S. Lung cancer is associated with decreased expression of perforin, granzyme b and interferon (Genome Bioinformatics Group)-γ by infiltrating lung tissue T cells, natural killer (NK) T-like and NK cells. Clin Exp Immunol. 2014; 178(1):79-85.

Tran H, **Barnawi J**, Roscioli1 E, Hodge G, Reynolds P.N, Pitson S, Jersmann H, Meech R, Haberberger R, Hodge S. Pulmonary macrophage efferocytosis in COPD is suppressed by disturbances to feedback mechanisms in sphingosine-1 phosphate signalling and reversed with FTY720 (manuscript under preparation).

Barnawi J, Tran H, Jersmann H, Pitson S, Roscioli1 E, Hodge G, Meech R, Haberberger R, Hodge S. Potential link between the sphingosine-1-phosphate (S1P) system and defective alveolar macrophage phagocytic function in chronic obstructive pulmonary disease (COPD). (Accepted for publication, Plos One, February 2015).

Barnawi J, Jersmann H, Moffat D, Haberberger R, Hodge S, Meech R.
Differential DNA methylation pattern of sphingosine-1 phosphate receptor 5 in lung macrophages in COPD with/without lung cancer: a potential link to failed efferocytosis. (Submitted to Plos One, July 2015).

Barnawi J, Tran H, Roscioli1 E, Hodge G, Jersmann H, Haberberger R, Hodge S. Pro-phagocytosis and anti-apoptotic effects of thymoquinone in the airways in chronic obstructive pulmonary disease: modulation of the sphingosine-1-phosphate signalling

system. (Submitted to Cellular Immunology September 2015; Manuscript Number CIMM-15-266).

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Khabour OF, **Barnawi JM.** Association of longevity with IL-10 -1082 G/A and TNF-alpha-308 G/A polymorphisms. Int J Immunogenet. 2010;37(4):293-8.

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Barnawi J, Holt P, Hodge G, Jersmann H, Pitson S, Haberberger R, Hodge S. Therapeutic modulation of the sphingosine signalling system with thymoquinine as a novel macrophage-targeted treatment for COPD. Respirology (2014); [TSANZ, Adelaide, Australia].

Holt P, **Barnawi J**, Hodge G, Jermann H, Reynolds P, Pitson S, Haberberger R, Hodge S. Impairment of efferocytosis by cigarette smoke can be reversed in THP-1 macrophages by stimulation with sphingosine-1-phosphate. Respirology (2014); [TSANZ, Adelaide, Australia].

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Barnawi J, Jersmann H, Tran H, Pitson S, Haberberger R, Meech R, Hodge S. Differential DNA methylation pattern of sphingosine-1 phosphate receptor 5 in alveolar macrophages in COPD – potential link to failed efferocytosis? Respirology (2015); [TSANZ, Gold Cost, Australia].

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Tran H, Zalewski P, Roscioli E, **Barnawi J**, Hodge G, Reynolds P, Hodge S. Zinc dyshomeostasis is linked to defective lung macrophage phagocytic function and airway inflammation in mice exposed to cigarette smoke. Respirology (2015); [TSANZ, Gold Cost, Australia].

ABSTRACT

Alveolar macrophages from patients with chronic obstructive pulmonary disease (COPD) are defective in their ability to phagocytose apoptotic bronchial epithelial cells (a process termed 'efferocytosis') and bacteria. These defects may contribute to COPD pathogenesis in several ways. Secondary necrosis of uncleared apoptotic material may result in chronic airways inflammation and perpetuation of COPD disease. A reduced alveolar macrophage phagocytic host response to bacteria, especially non-typeable *H influenzae* (NTHi), may contribute to neutrophilic inflammation and NTHi colonization of the lower airway. However, the exact mechanism that leads to the phagocytic dysfunction is still unknown. The sphingosine 1-phosphate (S1P) signalling system is known to regulate macrophage function. Experiments described in Chapter 2 of the thesis therefore applied a novel approach of measuring all S1P signalling system components in alveolar macrophages from COPD patients and healthy controls. Several components of the S1P system, in particular relative mRNA levels for sphingosine kinases *SPHK1* and S1P receptor *S1PR5*, were dysregulated in COPD and were strongly correlated with efferocytosis, suggesting a potential link to the defective alveolar macrophage phagocytic ability in COPD.

Oxidative stress and inflammation have been shown to contribute to many COPD characteristics, such as uncontrolled activation of cell signalling pathways, increased airway epithelial cell apoptosis, and defective alveolar macrophage phagocytic ability. Chapter 3 describes the effect of two models of oxidative stress and inflammation, cigarette smoke (potential oxidative conditions) and lipopolysaccharide (LPS) (potential inflammatory conditions) on components of S1P signalling and on efferocytosis and phagocytosis of NTHi, using a human macrophage cell line *in vitro*. Cigarette smoke and LPS increased the mRNA expression of *SPHK1* and *S1PR5* in macrophages,

extending the results in Chapter 2 and further supporting the potential link between the S1P signalling system and macrophage phagocytic ability. Cigarette smoke decreased the capacity of macrophages to phagocytose apoptotic cells and bacteria. However, LPS reduced phagocytosis of bacteria only. Treatment option for oxidative stress is anti-oxidants and thymoquinone (TQ) is anti-oxidant/anti-inflammatory agent that has been shown to modulate macrophage inflammatory responses and has successfully been trialled in human clinical studies. Chapter 3 further reports that TQ *per se* had a pro-phagocytic effect on macrophage phagocytic ability. TQ also rescued macrophages from the negative effects of cigarette smoke, and to lesser extent LPS, on macrophage efferocytosis and the mRNA expression respectively. In addition, TQ demonstrated a pro-survival effect on bronchial epithelial cells treated with cigarette smoke. The effects on relative mRNA expression of *SPHK1* and *S1PR5* in the cell line were mirrored using acutely isolated alveolar macrophages from COPD patients.

COPD patients are at increased risk for developing lung cancer and there is strong evidence that pulmonary macrophage dysfunction plays an important role in the pathogenesis of both diseases. DNA methylation has been shown to be modified in COPD and lung cancer. However, it unknown whether the change in mRNA expression of the S1P system (Chapter 2) are controlled by epigenetic modifications such as DNA methylation, and whether DNA methylation regulates macrophage efferocytosis. Data presented in Chapter 4 connect epigenetic modulation, mRNA expression and macrophage function. The results indicate that DNA methylation potentially regulates macrophage efferocytosis and is negatively correlated with the mRNA expression of S1P system components, in particular the *S1PR5* receptor, suggesting epigenetic regulation of macrophage efferocytosis in COPD and potentially lung cancer.

In conclusion, the data presented in this thesis have identified the cell- and disease-specific dysregulation of components of the S1P signalling. The combination of *in vitro* studies using a human macrophage cell line and the profiling of acutely isolated lung macrophages from patients and healthy subjects provide powerful evidence for the importance of S1P signalling for efferocytosis function of alveolar macrophages. In addition, it could be demonstrated that oxidative stress might be one cause for the dysregulation and treatment with TQ could rescue effects of the main cause of COPD, cigarette smoke. Furthermore, we could provide for the first time strong evidence for the epigenetic modulation of the *S1PR5* receptor in individual human cells (alveolar macrophages) under pathological conditions (COPD). The data generated as part the thesis are highly valuable for the understanding of macrophage function and S1P under conditions such as COPD. Furthermore, the data also provide possible treatment options for COPD such as anti-oxidants or epigenome modifying agents.

CHAPTER 1

Introduction and literature review

1.1 Chronic Obstructive Pulmonary Disease (COPD)

According to the Global Initiative for Chronic Obstructive Lung Disease (Vestbo, Hurd et al. 2013) chronic obstructive pulmonary (COPD) is defined "a preventable and treatable disease with some extra pulmonary effects that may contribute to the severity of the disease in individual patients". Cigarette smoking is the most recognised risk factor for the development of COPD. Other risk factors include occupational dust, biomass fuel, genetic background, ageing, nutrition, oxidative stress and asthma (Diaz-Guzman and Mannino 2014). COPD is characterized by chronic inflammation due to an abnormal inflammatory response to noxious agents in certain individuals that eventually leads to airflow limitation, the pathological characteristic of the disease. Airflow limitation is generally associated with chronic bronchitis and emphysema (destruction of the lung parenchyma).

These two features cause the classical symptoms of the disease; chronic productive cough and dyspnea. The diagnosis of COPD is usually carried out by spirometric assay, with chest X-ray and CT scan. There are four stages used to classify the disease severity in COPD patients; and these classifications provide valuable information about disease progression and treatment recommendation (Global Strategy for the Diagnosis 2010):

"Stage I: Mild COPD - Characterized by mild airflow limitation ($FEV_1/FVC < 0.70$ $FEV_1 \geq 80\%$ predicted). Symptoms of chronic cough and sputum production may be present, but are not always at this stage; the individual is usually unaware that his or her lung function is abnormal. Stage II: Moderate COPD - Characterized by worsening airflow limitation $FEV_1/FVC < 0.70$; $50\% \leq FEV_1 < 80\%$ predicted, with breathlessness typically developing on exertion. Cough and sputum production is

sometimes also present. This is the stage at which patients typically seek medical attention because of chronic respiratory symptoms and/or an exacerbation of their disease

Stage III: Severe COPD - Characterized by further worsening of airflow limitation $FEV_1/FVC < 0.70$; $30\% \leq FEV_1 < 50\%$ predicted, greater breathlessness,

Reduced exercise capacity, fatigue, and repeated exacerbations that usually have an impact on patients' quality of life.

Stage IV: Very Severe COPD - Characterized by severe airflow limitation $FEV_1/FVC < 0.70$; $FEV_1 < 30\%$ predicted or $FEV_1 < 50\%$ predicted plus the presence of chronic respiratory failure. Respiratory failure is defined as an arterial partial pressure of O_2 (PaO_2) less than 8.0 kPa (60 mm Hg), with or without arterial partial pressure of CO_2 ($PaCO_2$) greater than 6.7 kPa (50 mm Hg) while breathing air at sea level. Respiratory failure may also lead to effects on the heart such as cor pulmonale (right heart failure). Patients may have Stage IV: Very Severe COPD even if the FEV_1 is > 30% predicted whenever these complications are present. At this stage, quality of life is very appreciably impaired and exacerbations may be life threatening". COPD is a major cause of death worldwide. It has been anticipated by World Health Organisation that COPD will be the third leading cause of death by 2030 (World Health Organisation 2014). In the United States during 2000 over 23.6 billion adults over 18 years were diagnosed with COPD, resulting in 726.000 hospitalizations and 119.000 deaths (Mannino and Braman 2007). According to the Australian Lung Foundation, in Australia in 2008, over 2 million people were diagnosed with COPD and this number is predicted to climb to 4.5 million by 2050 in Australia. Already, 8.8 Billion Dollars per year is paid by the Australian community for COPD (Foundation 2008).

COPD is an incurable disease and existing treatments are largely symptomatic. COPD patients have an increased risk of bacterial colonisation of the airway, infective exacerbations and of developing lung cancer. Cessation of cigarette smoking is thus critical, however, once COPD is established, smoking cessation alone will not completely reverse the abnormal airway inflammation or improve physiological functioning. COPD mostly affects the aging population; although some reduction in the prevalence of smoking has recently been achieved, the lag time of 20-50 years means that there will be no reduction in incidence of COPD in the near future.

Thus, there is an urgent need for new treatments. Because macrophages are important players in many inflammatory diseases, and COPD is an inflammatory disease, macrophages are likely to be important in this disease. Indeed it has already been shown that the pathogenesis and pathology of COPD is closely linked to macrophage functions. Several important defects in macrophage function have been shown in the airway of patients with COPD (Hodge, Hodge et al. 2003, Taylor, Finney-Hayward et al. 2010). There is increasing evidence for the importance of signalling molecules in shaping macrophage function in many inflammatory diseases.

1.2 Pathogenesis and pathology of COPD

Most of the pathogenesis and pathology of COPD comes from the chronic inflammation in the airway due to stimulation and recruitment of many inflammatory cells including macrophages, neutrophils, dendritic cells, T cells and B cells. These inflammatory cells drive their effect through secretion of a network of inflammatory mediators including proteases, oxidants, chemotactic agents, cytokines, growth factors, lipid mediators and peptide mediators (Barnes 2014). It is proposed that the stimulation of inflammatory responses is initiated by direct contact of pulmonary macrophages and lung epithelial

cells with noxious agents (e.g. cigarette smoke particles) which activate and recruit other cells such as neutrophils, T cells, dendritic cells, and B cells to the lung (Figure 1.1). Other factors that are thought to contribute to the pathogenesis of COPD are protease anti-protease imbalance and oxidants anti-oxidant imbalance (Louhelainen, Rytila et al. 2009, Gorska, Maskey-Warzechowska et al. 2010, Barnes 2014).

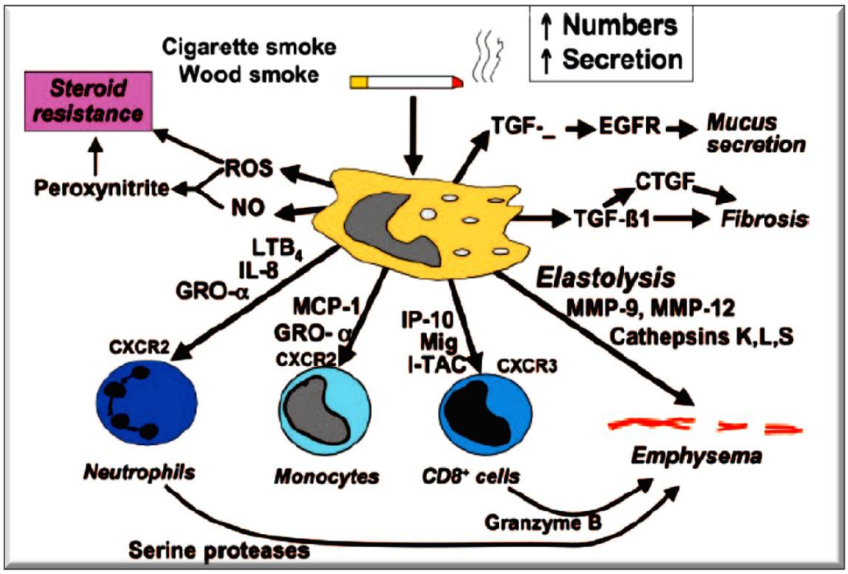

Figure 1.1: The potential roles of macrophages in COPD pathogenesis (Barnes 2004). Cigarette smoke activates alveolar macrophages to initiate inflammatory responses by secreting various cytokines and growth factors that activate and attract other inflammatory cells. Macrophages activate and attract 1) neutrophils via secretion of interleukin (El-Najjar, Chatila et al.)-8, growth related oncogene-a (GRO-a) and leukotriene B4 (LTB4); 2), monocytes via secretion of macrophage chemotactic protein-1 (MCP-1); 3) CD8 cells via secretion of interferon-g inducible protein (IP-10), monokine-induced by interferon-g (Mig) and interferon-inducible T-cell a-chemoattractant (I-TAC). In turn, neutrophil and CD8 cells secrete proteases and granzyme B that causes lung tissue damage. Macrophages also secrete elastolytic enzymes including matrix metalloproteinases (MMP) 9 and 12, and cathepsins which cause elastolysis, and secrete 1) transforming growth factor(TGF)-β1, that stimulates mucus hyper-secretion through the action of epithelial growth factor receptors (EGFR); 2) connective tissue growth factor (CTGF). Macrophages potentially contribute to steroid resistance by production of peroxynitrite via reactive oxygen species (ROS) and nitric oxide (NO).

COPD is a complex disease and characterized by many pathological features, thus investigating actual cells obtained from the airway or lung of COPD patients and comparing these cells to those from normal individuals is the most relevant method to study the disease. However, this is often impractical and alternative disease models that mimic the condition in humans are available to provide accurate information regarding the disease pathogenesis and promote effective therapeutic interventions. In this regard, two main COPD models have been applied; *in vivo* animal models that show several changes consistent with COPD including airway remodelling, chronic inflammation, mucus hypersecretion, emphysema, lung function, and systemic co-morbidities (Fricker, Deane et al. 2014, Vlahos and Bozinovski 2014). Because cigarette smoke is the main risk factor for COPD, it has been used as stimuli to mimic COPD pathogenic and pathological features in these models (Krimmer and Oliver 2011, Thorne and Adamson 2013, Fricker, Deane et al. 2014). *In vitro*models, which utilize various cells models such chronic inflammation, inflammatory / non inflammatory mediators associated with disease pathogenesis, as well as emphysema characteristics are also available (Krimmer and Oliver 2011, Thorne and Adamson 2013).

1.3 Macrophages, biology, development and role

1.3.1 Background

Innate immune response is the first line of defense in both humans and animals. Resident tissue macrophages are key cells of innate immunity and play a major role in tissue hemostasis by immediately initiating an inflammatory response to foreign, non-self-particles, such as pathogens, harmful chemical particles or dead or senescent cells. This response results in clearing the foreign particles (pathogens or other particles) or apoptotic cells. Once these pathogens or particles have been removed, macrophages actively start to regulate the inflammatory response by promoting resolution of the inflammation and repair any damage tissue. If the hemostatic role becomes uncontrolled for any reason, this can contribute to various pathological conditions (Krausgruber, Blazek et al. 2011, Sindrilaru, Peters et al. 2011).

1.3.2 Macrophage development

Macrophages first were discovered and proposed as cells maintaining tissue hemostasis in 1892, by Metchnikoff (Metchnikoff 1892). To date, two major embryonic hematopoiesis of macrophage development have been proposed. The first is primitive hematopoiesis (Samokhvalov 2014), which occurs in early gestation, between 6.5 and 8.5 embryonic days. At this stage, the yolk sac produces limited progenitor cells. Importantly, this stage is not dependent on myb transcription factors (myb-independent) that give hematopoietic progenitor cells the ability to form macrophages from monocytes (Cumano and Godin 2007). This takes place in the yolk sac and gives rise to macrophages without monocytes. The second stage is definitive hematopoiesis (Samokhvalov 2014), which occurs between 8.5 and 10 embryonic days.. This phase

produces macrophages from monocytes (myb-dependent), which require hematopoietic progenitor cell precursors (Cumano and Godin 2007). After birth and in adulthood, macrophages derived from monocytes by monocyte-derived macrophages come from bone marrow (BM) progenitors, myb-dependently.

1.4 Role of macrophages

1.4.1 Role of macrophages in development

Macrophages play an important role in innate immunity and tissue hemostasis, as mentioned above; however, macrophages also play an important role during development by maintaining tissue integrity and remodeling. For example, it has been shown that colony stimulating factor 1 (Csf1) null mutant mice, characterized by absence of the majority of macrophage populations, developed several abnormalities (Pollard 2009), notably, bone remodeling diseases, such as osteoporosis in which bone-reabsorbing macrophages, osteoclasts, are lost. In addition, absence of macrophages from breast mammary gland, kidney and pancreas tissue has been linked to remodeling diseases during developments (Pollard 2009, Stefater, Ren et al. 2011). Macrophage phagocytic function plays a well-known role in innate immunity, and in addition, macrophage phagocytic function plays a key role during development. This role has been demonstrated in various models, for example, the process of erythrocyte maturation, erythropoiesis. In this process, erythroblasts (after mutation and before leaving the bone marrow extrude their nuclei into the surrounding environment where macrophages are readily available to phagocytose these nuclei. If macrophages are absent during this process, remarkably, erythropoiesis is inhibited with subsequent lethality (Kawane, Fukuyama et al. 2001). Furthermore, macrophages also guard hematopoietic egress from the bone marrow by ensuring that cells egressing from the

bone marrow express CD47 ligand (Jaiswal, Jamieson et al. 2009) that prevents the elimination of the cell through the process of phagocytosis, as CD47, which is indication of healthy and normal cell, interact with SIRPα (Jiang, Lagenaur et al. 1999) on macrophages and suppress phagocytosis (Brown and Frazier 2001). Moreover, macrophage phagocytic function is important for maintaining the balance of hematopoietic via eliminating neutrophils and erythrocytes in the spleen and liver. Deficiency in this function has been associated with neutropenia, splenomegaly and reduced body weight (Gordy, Pua et al. 2011). Macrophage efferocytosis (phagocytosis of apoptotic cells) has been shown to play an important role during development, where decreased macrophage numbers has been linked to an increased number of apoptotic cells (Dai, Ryan et al. 2002).

In addition, angiogenesis, formation of new blood vessels, is regulated by macrophages. One example is eye development. Soon after birth, the hyaloid vasculature regresses, and macrophages release an apoptotic signal (Wnt7b), which identifies, activates and instructs vascular endothelial cells (VECs) for apoptosis. The absence of the macrophages or this signal result in persistent survival of these cells, and vascular over-growth occurs (Rao, Lobov et al. 2007).

1.4.2 Role of macrophages in adults

1.4.2.1 Background

Although macrophages play a central role in tissue housekeeping by performing a variety of haemostatic functions in the body, these cells show great variability in phenotypic features and functional activities, according to cellular localization and environmental stimuli. Therefore, macrophages have distinct names according to their tissue localization, such as brain macrophages (microglial cells), bone macrophages

(osteoclasts), lung macrophages (alveolar macrophages and other lung macrophages), histiocytes (connective tissue), skin macrophages (Langerhans cells) and liver macrophages (Kupffer cells). In addition, macrophages can be classified further according to their activation status. Classically activated macrophages ('M1') are involved in mediating host defence mechnisms and antitumor immunity. Alternatively activated macrophages ('M2') are involved in inflammatory suppression and regulating wound healing. Regulatory macrophages (IL-10 macrophages) are involved in inflammatory suppression and act as anti-inflammatory macrophages(Mosser and Edwards 2008)

1.4.2.2 Role of macrophages in innate immunity

During innate immune responses, macrophages play a central role. They perform immune surveillance functions by expressing numerous macrophage recognition receptors, such as pathogen-associated molecular patterns (PAMPs), which recognize pathogen molecules, and danger-associated molecular patterns (DAMPs), which recognize non-pathogen molecules and dead cells. Examples of these receptors are Toll-like receptors, NOD-like receptors, the RIG-I family, lectins and scavenger receptors (Akira, Takeda et al. 2001, Inohara and Nunez 2003, Taylor, Martinez-Pomares et al. 2005). In addition, macrophages play a role in inducing the inflammatory response following recognition of pathogenic or non-pathogenic molecules (Ajuebor, Das et al. 1999, Maus, Koay et al. 2002, Cailhier, Partolina et al. 2005); however, this activation depends on the type of damage caused (Ajuebor, Das et al. 1999) and and how strong it is (Rosas, Liddiard et al. 2008). Another important role of macrophages in innate immune response is that after inflammation induction and during the resolution phase, macrophages play a critical role in wound healing. Macrophages are

rapidly recruited to wounds after platelet degranulation, which means macrophages respond to platelet growth factors. In addition, macrophages are involved in regulating recruitment deposition of extracellular matrix

1.5 Macrophages in health and disease (COPD)

1.5.1 Background

The innate immune response in the human lung plays a major role in lung haemostasis and performs different tasks, ranging from engaging inflammatory responses against foreign particles to the clearance of apoptotic cell material and the resolution of the inflammatory response. Alveolar macrophages are the first line of defence in the airways. They play a key role in the innate and adaptive immune responses (Barnes 2004) and in the pathogenesis of many lung inflammatory diseases, such as COPD and lung cancer. Thus, the elevated numbers of alveolar macrophages that have been reported in the lungs of COPD patients (Gorska, Krenke et al. 2008) is expected if we consider the important role of these cells in the lung immune response. In addition, the strong role of alveolar macrophages in COPD pathogenesis is further highlighted by the finding of cigarette smoke particles in these cells after years of smoke cessation (Marques, Teschler et al. 1997). This finding might also provide the pathogenic mechanism for persistent chronic inflammation after smoking cessation that has been reported in COPD patients.

1.5.2 Phagocytosis of bacteria and apoptotic cells by pulmonary macrophages

Normally, lung cells undergo turn over (cell death, apoptosis) as a process to maintain lung haemostasis and the resolution of an inflammatory response at the resolution phase of the inflammation, as well as under normal physiological conditions. Macrophages in

the lung play a key role in this process by removing these apoptotic cells (a process termed ‘efferocytosis’). When cells enter the apoptotic state, they share and become smaller in size, then their chromatin condensate and finally, apoptotic bodies with intact cell membranes form. Clearance of these cells by macrophages can be divided into several stages: recognition of apoptotic cells (responding to ‘come and get me’ signals), the activation of signalling molecules molecules on the surface of the apoptotic cells and specific receptors in macrophages. A clear example of this is phosphatidyl serine, which is present in the outer member surface of apoptotic cells and recognised by alveolar macrophage-specific recognition molecules. This process is also accompanied by the secretion of anti-inflammatory mediators IL-10 and transforming growth factor (TGF)-β1 by the macrophages that inhibit the secretion of pro-inflammatory mediators by other immune cell types and activate tissue repair machinery for the damaged area (Fadok, Bratton et al. 1998). Any defect in this process will lead to an accumulation of these apoptotic bodies that may eventually undergo secondary necrosis and release their toxic cell contents into the surrounding tissue area, further augmenting the ongoing inflammation and preventing the resolution of inflammation (Hagimoto, Kuwano et al. 1997, Kuwano, Hagimoto et al. 1999). Thus, macrophage efferocytosis is likely to play a key role in the pathogenesis of chronic inflammatory lung diseases, such as COPD.

In addition to the role of alveolar macrophages in apoptotic cell clearance, macrophages also play a major role in defence against the invasion of pathogens, while also maintaining sterility and haemostasis of the lung. Although the process of the phagocytosis of bacteria shares a similarity with efferocytosis, its distinct mechanism also involves several steps: recognition of the invading microbe as a foreigner, usually the serum coating of the microbe (process term “opsonisation”), followed by the activation of complement receptors or, alternatively, immunoglobulin receptor activation with active signalling pathways that eventually lead to pathogen engulfment.

However, because airways are not a serum-rich environment, a second non-opsonising mechanism is predominant. It usually involves the recognition of the microbe by specific receptors called scavenger receptors; most noticeable scavenger receptors for alveolar macrophages are macrophage receptors with collagenous structure (MARCO) (Arredouani, Yang et al. 2004, Arredouani, Palecanda et al. 2005). Other scavenger receptors that have been linked to alveolar macrophages are the mannose receptor (CD206), CD136, CD163 and CD36 (Palecanda and Kobzik 2001, Stuart and Ezekowitz 2005). After the recognition of a foreign pathogen by these receptors, they activate singling pathways that lead to bacteria engulfment. An interaction between efferocytosis and bacteria phagocytosis has been suggested (Martin, Peters et al. 2014), and there is growing evidence that efferocytosis plays a role in controlling bacterial infection (Martin, Peters et al. 2014). A clear example for this interaction is mycobacterium tuberculosis, a highly successful intercellular organism that survive in macrophage and avoid immune clearance (Behar, Divangahi et al. 2010), However, for non-obvious reason some macrophages undergo apoptosis after engulfment of this organism, which successfully cleared by efferocytosis, this mechanism has been shown to suppress bacterial growth (Levy, Khan et al. 2009, Schweitzer, Hatoum et al. 2011, Kamocki, Van Demark et al. 2013). Thus, efferocytosis has additional role in bacterial infection control, besides its role in apoptotic cell removal, which demonstrates the strong and important influence of macrophage efferocytosis on overall lung haemostasis.

1.6 Sphingolipids

1.6.1 Sphingolipids: Overview

Nearly 10^9 bioactive lipid molecules have been identified thus far, among them, Sphingolipids. Sphingolipids are integral components of the cell membrane and are

present in nearly all cells. Although their exact function is not fully understood, they have become known recently as molecules that are important mediators of many cell biological functions, such as stress response, apoptosis innate and adaptive immunity and vascular integrity. The most studied and well-known sphingolipid molecules are sphingosine 1-phosphate (S1P) and ceramide. Sphingosine is the building block of sphingolipid synthesis by either phosphorylation to produce S1P or acylation to produce ceramide. S1P metabolism has a complex network of reaction, enzymes and intermediate products (Figure 1.2). S1P is one of the most studied sphingolipid molecules and has been implicated in many diseases. S1P is synthesised by phosphorylation of sphingosine by two enzyme isoforms: sphingosine kinase 1 (*SPHK1*) and sphingosine kinase 2 (*SPHK2*), and it is degraded by two enzymes, reversibly by S1P phosphatases with covert S1P back to sphingosine or irreversibly by S1P lyase (Mendelson, Evans et al. 2014). Although these two enzymes produce the same product, S1P, they have an opposing functional role in the cell. *SPHK1* is activated by various stimuli, such as growth factors and cytokines. The expression of *SPHK1* has been shown to promote cell survival and growth (Olivera, Kohama et al. 1999, Xia, Gamble et al. 2000, Olivera, Rosenfeldt et al. 2003). On the other hand, *SPHK2*, which is lesser known, has been shown to promote apoptosis and growth arrests (Jessup, Bonder et al. 2011, Chan and Pitson 2013, Neubauer and Pitson 2013)

The importance of S1P is evident by its diverse role in many biological cell functions, such as survival (Cuvillier, Pirianov et al. 1996), apoptosis and cell growth (Zhang, Desai et al. 1991, Olivera and Spiegel 1993). Ceramide has an opposing role to S1P (Spiegel, Foster et al. 1996, Huwiler, Kolter et al. 2000, Young, Kester et al. 2013), promoting cell apoptosis, growth arrest and apoptosis (Mathias, Pena et al. 1998); thus, their balance is important to maintaining the S1P/ ceramide ratio (Spiegel, Foster et al.

1996, Huwiler, Kolter et al. 2000, Young, Kester et al. 2013). S1P produced by *SPHK1* is transported outside the cell to bind to four G-coupled receptors, *S1PRs* 1-5, and these receptors have been shown to be partially redundant and cell-specific. They are mostly involved in cell migration, during inflammation, in which S1P production increases in the inflamed tissue, and a S1P gradient is established to lead to the activation and migration of the immune cells to the inflammation site.

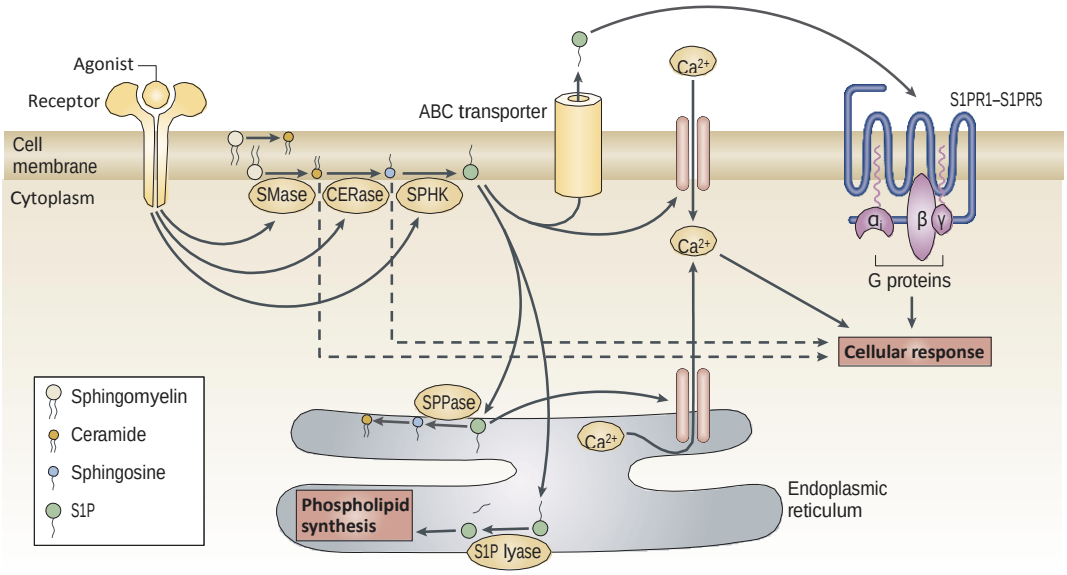

Figure 1.2: Sphingosine 1-phosphate (S1P) metabolism (Rivera, Proia et al. 2008)

Stimulation of various type of cell membrane receptors activates sphingomyelinases (SMase), which produce ceramide by cleavage of sphingomyelin; ceramide is then used as intermediate for production of sphingosine by ceramidases (CERase); sphingosine is phosphorylated to form sphingosine 1-phosphate (S1P) via sphingosine kinases (*SPHK*). Synthesised S1P can act as intercellular second messenger or can be transported outside of the cell by ABC transporters to bind a family of five plasma membrane G-protein-coupled receptors *S1PRs* (1–5) that have shown to be cell specific. S1P can be degraded by S1P lyase or converted back to sphingosine by S1P phosphatases (SPPase). S1P can affect calcium (Ca2+) fluxes in the cell.

1.6.2 Sphingolipids in health and lung diseases

Current data clearly imply the importance of sphingolipids in various cellular process and tissue haemostasis. Most knowledge of sphingolipids comes from S1P and ceramide, the most extensively studied sphingolipid components. Ceramide plays a central role in sphingolipid metabolism, giving rise to all other sphingolipid molecules. Ceramide is activated by various cellular stressors such as radiation, ischemia and reperfusion, chemotherapeutic agents, inflammatory mediators and growth factor (Hannun 1996, Nikolova-Karakashian and Rozenova 2010). In addition, ceramide promotes apoptosis and cell growth arrest (Hannun and Obeid). In contrast, S1P, which also plays a key role in maintaining cellular homeostasis, has an opposing role and functions. It promotes survival, migration, cell-to-cell adhesion and proliferations (Olivera and Spiegel 1993, Pyne and Pyne 2000). These opposing roles imply the importance of the balance between these mediators in determining proper cellular function and fate through this dynamic balance (rheostat) (Spiegel, Foster et al. 1996, Huwiler, Kolter et al. 2000, Young, Kester et al. 2013). Numerous disease models have been linked to abnormal sphingolipids. In the lungs, unbalanced sphingolipid levels have been associated with lung injury, either due to irradiation (Mathew, Jacobson et al. 2011), hyperoxia (Tibboel, Joza et al. 2013) or cigarette smoke (Levy, Khan et al. 2009, Schweitzer, Hatoum et al. 2011, Kamocki, Van Demark et al. 2013). In addition, abnormal sphingolipids have been shown to play a role in cystic fibrosis (Becker, Tummler et al. 2010), asthma (Sawicka, Zuany-Amorim et al. 2003, Idzko, Hammad et al. 2006) and COPD (Petrache and Petrusca 2013).

1.7 S1P system

1.7.1 S1P system: Overview

S1P is present mostly in plasma and tissue, with plasma concentrations several times higher than tissue concentrations. This concentration difference maintains a gradient between tissue and plasma, which is important for body homeostasis and proper functioning of bodily organs. Several sources contribute to S1P plasma levels, including platelets, erythrocytes and vascular endothelial cells (ECs). In plasma, S1P is bound to protein carriers, which are involved in determining S1P levels. These protein carriers include high-density lipoprotein (HDL), which carries about 60% of S1P, and albumin proteins, which carry about 30%. Small amounts are carried by low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) (Argraves and Argraves 2007). Although S1P can act intracellularly and extracellularly, the majority of its biological functions are attributed to it extracellular secretions via binding to specific G-protein-coupled receptors *S1PRs* (1–5) (Blaho and Hla 2014). Although all these receptors are expressed in bodily tissues, *S1PR1* and *S1PR3* are more widely expressed (Regard, Sato et al. 2008). In addition, expression and function of each of these receptors is cell specific. Upon ligation of S1P with its target receptor, it will activate signal transduction downstream from the targeted receptor, resulting in cellular responses (Chun, Hla et al. 2010, Blaho and Hla 2014). Maintaining the S1P gradient difference between tissue and plasma requires a tight control mechanism. S1P has been shown to play a key role in this mechanism, as it absence results in increased S1P levels in the tissue (Schwab, Pereira et al. 2005, Allende, Bektas et al. 2011).

1.7.2 S1P system: Role in lung biology

Endothelial cells lining the vasculature regulate a variety of functions, such as vascular tone, blood coagulation, inflammation, angiogenesis, and tissue fluid homeostasis (Garcia, Liu et al. 2001, Mehta and Malik 2006, Wang and Dudek 2009). S1P's role in the lungs include its effects on lung structural cells, endothelial cells and other immune cells. S1P also mediates *S1PR1*, *2* and *3*,expression which have been shown to regulate vascular development and function (Kono, Mi et al. 2004, Mendelson, Zygmunt et al. 2013). In adult mice lacking plasma S1P, vascular leakage occurs; this leakage can be reversed with a *S1PR1* agonist, implying the important role of S1P. Similarly, S1PR2 on mice lung endothelial cells has been shown to be important in vascular integrity by inhibiting endothelial nitric oxide synthase (Mathew, Jacobson et al.) (Cui, Okamoto et al. 2013). The role of S1P in endothelial function is mediated by HDL-associated apolipoprotein M in mice and humans (Christoffersen, Obinata et al. 2011). Extracellular S1P regulates human endothelial cell motility via the *S1PR1* axis. This process is regulated in part by SPHK1 and S1P lyase (Berdyshev, Gorshkova et al. 2011). Repair after injury to lung structural cells is a process essential to lung haemostasis. Recently, it has been shown that this process is regulated by S1P/*S1PR2* via its effect on human lung fibroblast chemotaxis (Hashimoto, Wang et al. 2008).

S1P plays a further major role in lung immune responses by affecting the functions of various immune cells required for innate and adaptive immune response. One study showed that activation of S1P/*S1PR2* in murine mast cells regulated T cell infiltration in lung (Oskeritzian, Hait et al. 2015), while a further study showed that S1P had a homeostatic role in LPS-induced acute lung injury in a murine model (Zhao, Gorshkova et al. 2011). There is a growing body of evidence of the role of S1P in alveolar macrophage phagocytosis (McQuiston, Luberto et al. 2010, McQuiston, Luberto et al. 2011, Farnoud, Bryan et al. 2015). Immune cell adhesion is a critical process in immune

response. A recent study demonstrated that S1P regulates eosinophil adherence to human lung epithelium (Sashio, Kume et al. 2012). In addition, involvement of S1P in mediating inflammatory secretions has been investigated in the lung, where activation of the SPHK1/S1P/S1P1 signalling pathway in response to inflammatory mediators in endothelial cells regulated endothelial barrier homeostasis (Tauseef, Kini et al. 2008). Similarly, S1P promoted IL-8 secretion and proliferation of human lung epithelial cells in vitro. In addition, the supernatant of these S1P-treated cells increased neutrophil chemotaxis, further implicating S1P in cell-cell interactions (Milara, Mata et al. 2009).

1.8 Potential role of the Sphingosine-1-phosphate (S1P) system in defective macrophage function in COPD.

1.8.1 S1P system: a potential cause of macrophage dysfunction in COPD.

S1P acts by binding a family of five G-protein-coupled receptors known as S1P receptors (*S1PR1–S1PR5*). *S1PR1*, 2 and 3 have been identified as major contributors in macrophage function (Kuehnel, Reiss et al. 2009, Michaud, Im et al. 2010). Moreover, it has been shown the *S1PR2* plays an important role in the ability of alveolar macrophages to phagocytose Cryptococcus neoformans (McQuiston, Luberto et al. 2011). Our lab also has reported that *S1PR5* is decreased in the lung tissue of COPD patients (Cordts, Pitson et al. 2011). However, to date, no studies have fully addressed and characterized this system in airway/lung tissue macrophages of COPD patients.

1.8.2 S1P system: potential effect on macrophage phenotype

Macrophages are a heterogeneous population of cells with significant phenotypic plasticity. Depending on the microenvironment they may undergo reprogramming to acquire polarised phenotypes that have broadly been classified as classically activated

'M1' (pro-inflammatory; regulation of antigen presentation) or alternatively activated 'M2' (poor antigen presentation; improved efferocytosis). This is a growing area of research that could lead to selective targeting of the appropriate macrophage population or promoting phenotype switching in diseases including COPD. Our group has shown that treating COPD patients with the macrolide antibiotic, azithromycin improves efferocytosis function of Alveolar macrophages (Hodge, Hodge et al. 2008). This improvement was associated with a change in macrophage activation suggestive of an 'M2' phenotype (Hodge, Hodge et al. 2008) although these results are exciting, the use of long-term antibiotic therapy has obvious draw-backs and identification of more downstream targets are warranted.

Both S1P and *SPHK* may play a role in macrophage polarisation and have been shown to mediate an increase in the ratio of 'M2' activated macrophages mediated by interaction with *S1PR1* (Rivera, Proia et al. 2008). *SPHK1* is likely to be more important in this regard than *SPHK2*, as *SPHK1* deficiency enhanced LPS induced production (a 'M1' function) despite the presence of *SPHK2* (Schroder, Richter et al. 2011). We have reported that alveolar macrophages from COPD patients are a mixed phenotype, with an 'M1' predominance (Hodge, Matthews et al. 2011), thus, we would expect that S1P and *SPHK* and associated receptors may be involved in these cells regulation and a target for therapeutic intervention.

1.8.3 S1P system: potential effect on reorganization of the actin skeleton and activation of Rac that occurs during the efferocytosis

The signaling pathways elicited by the various phagocytic systems (AFcg receptor, complement receptor, or efferocytosis) are still under active investigation; however, all systems have been shown to involve activation of cytoskeletal-remodeling molecules.

Efferocytosis has been shown to occur with extensive pseudopod extensions, and the early steps in signaling after interaction with apoptotic cells are dependent on activation of Rac, a GTPase of the Rho family and downstream actin polymerization and formation of lamellipodia. The effectiveness of efferocytosis is mediated by a tight balance between the expression of Rac and Rho. The S1P system is likely to be important in these processes as (a) increased macrophage expression of S1P is involved in phagosome maturation (b) *S1PR2* couples mainly to $G_{12/13}$ to activate Rho and inhibit Rac and is therefore likely to play an inhibitory role (c) TNFα (which is increased in COPD and has been shown to be activator of *SPHK*) inhibits efferocytosis in human and murine macrophages through an oxidation dependant pathway and activation of Rho, which is known to be important in efferocytosis (McPhillips, Janssen et al. 2007). Second, it has been shown that cigarette smoke mediates activation of the RhoA–Rho kinase pathway in an oxidation dependant (Richens, Linderman et al. 2009). Taken together these findings demonstrate a potential role for the S1P system in the inhibitory effect of TNFα, Rho, oxidative stress, and cigarette smoke on macrophage function and suggest a potential link between pulmonary macrophage phagocytic ability and S1P system in COPD.

1.8.4 Epigenetic regulation of the S1P signalling system and potential role in defective macrophage functions in COPD.

Multicellular organisms have tissue specific gene expression even though we have identical genotype in all tissue cells in the body. The specificity in gene expression is maintained by a mechanism termed “epigenetic modification". Epigenetic is defined as “heritable change in DNA which can be transmitted from one generation to another without interfering with DNA sequence (Yang and Schwartz 2011).

Importantly, this mechanism is affected by different environmental stimuli such as diet, smoking, radiation and diseases. Therefore, exposure to environmental stimuli could lead to shaping gene expression during development or adulthood and this could lead to serious diseases. There are three main epigenetic changes in our body. The first is DNA methylation, which is carried out by DNA methyltransferases (DNMTs) enzymes which carry out the methylation of DNA in CpG islands in the promoter region of the gene.

The second epigenetic modification is histone modification by histone acetyltransferases (HATs), deacetylation by histone deacetylases (HDACs), histone methylation by histone methyltransferases (HMTs), and histone demethylation by histone demethylases (HDMs). The third is non-coding RNAS, mostly MicroRNAs that bind to 3` untranslated regions of messenger RNA (mRNA) that result in untranslatable mRNA. As epigenetic modifications can be the result of cellular response to oxidative stress, diet, disease and environmental stimuli and because cigarette smoke is the main risk factor in COPD, this disease could be regulated by epigenetic mechanisms. Some studies have already reported epigenetic changes in COPD. For examples, HDACs, the key enzymes in chromatin remodelling have been shown to be reduced in COPD (Ito, Ito et al. 2005). Moreover, it has been demonstrated that HDAC2 and HDAC1 are associated with the *SPHK2*/S1P system (Hait, Allegood et al. 2009). In macrophages it has been shown that function could be epigenetically regulated (Takeuch and Akira 2011). Although epigenetic changes in airway macrophages in this disease have not been described, these findings strongly suggest epigenetic role S1P system in pulmonary macrophage function in COPD.

1.9 Summary and project rational.

COPD is chronic inflammatory disease and anticipated to be the third leading cause death. In addition, COPD is major risk factor for lung cancer independent of smoking history. The treatment available for COPD is ineffective in full disease management. Thus, there is an urgent need for better understanding of disease pathogenesis in order to develop new effective treatments. Defective alveolar macrophages efferocytosis has been considered to play a key role in disease progression and development, potentially by increasing the accumulation of apoptotic cells and bacterial colonization in the airways of COPD patients, which in turn can promote inflammation and increased oxidative stress that eventually lead to lung damage. The exact mechanism for this defect is still unknown, despite the identification of numerous molecules as potential candidates, which suggest that a network of molecules rather than single molecules may cause this defect. In addition, many of the molecules identified so far have been extrinsic macrophage receptors. The overall hypothesis of this thesis is therefore that network of closely molecules in pathways such as the S1P system may be involved in the phagocytic defects of alveolar macrophages in COPD.

The metabolism of S1P is tightly regulated in by complex network of proteins and molecules, and the S1P system has been implicated in many cell functions, including regulation of macrophage trafficking, migration, phenotype polarization and phagosome maturation; thus, it is likely that that the defective macrophages phagocytic capacity in COPD may be linked to dysregulation of the S1P system.

The work presented in this thesis used novel approaches to determine the candidate molecules of defective alveolar macrophage efferocytosis, by firstly evaluating the whole S1P system in alveolar macrophages from COPD patient and healthy smokers, and controls. The effects of a novel therapeutic agent that is able to modulate the S1P

system in order to increase macrophage efferocytic ability was then investigated. Finally, the potential role of epigenetic modification (such as DNA methylation) in alveolar and lung tissue macrophages, and its effect on efferocytosis and expression of S1P system was investigated.

The overall Aims of the thesis are:

1. To characterise expression and function of the S1P system in human alveolar macrophages in COPD using primary airway samples.

2. To target the S1P system in airway macrophages with a candidate therapeutic agent *in vitro.*

3. To determine the possible role of epigenetic modification, such as DNA methylation, in the regulation of macrophage efferocytosis and expression of S1P system genes, in human alveolar macrophages and lung tissue macrophages in COPD.

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CHAPTER 2

Potential link between the sphingosine-1-phosphate (S1P) system and defective alveolar macrophage phagocytic function in chronic obstructive pulmonary disease (COPD).

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Abstract

We previously reported that alveolar macrophages from patients with chronic obstructive pulmonary disease (COPD) are defective in their ability to phagocytose apoptotic cells, with a similar defect in response to cigarette smoke. The exact mechanisms for this defect are unknown. Sphingolipids including ceramide, sphingosine and sphingosine-1-phosphate (S1P) are involved in diverse cellular processes and we hypothesised that a comprehensive analysis of this system in alveolar macrophages in COPD may help to delineate the reasons for defective phagocytic function. **Methods:** We compared mRNA expression of sphingosine kinases (*SPHK1/2*), S1P receptors *(S1PR1-5)* and S1P-degrading enzymes (*SGPP1*, *SGPP2* and *SGPL1*) in bronchoalveolar lavage-derived alveolar macrophages from 10 healthy controls, 7 healthy smokers and 20 COPD patients (10 current- and 10 ex-smokers) using Real-Time PCR. Phagocytosis of apoptotic cells was investigated using flow cytometry. Functional associations were assessed between sphingosine signalling system components and alveolar macrophage phagocytic ability in COPD

To elucidate functional effects of increased *S1PR5* on macrophage phagocytic ability, we performed the phagocytosis assay in the presence of varying concentrations of suramin, an antagonist of *S1PR3* and *S1PR5.* The effects of cigarette smoking on the S1P system were investigated using a THP-1 macrophage cell line model. **Results:** We found significant increases in *SPHK1/2* (3.4- and 2.1-fold increases respectively), *S1PR2* and *5* (4.3- and 14.6-fold increases respectively), and *SGPL1* (4.5-fold increase) in COPD vs. controls. *S1PR5* and *SGPL1* expression was unaffected by smoking status, suggesting a COPD "disease effect" rather than smoke effect *per se*. Significant associations were noted between *S1PR5* and both lung function and phagocytosis. Cigarette smoke extract significantly increased mRNA expression of *SPHK1*, *SPHK2*, *S1PR2* and *S1PR5* by THP-1 macrophages, confirming the results in patient-derived

macrophages. Antagonising *S1PR5* significantly improved phagocytosis. **Conclusion:** Our results suggest a potential link between the S1P signalling system and defective macrophage phagocytic function in COPD and advise therapeutic targets.

Introduction

Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality worldwide. Cigarette smoking is a major cause of COPD, yet despite the huge campaign that encourages smoking cessation worldwide, the smoking incidence is only slowly decreasing in developed countries and still increasing in developing countries. COPD is an incurable disease and currently available treatments are largely ineffective (Pauwels, Lofdahl et al. 1999, Pauwels, Buist et al. 2001). There is therefore an urgent need for further understanding the pathophysiology of COPD to advise effective new therapies.

In previous studies our group has shown that alveolar macrophages from COPD patients are defective in their ability to phagocytose apoptotic cells despite smoking cessation (defective efferocytosis) (Hodge, Hodge et al. 2003, Hodge, Hodge et al. 2007, Hodge, Hodge et al. 2008, Hodge, Matthews et al. 2010, Hodge, Matthews et al. 2011). We and others have shown that if these apoptotic cells are not cleared effectively by alveolar macrophages, they may undergo secondary necrosis which can further promote the inflammation in the lung (Hart, Haslett et al. 1996, Bertelmann, Knapp et al. 2003).We have also shown that both alveolar macrophages and monocyte-derived macrophages from COPD patients are impaired in their ability to phagocytose bacteria which might potentially contribute to bacterial colonization in COPD (Hodge and Reynolds 2012). Numerous molecules have been identified as possible contributors to these macrophage phenomena in COPD (Mukaro and Hodge 2011), but the exact mechanism is yet unknown.

Sphingolipid metabolites including ceramide, sphingosine and sphingosine-1-phosphate (S1P) are involved in diverse cellular processes. Phosphorylation of sphingosine by the sphingosine kinases (*SPHK*s) results in the production of S1P, while its acetylation by ceramide synthase produces ceramide, and then sphingomyelin after sphingomyelin synthase catalysed coupling to phosphocholine. Structural changes in these molecules have been proven to result in modulation of cellular function (Uhlig and Gulbins 2008). Many sphingolipid molecules have been linked to inflammatory lung diseases and used as potential therapeutic targets, such as acid sphingomyelinase in acute lung injury (Kuebler, Yang et al. 2010), neutral sphingomyelinase-2 in COPD (Filosto, Castillo et al. 2011), and ceramide in cystic fibrosis (Becker, Riethmuller et al. 2010).

We have previously shown that human lung tissue comprises a complex expression profile for the individual components of the S1P signalling system including synthesising enzymes, receptors and degrading enzymes (Cordts, Pitson et al. 2011). In COPD, we found correlations between mRNA expression levels of several receptors and enzymes involved in the S1P signalling system in the lung suggesting common regulatory mechanisms. However, we did not assess individual lung cells such as alveolar macrophages, which are likely to be of importance in COPD. S1P is one of the most important sphingolipid molecules and its role in immune cell function has been shown in many studies (Rivera, Proia et al. 2008, Spiegel and Milstien 2011). It has been shown to be involved in a multitude of cellular signalling pathways and responses, such as proliferation, survival and growth (Spiegel and Milstien 2003) and exerts its function through five G-protein coupled receptors (*S1PR1-5*) (Sanchez and Hla 2004).

The alveolar macrophage is a very interesting target for our investigations since it has been shown that S1P regulates macrophage function, phagosome maturation and migration (Weigert, Weis et al. 2009).The phagocytic function of macrophages has also been shown to be modulated by the sphingosine system; for example, it has been shown

that phagocytosis of *M.tuberculosis* bacteria depends on *SPHK1* (Bauer, Hatzivassiliou et al. 2005). S1P which has been implicated in macrophage actin assembly and phagosome function (Treede, Braun et al. 2007) was shown to improve phagocytosis of *Cryptococcus neoformans* when exogenously added to wild-type alveolar macrophages (McQuiston, Luberto et al. 2011). Furthermore, ceramide, the precursor of S1P, has an opposing role to S1P and has been found to decrease macrophage efferocytosis in COPD (Petrusca, Gu et al. 2010). Taken together, these studies suggest that defective efferocytosis in alveolar macrophages in COPD may be associated with the S1P system. Despite the numerous studies on this system and its role in inflammation and diseases, to the best of our knowledge, there have been no comprehensive studies of the S1P system in macrophages from healthy controls, or of the role of this system in the defective macrophage function in COPD. In this study, we compared the expression of components of the S1P-signalling system in alveolar macrophages from healthy control volunteers and COPD patients. We determined the expression profiles of *SPHK1*, *SPHK2*, receptors (*S1PR1*-5) and the S1P-degrading enzymes, sphingosine-1-phosphate phosphatase 1 and 2 (*SGPP1* and *SGPP2*) and sphingosine-1-phosphate lyase 1 (*SGPL1*) and their correlation with the ability of alveolar macrophages to phagocytose apoptotic cells. We further investigated functional consequences of smoking on the expression profile of the S1P system in current- or ex-smoking COPD subjects and by using an *in vitro* THP-1 macrophage cell line model.

Material and methods:

Categorization of patients

This study was approved by the Royal Adelaide Hospital Ethics Committee (Adelaide, Australia) and informed written consent was obtained for each subject in this study.

Patients were categorized based on gender, smoking status, age, the presence of lung cancer and lung function (Table 1). The diagnosis of COPD was carried out according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) standards. Any subject who had ceased smoking within the previous 6 months was included in the 'current smoker' groups. Exclusion criteria included diagnosis of other inflammatory lung diseases, blood malignancy and current infection.

Table 1: Patients demographic

	Controls	Control Cur-Smoker	COPD	COPD Cur-smoker	COPD Ex-Smoker
Number (n)	10	7	20	10	10
Age (yr)	56.5(26-71)	36(28-46)	66.5(42-86)	60(42-75)	74(49-86)
Smoking(cur/ex/no)	0/6/4	7/0/0	10/10/0	10/0/0	0/10/0
Gender (M/F)	6/4	2/5	14/6	5/5	9/1
Lung Cancer (y/n)	0/10	0/7	10/10	4/6	6/4
Type of Lung Cancer (NSCLC/SCLC)	0/0	0/0	10/0	4/0	6/0
Type of NSCLC (adenos /squa/ larg)	0/0	0/0	3/7/0	1/3/0	2/4/0
Radiation therapy (cur/pre/no)	0/0/10	0/0/7	1/2/7	0/1/3	1/1/4
Chemotherapy (cur/pre/no)	0/0/10	0/0/7	0/1/9	0/0/4	0/1/5
Glucocorticoid treatment (y/n)	0/10	0/0/7	0/0/10	0/0/4	0/0/6
Pky	4(0-28)	17.5(4-35)	40(10-75)*	50(10-50)*	25(17-75)*
FEV_1	106(71-120)	97(82-107)	76.5(47-113)*	78(47-113)*	76(67-90)*
FEV_1/FVC ratio	80(70-88)	84(72-87)	63(49-69)*	62(50-67)*	64(49-69)*

Data are presented as number, or median and data range. COPD: chronic obstructive pulmonary disease;

Smoking history: cur: current, ex: ex-smokers, no: never smoked; Lung Cancer: presence of lung cancer; NSCLC: Non-small cell lung cancer; SCLC: Small cell lung cancer; Type of NSCLC: adeno: adenocarcinoma, squa: squamous cell carcinoma, larg: large cell carcinoma; Radiation therapy: cur: current, pre: previous exposure, no: never exposed; Chemotherapy: cur: current, pre: previous exposure, no: never exposed; Pky: smoking pack years;fev1: forced expiratory volume in one second, % pred: percentage of predicted; FVC : forced vital capacity. *: significant difference from control group, $p<0.05$. Note that one COPD patient was classified as 'mild COPD', with a FEV_1/FVC of 67.

Bronchoalveolar lavage (BAL) and isolation of macrophages

Bronchoscopy was performed according to American Thoracic Society recommendations for the performance of bronchoscopy for investigative purposes, as previously described (Hodge, Hodge et al. 2005). BAL was collected then centrifuged at 1600 RPM for 5 min and the supernatant frozen for later use. Macrophages were isolated by adhering to plastic as previously described, concentration adjusted to 4×10^5 cells/ml (Hodge, Hodge et al. 2003) then either used for phagocytosis or frozen immediately at -80°C for gene expression analysis.

Cell culture and stimulation

THP-1 monocytes (ATCC, Manassas, VA, USA) were differentiated into macrophages by seeding the cells at a density of 4×10^5 cells/mL in 24-well plastic plates, then stimulating with 100 nM phorbol 12-myristate 13-acetate (Herbst, Heymach et al.) in

RPMI 1640 medium at 37°C with 5% CO_2 for 72 h as previously described (Dehle, Mukaro et al. 2013). Experiments were carried out between passages 6 and 20.

Exposure of THP-1 macrophages to cigarette smoke extract was carried as previously described (Hodge, Hodge et al. 2004, Hodge, Hodge et al. 2006, Hodge, Hodge et al. 2007, Hodge, Hodge et al. 2008). After cigarette smoke extract exposure, non-adherent cells were discarded, and adherent cells were incubated with 500 μL cold PBS for 5 min, then harvested by scraping the adherent cells with a plastic scraper into a 1.5 mL tubes prior to centrifugation at 4°C at 1600 RPM. Supernatant was removed and cell pellet stored at – 80°C for mRNA expression analysis.

Reverse transcription and quantitative real-time PCR (qPCR)

The total RNA was extracted from snap frozen alveolar macrophages or THP-1 macrophages using the RNeasy Mini Kit (Qiagen, Doncaster, Australia) according to the manufacturer's instructions. The concentration of total RNA was determined using standard photospectometry. The RNA quality expressed as RIN number was determined using the RNA Pico-Chip and Agilent 2100 Bioanalyzer (Agilent Technologies, Forest Hill, Australia). Complementary DNA (cDNA) was synthesized using RT² HT First Strand Kit (Qiagen). Synthesized cDNA was stored at −20°C. The qPCR analyses of the samples were performed using Custom RT2 Profiler PCR arrays (Qiagen) and the ABI sequence detection system (StepOne Plus) as previously described (Cordts, Pitson et al. 2011), with modifications. Briefly, a 25 μL reaction mixture into 96-well plates coated with primer pairs directed against components of the S1P system (*SPHK1* and *SPHK2*, S1P receptors (*S1PR1*, *S1PR2*, *S1PR3*, *S1PR4* and *S1PR5*) and the S1P-degrading enzymes *SGPP1*, *SGPP2* and *SGPL1*. Three reference genes, glucose 6-phosphate dehydrogenase (*G6PD*), hypoxanthine phosphoribosyl transferase 1 (*HPRT1*) and ribosomal protein L13a (*RPL13A*) were used in the array for data normalization. The

efficiency and reliability of the PCR reaction was assessed by determining the efficiency of the reverse transcriptase reaction by Reverse-transcription control (RTC) determining, the efficiency of polymerase chain reaction itself by positive PCR control (PPC), and DNA contamination in the reaction by genomic DNA control (HGDC).

Functional associations between sphingosine signalling system components and alveolar macrophage phagocytic ability in COPD

Phagocytosis of apoptotic cells was measured as previously reported (Hodge, Hodge et al. 2003, Hodge, Hodge et al. 2007, Tran, Ahern et al. 2014). Briefly, 16HBE bronchial epithelial cell targets were maintained in continuous culture, induced to apoptosis using UV, then labelled with sytox orange (Molecular Probes, Oregon, USA). The apoptotic cells were incubated with macrophages at a ratio of 10:1 for 1.5 h. Non-adhered cells were removed and macrophages removed by gentle pipetting, following 5 min incubation with 500 uL ice-cold phosphate buffered saline (PBS). Macrophages that had ingested apoptotic cells were stained with CD13 phycoerythrin cyanine-7 (PE-Cy7) (BD Biosciences, San Jose CA, USA), autofluorescence was quenched with trypan blue and 30,000 total events per tube were acquired immediately using a FACSCanto II Flow Cytometer (BD Biosciences). Macrophages were identified based on autofluorescence properties and staining with CD13. The percentage of the macrophages ingesting apoptotic cells was recorded.

To further elucidate functional effect of increased *S1PR5* on macrophage phagocytic ability, we performed the phagocytosis assay in the presence of varying concentrations of Suramin (Sigma Aldrich, Castle Hill, NSW, Australia), an antagonist of *S1PR3* and *S1PR5* (Miron, Jung et al. 2008). Suramin at concentrations of 10 nM to 10 µM was added for 30 min prior to the phagocytosis assay.

Statistical analysis

Analysis was performed using SPSS statistic software (SPSS Inc. IBM Chicago, USA) and the two-sample Wilcoxon rank sum test, or the Kruskal-Wallis test for analyses of more than two groups. Correlation with lung function, smoking, age, gender and presence of lung cancer on mRNA expression levels of S1P system genes were determined using data from all subjects and Pearson's correlation coefficient with significance set at $p<0.05$.

Results

Characterization of expression profile of the S1P system in alveolar macrophages in healthy subjects

The mRNA expression levels of the components of the S1P system in alveolar macrophages from healthy individuals are unknown. We therefore compared the relative mRNA expression levels of the S1P system genes in alveolar macrophages obtained from healthy control subjects (n =10, Figure 1). All components of the S1P system were expressed in alveolar macrophages. We found no significant difference in the relative mRNA expression levels between the S1P synthesizing enzymes, *SPHK1* and *2*. In contrast, the S1P degradation enzymes, *SGPP1*, *SGPP2* and *SPGL1* were differentially expressed. *SGPP1* showed the highest, while *SGPP2* had the lowest relative expression level. There were large differences in relative-mRNA expression levels among S1P receptors, with *S1PR4* being the predominant subtype while *S1PR5* showed the lowest expression level. The rank order of mRNA expression levels of all receptors from the high to low was *S1PR4>S1PR2>S1PR1>S1PR3>S1PR5*.

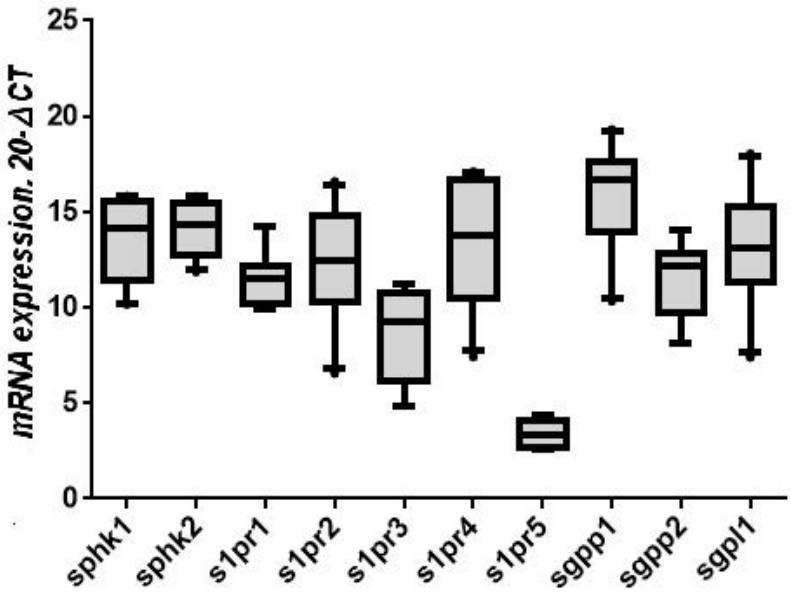

Figure 1: Relative mRNA expression levels of S1P system genes in alveolar macrophages across normal subjects (N=10). Box plots present median ± 25th and 75th percentiles (solid box) with the 10th and 90th percentiles shown by whiskers outside the box. The Ct values were subtracted from 20, so that higher values represent higher mRNA expression levels.

Characterization of expression profile of the sphingosine system in alveolar macrophages in COPD

There was significantly higher mRNA-expression of both *SPHK1* & *SPHK2* in alveolar macrophages from COPD patients (3.5- and 2.1-fold increase respectively) compared to healthy controls (Figure 2). To assess the effect of smoking, the expression of *SPHK* was assessed in alveolar macrophages of healthy current smokers and current- or ex-smoker COPD patients. Compared to non-smoking individuals, a significantly higher relative mRNA expression of *SPHK1* was found in current healthy smokers (6-fold increase) and current-smoker COPD patients (4.8-fold increase) compared to control subjects (Figure 2A). Significantly higher mRNA expression levels for *SPHK2* were also found in current healthy smokers (4.4-fold increase) and current-smoker COPD subjects (2.3-fold increase) (Figure 2B). No significant changes in *SPHK1* or *SPHK2* expression were found in ex-smoker COPD patients, consistent with an effect of smoke rather than COPD disease on the relative expression levels of this *SPHK* isoform in alveolar macrophages.

Significantly higher mRNA expression levels for *S1PR2* and *S1PR5* were present in alveolar macrophages from COPD subjects (4.3- and 14.6-fold increase respectively) compared to healthy controls (Figure 3). No significant differences were found between *S1PR1, S1PR3* and *S1PR4,* and healthy controls (data not shown).

To assess the effect of smoking, the expression of S1P receptors was assessed in alveolar macrophages from healthy current smokers and current- or ex- smoker COPD patients. We found significantly higher mRNA expression for *S1PR2* in the healthy current smokers (9.3-fold increase $p<0.05$ vs control) and current-smoker COPD patients (6.1-fold increase $p<0.05$ vs control) compared to healthy controls (Figure 3A). Interestingly, no difference was found between ex-smoker COPD subjects and control subjects. In contrast, we observed significantly higher *S1PR5* mRNA levels in alveolar macrophages

from both current- and ex-smoker COPD patients compared to control subjects (n = 10, $p<0.05$ vs control, Figure 3B). The basal very low *S1PR5* expression was 32-fold higher in alveolar macrophages from ex-smoker COPD patients and 5.4-fold higher from current-smoker COPD subjects compared to health controls. In addition there was no difference in the relative *S1PR5* expression levels between healthy smokers and non-smokers (Figure 3B), suggesting a COPD disease-specific effect rather than a cigarette smoke effect. No difference was found in *S1PR1*, *S1PR3* and *S1PR4* mRNA expression in healthy current smokers and current- or ex- smoker COPD patients compared to healthy controls (data not shown).

Significantly higher mRNA expression of *SGPL1* was found in COPD alveolar macrophages compared to cells from control subjects (4.5-fold increase $p<0.05$ COPD vs. control, Figure 4A). No significant differences were found between groups for either *SGPP1* or *SGPP2* (Figures 4B & C). However, when we split the COPD on the basis of their smoking status we found significantly higher expression of *SGPP2* mRNA in alveolar macrophages from current-smoker COPD subjects compared to healthy control subjects (6.1-fold increase $p<0.05$ vs control, Figure 4). In contrast, no significant differences were found in *SGPP2* expression in either the ex-smoker COPD group or healthy current smokers vs. healthy non-smoking controls.

A non-significant trend toward higher expression of *SGPL1* mRNA was found in healthy current smokers ($p=0.08$) and in both ex-smoker and current-smoker COPD subjects ($p=0.06$ for both) compared to control subjects. No significant differences were found in relative mRNA expression for *SGPP1* between healthy current smokers, ex-smoker COPD subjects and current smoker COPD subjects compared to control subjects (Data not shown).

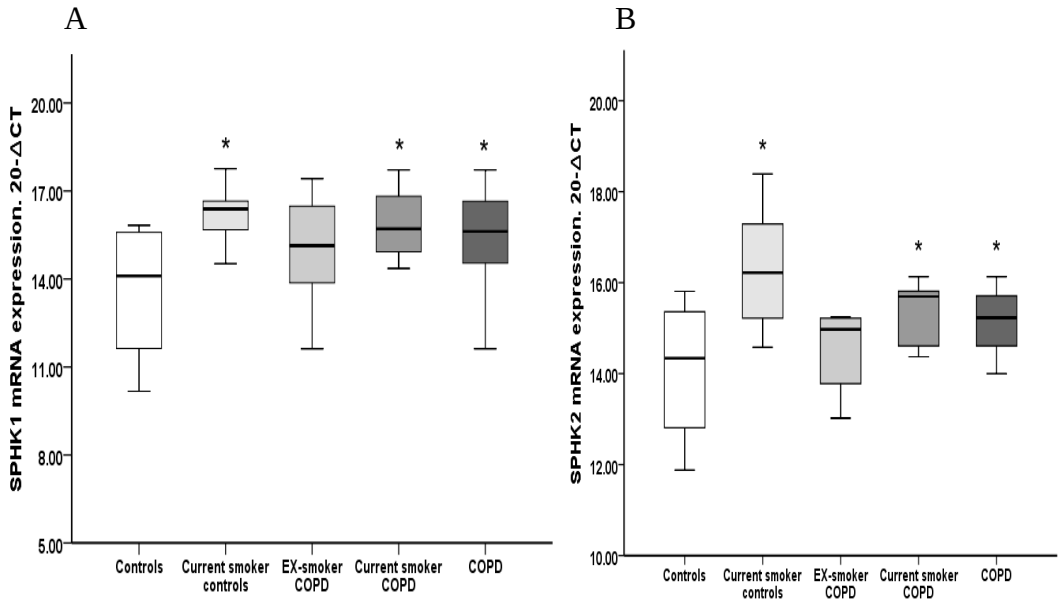

Figure 2: Relative mRNA expression of the S1P synthesizing enzymes, *SPHK1*, and *SPHK2* in alveolar macrophages. (A) Significantly higher mRNA expression of *SPHK1* was noted in current healthy smokers (6-fold increase), current smoker COPD (4.8-fold increase) and total COPD (3.5-fold increase) compared to control subjects (*$p<0.05$ vs control). No significant increase of *SPHK1* was found in alveolar macrophages isolated from ex-smoker COPD patients**.** (B) Significantly higher mRNA expression of *SPHK2* in current healthy smokers (4.4-fold increase), current smoker COPD (2.3-fold increase) and in the total COPD group (2.1-fold increase) versus control subjects (*$p<0.05$ vs control) was found. No significant increase in *SPHK2* was found in ex-smoker COPD subjects compared to control subjects. Data presented as box plots as described in Figure 1. The Ct values were subtracted from 20 so that higher values mean higher mRNA expression levels. Data presented as box plots as described in Figure 1. The Ct values were subtracted from 20 so that higher values represent higher mRNA expression levels.

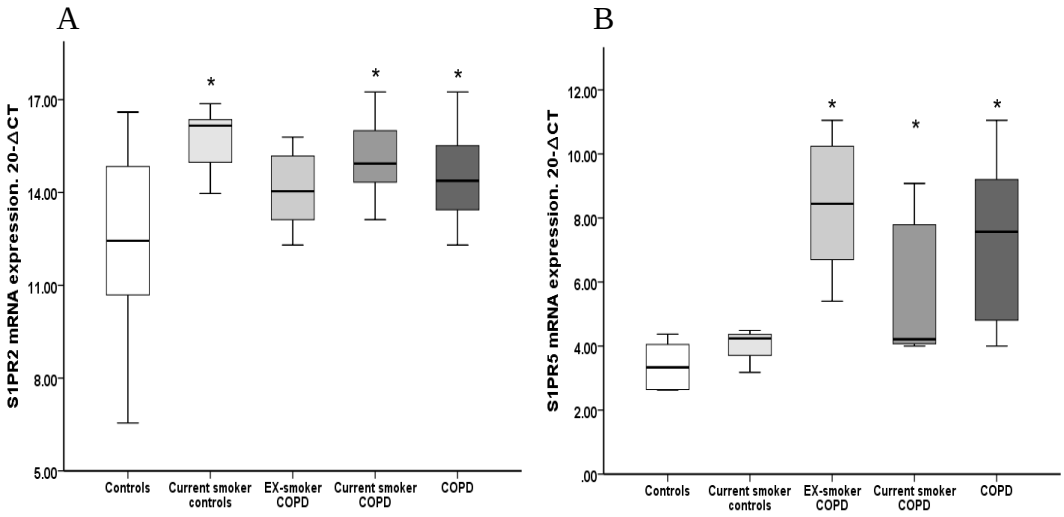

Figure 3: Relative mRNA expression levels of *S1PR2* and *S1PR5* in alveolar macrophages. (A) Significantly higher mRNA expression levels of *S1PR2* in current healthy smokers (9.3-fold increase), current smoker COPD subjects (6.1-fold increase) and in the total COPD group (4.3-fold increase) versus control subjects (*$p<0.05$ vs control) was found. No significant increase of *S1PR2* was found in ex-smoker COPD patients compared to control subjects. (B) Significantly higher mRNA expression of *S1PR5* was noted in in ex-smoker COPD subjects (32-fold increase), current smoker COPD subjects (5.4-fold increase) and in the COPD group (14.6-fold increase) versus healthy control subjects (*$p<0.05$ vs control). Data presented as box plots as described in Figure 1. The Ct values were subtracted from 20 so that higher values mean higher mRNA expression levels.

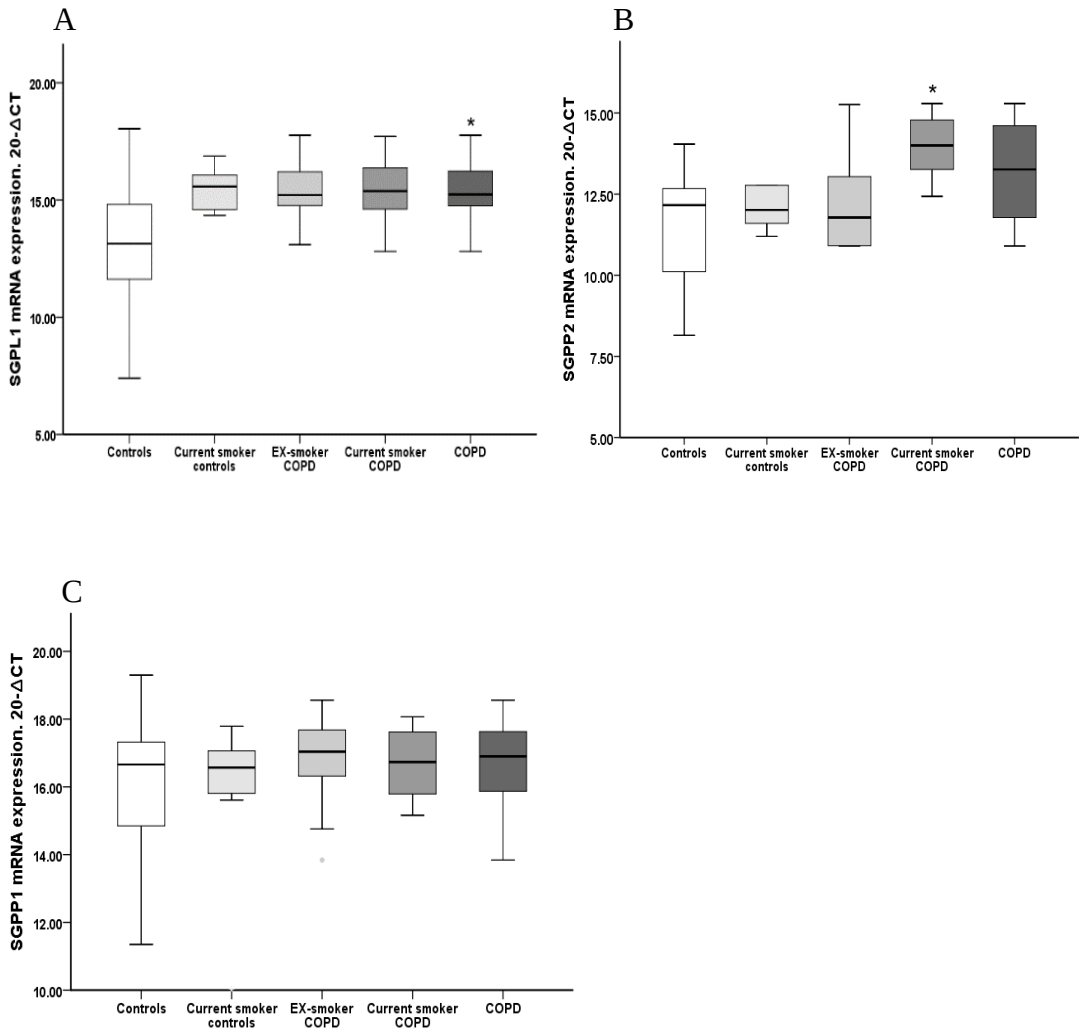

Figure 4: Relative mRNA expression of S1P degradation enzymes *in* alveolar macrophages. (A) Significantly higher mRNA expression of *SGPL1* in COPD patients (4.5-fold increase) versus control subjects (*p<0.05 vs control). No significant increase of *SGPL1*was found in current smoker COPD, ex-smoker COPD or healthy current smokers**.** (B) Significantly higher mRNA expression levels of *SGPP2* in current smoker COPD patients (6.1-fold increase) versus control subjects (*p<0.05 vs control) was noted. No significant increase of *SGPP2* was found in ex-smoker COPD subjects or healthy current smokers Data presented as box plots as described in Figure 1. The Ct values were subtracted from 20 so that higher values mean higher mRNA expression levels.

Functional associations between S1P signalling system components and alveolar macrophage phagocytic ability in COPD

To assess the functional relevance of our findings with regard to macrophage phagocytic function we investigated the association between phagocytosis and expression pattern of the S1P signalling system components. Consistent with our previous reports (Hodge, Hodge et al. 2003, Hodge, Hodge et al. 2007, Hodge, Hodge et al. 2008, Hodge, Matthews et al. 2010, Hodge, Matthews et al. 2011), a significantly reduced ability to phagocytose apoptotic cells was observed in alveolar macrophages from COPD patients in comparison to healthy control macrophages (mean±SEM, COPD 12.62±2.43% vs controls 20.98±1.98% p=0.004, Figure 5A). For 13 COPD subjects, a strong negative correlation was found between *SPHK1, S1PR3* and *S1PR5* mRNA expression levels and the ability of alveolar macrophages to phagocytose apoptotic cells (*SPHK1* r = -0.59, $p<0.05$; *S1PR3* r = -0.70, $p<0.05$; *S1PR5* r = -0.87, $p<0.05$; *SPHK1* data presented in Figure 5B). No significant associations were found between the presence of lung cancer and macrophage phagocytic ability (r=-0.471, p=0.098).

Phagocytosis of apoptotic cells was dose dependently increased by the addition of the *S1PR3* and *S1PR5* agonist Suramin, with a maximum effect at a concentration of 1μM (Figure 6).

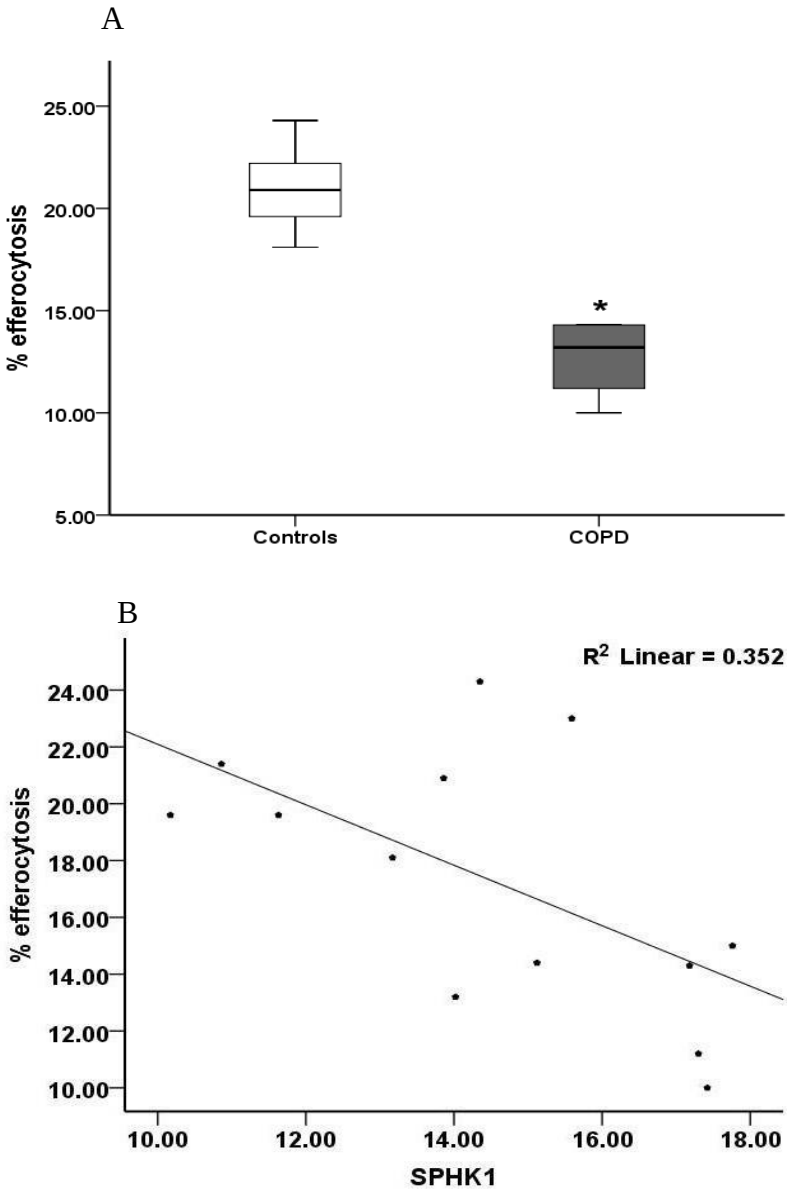

Figure 5: (A) Phagocytosis of apoptotic 16HBE airway epithelial cells (AEC) by alveolar macrophages from chronic obstructive pulmonary disease (COPD) subjects and controls. Compared to alveolar macrophages from healthy controls, macrophages from COPD patients had a significantly reduced ability to phagocytose apoptotic 16HBE epithelial cells (*$p<0.05$ vs control). (B) Correlation between the relative mRNA expression of *SPHK1* and % efferocytosis. Data shows a significant correlation between efferocytosis and mRNA expression of *SPHK1* ($p<0.05$). The correlation coefficients are listed in the results.

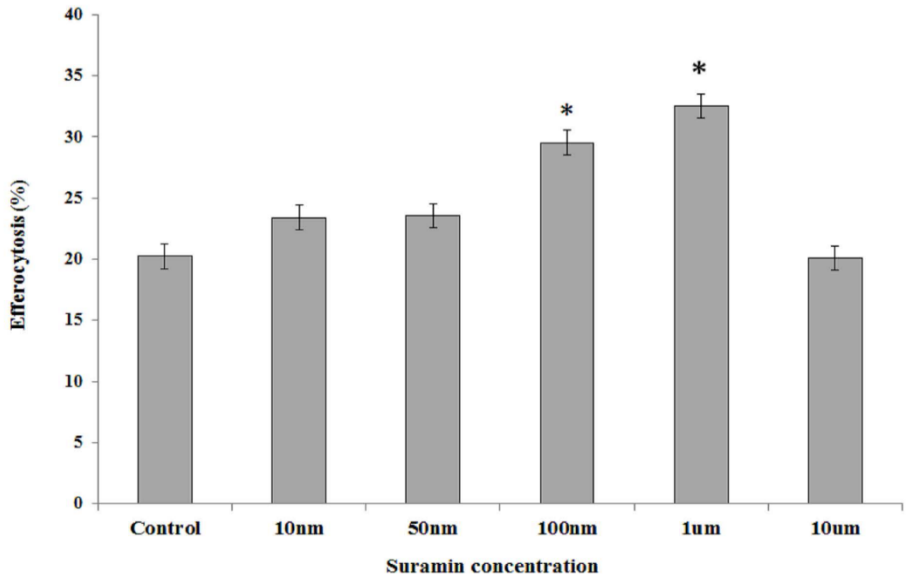

Figure 6: Effects of antagonising *S1PR3* and *S1PR5* with Suramin. To further elucidate functional effect of increased *S1PR5* on macrophage phagocytic ability, we performed the phagocytosis assay in the presence of varying concentrations of Suramin, an antagonist of *S1PR3* and *S1PR5*. Suramin at concentrations of 10 nM to 10 µM were added for 30 min prior to the phagocytosis assay. We noted a significant ($p<0.05$) increase in phagocytosis of apoptotic cells in the presence of Suramin.

Associations between S1P signalling system components in alveolar macrophages

Since the components of the S1P system are part of a combined signalling system, we analysed correlations between expressions of the various components. A strong positive correlation between the relative mRNA expression levels was observed for *SPHK1* and *SPHK2* ($r =0.62$, $p<0.05$) (Figure 7A). In addition a strong positive correlation was found between S1P receptor subtypes e.g. *S1PR1* and *S1PR5* ($r =0.55$, $p<0.05$); *S1PR2* and *S1PR4* ($r =0.79$, $p<0.05$; Figures. 7B & C). We also noted a strong positive correlation between *SPHK1* and *S1PR2* ($r =0.81$, $p<0.05$). There were positive correlations between the S1P degradation enzymes; *SGPP1* significantly correlated with

SGPP2 ($r = 0.38$, $p<0.05$) (Figure 7D); *SGPL1* significantly correlated with both *SGPP1* and *SGPP2* ($r = 0.60$, $p<0.05$ and $r = 0.54$, $p<0.05$ respectively; Figures 7E & F).

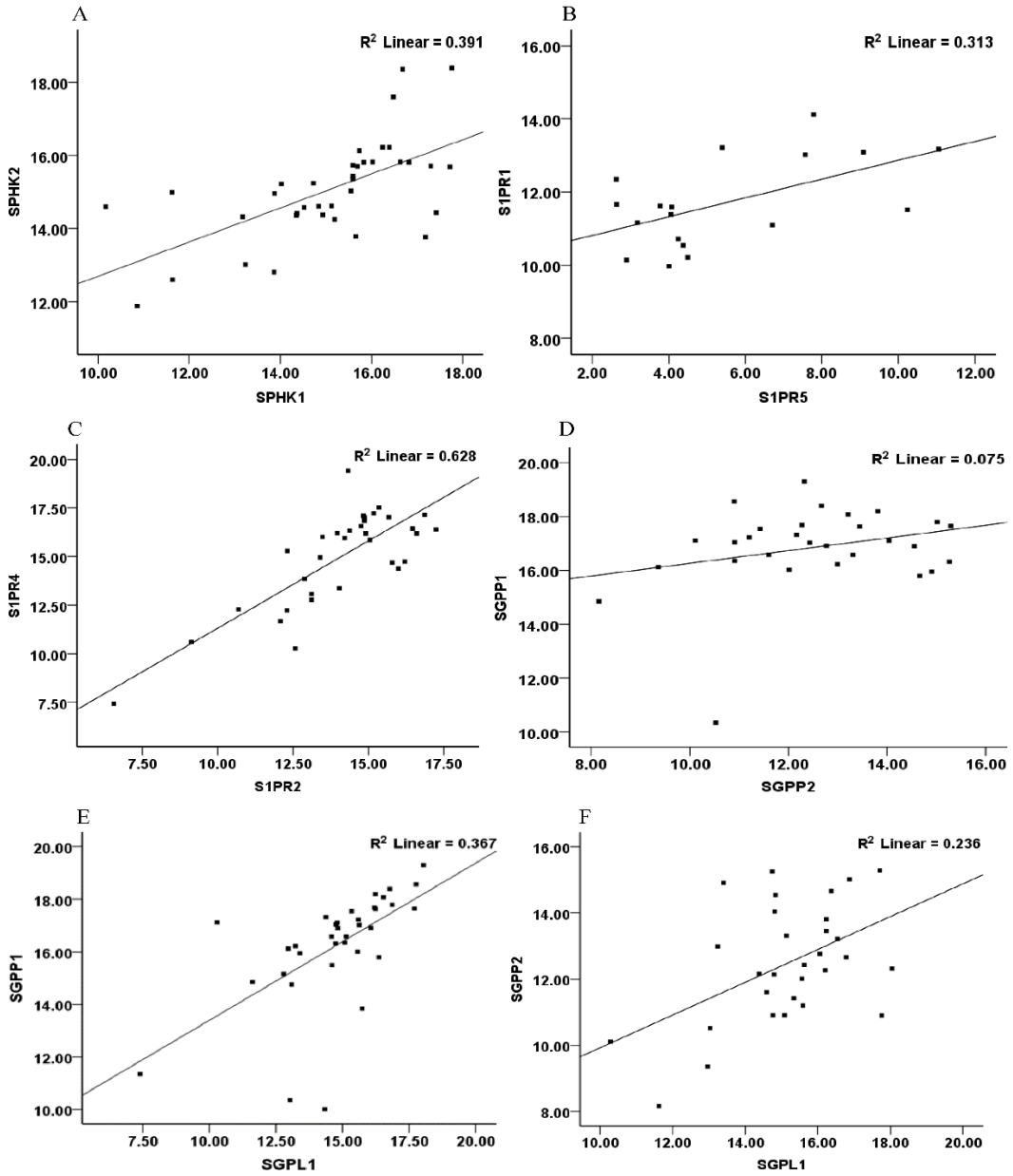

Figure 7: Correlations between relative mRNA expression of various components of the S1P signalling system in alveolar macrophages. (A) Correlation between *SPHK1 and* 2, $p<0.05$; (B) Correlation between *S1PR1 and S1PR5*, $p<0.05$; (C) Correlation between *S1PR2 and S1PR4*, $p<0.05$; (D) Correlation between *SGPP1 and SGPP2* $p<0.05$; (E) Correlation between *SGPL1* and *SGPP1* $p<0.05$; (F) *SGPL1* and *SGPP2*. The correlation coefficients are listed in the results.

Associations between S1P signalling system components and FEV_1, FEV_1/FVC ratio , smoking status, pack years, age, gender, presence of cancer, type of cancer and previous or ongoing chemotherapy and/or radiotherapy

A strong negative correlation was found between lung function (FEV_1) and mRNA expression levels of *SPHK1*, *S1PR1*, *S1PR3* and *S1PR5* in alveolar macrophages (*SPHK1* r= -0.49, p<0.05; *S1PR1* r= -0.44, p<0.05; *S1PR3*, r=-0.45, p<0.05; *S1PR5* r= -0.55, p<0.05; Figure 8), with a trend observed for both *SGPL1* and *SGPP2* with FEV_1 (*SGPL1* r = -0.32, p=.078; *SGPP2* r = -0.37, p=.091; Figure 8). Furthermore a negative correlation was observed between the FEV_1/FVC ratio, a further indicator of lung function, and mRNA expression levels of *S1PR5* and *S1PR3* (*S1PR5* r= -0.61, p<0.05; *S1PR3* r= -0.52, p<0.05), with a trend observed between *SPHK1* and FEV_1/FVC (r= -0.33, p=.063).

We found a significant positive correlation between smoking status and relative mRNA expression levels of both *SPHK* isoforms, *S1PR2*, *S1PR4* and *SGPP2* (*SPHK1* r=0.41, p<0.05; *SPHK2* r = 0.38, p<0.05; *S1PR2* r = 0.36, p<0.05; *S1PR4* r = 0.35, p<0.05; *SGPP2* r = 0.39, p<0.05). There were significant positive correlations between age and mRNA expression levels of *S1PR1* and *S1PR3* (*S1PR1* r = 0.39, p<0.05; *S1PR3* r = 0.45, p<0.05). Moreover a high positive correlation was revealed between mRNA expression levels of *SPHK2* and *S1PR5* and smoking pack years (*SPHK2* r = 0.36, p<0.05; *S1PR5* r = 0.54, p<0.05). No correlation was found between gender and any of the S1P system mRNA levels. The only significant correlation between the presence of cancer and S1P system mRNA expression levels was for *S1PR3* that showed a positive correlation with the presence of cancer independent of COPD status (r = 0.41, p<0.05). Moreover, no

significant associations were found between S1P system mRNA expression levels with types of lung cancer or exposure to chemotherapy and/or radiotherapy.

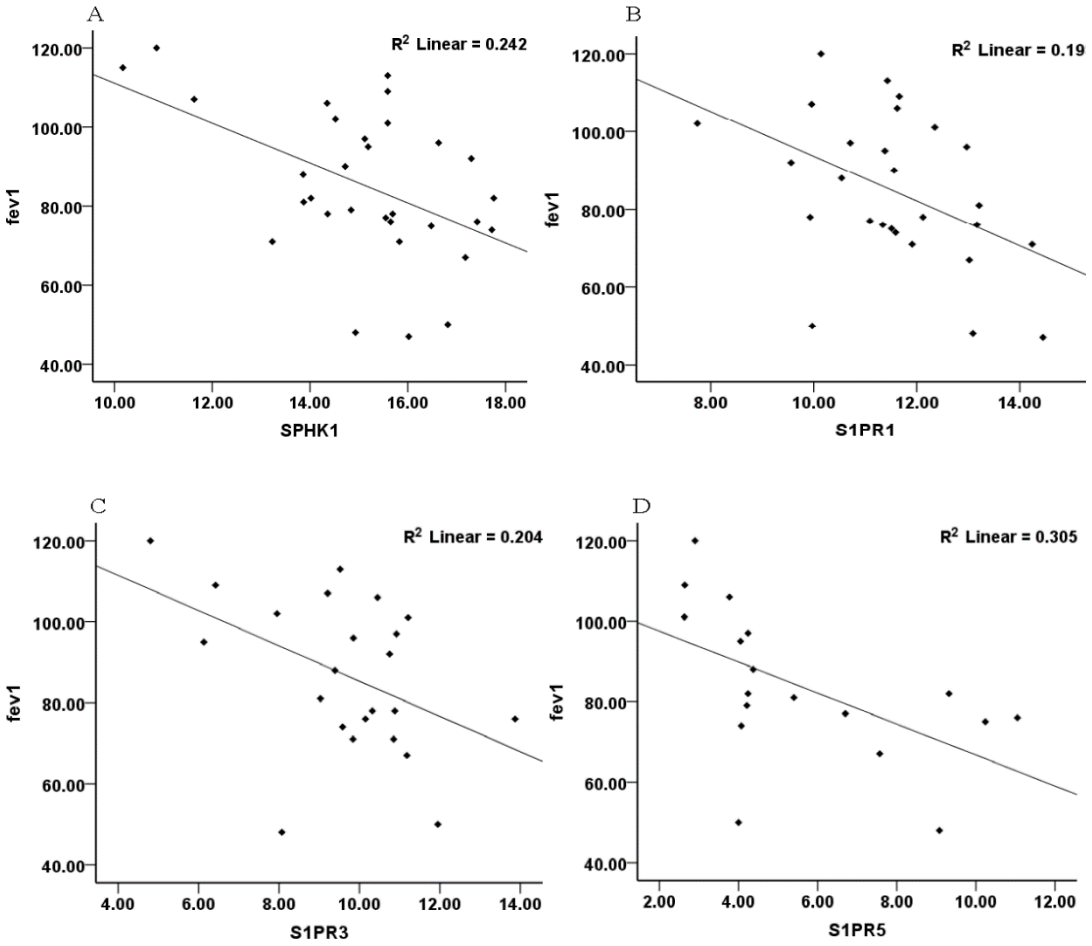

Figure 8: Correlations between the relative mRNA expression of various components of the S1P signalling system and lung function (FEV_1). Significant ($p<0.05$) correlations were found between FEV_1 and (A) *SPHK1* (B) *S1PR1* (C) *S1PR3*, (D) *S1PR5*, $p<0.05$. The correlation coefficients are listed in the results.

Investigation of the effect of cigarette smoke extract on the expression of *SPHK1*, *SPHK2*, *S1PR2*, *S1PR5* and *SGPP2* in THP-1 macrophages in vitro

Assessment of S1P system genes showed variation according to their passages, with expression pattern comparable to normal human alveolar macrophages between

passages 6 to 20. To confirm the effects of cigarette smoke on macrophage expression of *SPHK1*, *SPHK2*, *S1PR2*, *S1PR5* and *SGPP2* we determined the expression levels of the S1P system in THP-1 macrophages exposed to 10% cigarette smoke extract for 24 h. Consistent with the alveolar macrophages from human smoker COPD subjects, we noted significantly higher relative mRNA expression of *SPHK1*, *SPHK2*, *S1PR5* and *S1PR2* whereas there was no increase in expression of *SGPP2* and *SGPL1* (n =7 experiments $p<0.05$, Figure 9**).**

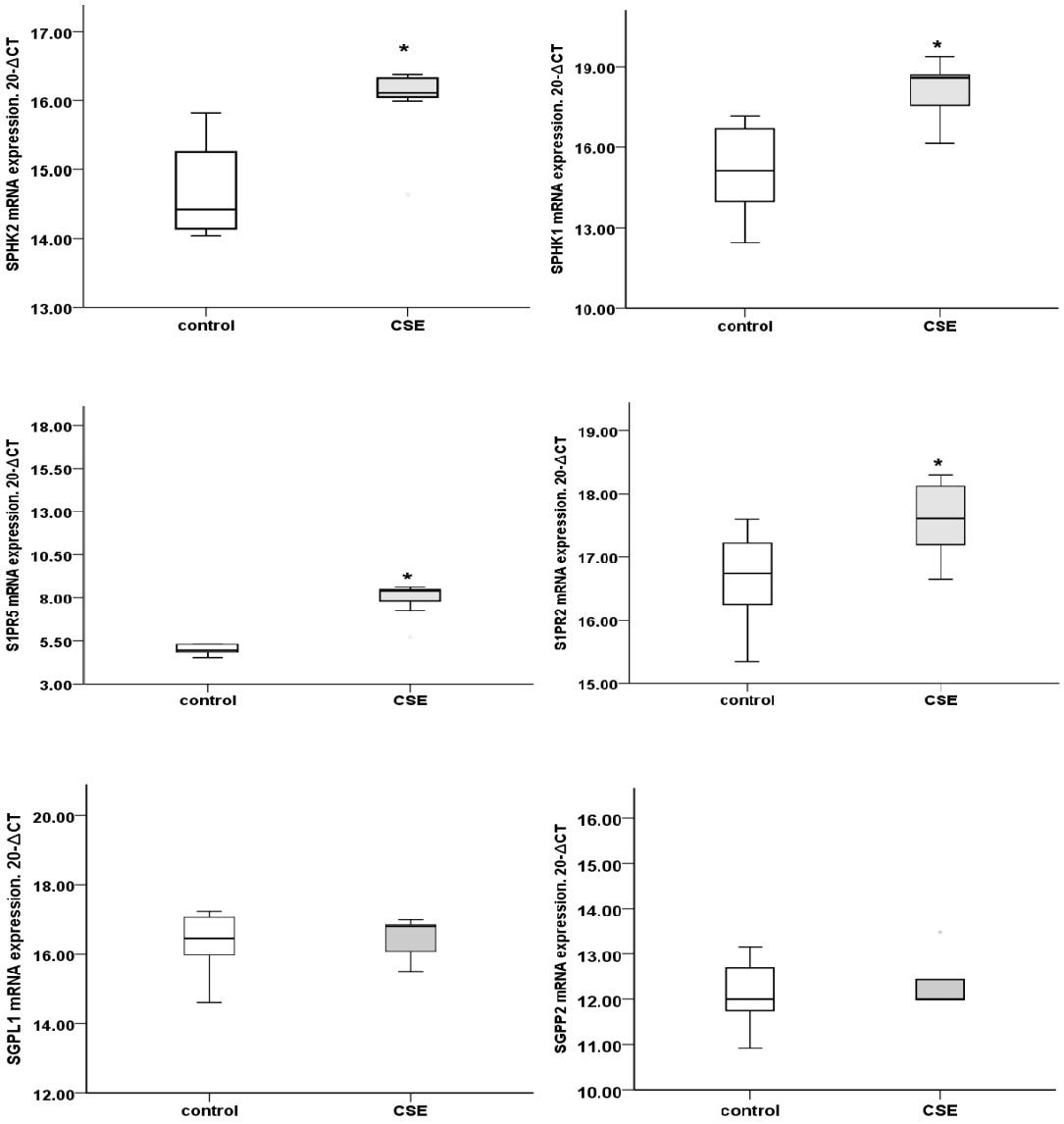

Figure 9: mRNA expression of various components of the S1P signalling system in THP-1 macrophages following stimulation with 10% cigarette smoke extract for 24 h. *:Significant increase in mRNA expression of *SPHK1*, *SPHK2*, *S1PR2* and *S1PR5*, but no significant changes were found in *SGPL1* and *SGPP2* compared to non-stimulated macrophages ($p<0.05$).

Discussion

In recent years it has become evident that alveolar macrophages play a vital role in the pathophysiology and development of COPD (Barnes 2004). Macrophages modulate and control levels of inflammation; however, the macrophage-dependent inflammatory haemostasis is defective in COPD (Barnes 2004). One of the major causes of this defect is thought to be a reduced ability of alveolar macrophages to phagocytose apoptotic cells and bacteria (Hodge, Hodge et al. 2003, Taylor, Finney-Hayward et al. 2010) which contributes to ongoing inflammation in lungs of these patients (Hart, Haslett et al. 1996, Knapp, Leemans et al. 2003). Several studies have reported changes in candidate molecules that may at least partially contribute to the defective efferocytosis in COPD, although it is becoming clear that a more complex network of molecules rather than a single molecule is the more likely cause of the reduced efferocytosis (Erwig and Henson 2008, Mukaro and Hodge 2011). In particular, identification of molecule(s) that are affected in COPD irrespective of smoking status would be an important strategy toward identification of new macrophage-targeted treatments for COPD.

Previous studies have identified the S1P system as a candidate for macrophage dysfunction. McQuiston et al. showed that the S1P-mediated increase in phagocytosis occurred in an antibody-dependent manner (McQuiston, Luberto et al. 2010, McQuiston, Luberto et al. 2011). The antibody-mediated pathway is commonly linked to phagocytosis of pathogen; however, it became clear in the last decade that apoptotic cells can also become opsonized with naturally occurring IgM auto antibodies which facilitate their internalization (Hart, Smith et al. 2004, Ogden, Kowalewski et al. 2005, Peng, Kowalewski et al. 2005, Chen, Khanna et al. 2009) This mechanism is particularly efficient when combined with the complement (Ogden, Kowalewski et al. 2005).

Petrusca et al. reported that up-regulation of the S1P precursor ceramide inhibited the alveolar macrophage-mediated clearance of apoptotic cells (Petrusca, Gu et al. 2010). A further study on murine microglia, resident macrophage-like cells of the CNS which are involved in phagocytosis and inflammatory responses, showed the involvement of *SPHK1* signalling in their functions (Nayak, Huo et al. 2010). These studies, however, were focused on single components of the S1P system, and to the best of our knowledge, there is no study that has comprehensively studied the combination of synthesizing, degrading enzymes and receptors in human alveolar macrophages in COPD.

S1P, the product of *SPHK1* and 2 has been implicated in many biological functions in the cell, including survival, proliferation and migration, and macrophage phagocytic function (Chun, Goetzl et al. 2002, Rivera, Proia et al. 2008, Weigert, Weis et al. 2009, Blaho and Hla 2011). In the present study, the expression of both *SPHK1* and *SPHK2* mRNAs was significantly increased in alveolar macrophages from healthy smokers and current-smoker COPD subjects, with no changes found in macrophages obtained from ex-smoker COPD subjects. This indicates a close association between smoking and expression of S1P synthesizing enzymes. The effect of cigarette smoke was further highlighted by a significant increase in *SPHK1* in the model of cigarette smoke-exposed THP-1 macrophages. Furthermore analysis of expression and patient data showed and a positive correlation between *SPHK1* and smoking status. Smoking causes inflammation and inflammation is known to modulate *SPHK1* mRNA expression in animal macrophages (Hammad, Crellin et al. 2008, Nayak, Huo et al. 2010, Fischer, Alliod et al. 2011).

In this study, we showed for the first time the response in *SPHK1* in the context of COPD or cigarette smoking. Increased ceramide levels in alveolar macrophages have been shown to impair their ability to phagocytose apoptotic cells (Petrusca, Gu et al. 2010). Ceramide is converted to sphingosine which is phosphorylated via *SPHK1* and *SPHK2*

to S1P (Igarashi 1997). It is thus possible that the higher mRNA expression levels of *SPHK1* or *SPHK2* in alveolar macrophages in response to smoking reflect the response to high ceramide levels with the aim to reduce ceramide concentration.

A finely-tuned balance between *SPHK1* and *SPHK2* expression and functions, with tissue specificity has been reported (Maceyka, Sankala et al. 2005). For example, in rodent neuronal tissue there are higher levels of *SPHK2* compared to *SPHK1* (Blondeau, Lai et al. 2007), whereas in murine lungs there is higher expression and activity of *SPHK1* (Fukuda, Kihara et al. 2003, Wadgaonkar, Patel et al. 2009). It should be noted that the expression in the lung reflects many cell types including endothelial, epithelial cells and macrophages. Interestingly in the present study, we observed equal relative mRNA-expression levels for *SPHK1* and *SPHK2*, and a correlation between the two in alveolar macrophages from healthy controls. In contrast, the two kinases were differentially expressed in alveolar macrophages from COPD subjects and although both *SPHK1* and *SPHK2* separately correlated positively with smoking status**,** only *SPHK1* was found to be correlated with lung function suggesting a changed mRNA expression and presumably a change in balance between the kinases and the potential involvement of *SPHK1* in smoking-related COPD disease progression.

S1P receptors are essential for S1P function (Sanchez and Hla 2004). Changes in expression of these receptors have been reported in many cell types in various lung diseases including acute lung injury, asthma, cystic fibrosis and COPD (Yang and Uhlig 2011). The distribution of the receptors has been shown to be cell specific and affected differently in the various disease conditions (Rivera, Proia et al. 2008). The presence of S1P receptors in macrophages has been well documented (Weigert, Weis et al. 2009, Weigert, Weichand et al. 2011); however, the presence and expression in human alveolar macrophages is less studied. The *S1PR4* subtype showed the highest relative mRNA expression followed by the *S1PR2* subtype. Consistent with our data for *SPHK*s, we

found that expression of *S1PR2* mRNA in alveolar macrophages increased with smoking, and increased in both healthy current smokers and current-smoker COPD subjects, but was unchanged in ex-smoker COPD subjects. In addition, our *in vitro* investigations revealed a significant increase in *S1PR2* in cigarette smoke extract exposed THP-1 macrophages, confirming the direct effects of cigarette smoke on expression of this receptor. A potential role for this receptor in phagocytosis was also shown by McQuiston et al who showed that treatment with the dual *S1PR2*/4 antagonist JTE-013 affected the phagocytosis of *Cryptococcus neoformans* by wild-type alveolar macrophages in contrast with the *S1PR1* antagonists W146 or VPC 23901 (McQuiston, Luberto et al. 2010, McQuiston, Luberto et al. 2011). In non-alveolar macrophages/monocytes activation of *S1PR2* attenuated migration (Weichand, Weis et al. 2013). This could relate to cigarette smoke-induced inhibition of macrophage function in alveoli. *S1PR2* may also play a role in the activation of cytoskeletal-remodelling that occurs during efferocytosis. The increased cigarette smoke-related *S1PR2* expression by alveolar macrophages found in our study was therefore surprising; however, we also noted a positive correlation between *S1PR2* and *S1PR4*. Efferocytosis occurs with extensive pseudopod extensions and is dependent on the relative activation of Rho GTPases, Rac-1 and RhoA (Ndozangue-Touriguine, Hamelin et al. 2008) that control actin polymerisation and formation of lamellipodia that is required for macrophage engulfment of apoptotic cells. We and others have shown that Rho activity is increased and Rac-1 activity decreased, in macrophages in response to cigarette smoke (Richens, Linderman et al. 2009, Hodge, Matthews et al. 2010, Mukaro, Bylund et al. 2013). Interestingly, expression of *S1PR4* protein has also been shown to activate RhoA in response to S1P stimulation in Chinese hamster ovary (CHO) cells (Graler, Grosse et al. 2003), and S1P has been shown to promote migration of cells expressing *S1PR4* through activation Cdc42, a Rho family member (Kohno, Matsuyuki et al. 2003). The

positive correlation we found between *S1PR2* and *S1PR4* thus suggests that these receptors may potentially work in concert to negatively affect the efferocytosis process in response to cigarette smoke. *S1PR2* expression also induces increased macrophage accumulation in atherosclerosis plaque and promotion of inflammation in mice (Wang, Okamoto et al. 2010, Skoura, Michaud et al. 2011), and its increased expression could thus also contribute to the macrophage accumulation that has been reported in current smokers with or without COPD (Finkelstein, Fraser et al. 1995, Retamales, Elliott et al. 2001).

In contrast to the findings with *SPHK1, SPHK2* and *S1PR2,* we found that *S1PR5* was higher in alveolar macrophages from both ex-smoker COPD and current smoker COPD subjects and negatively correlated with both FEV_1 and FEV_1/FVC ratio indicating a possible involvement in COPD progression that is at least to some extent independent of cigarette smoking. Unlike *S1PR2,* there were no correlations between *S1PR5* and smoking status, or with *SPHK1* or *SPHK2* or *S1PR2*. Currently, the role of *S1PR5* in macrophages is unclear, although our findings of a strong negative correlation between expression of *S1PR5* and phagocytosis of apoptotic cells suggest that it may play an important role in this function and contribute to the defective macrophage function in COPD.

Several studies have shown that mRNA levels not always predict protein expression. We therefore utilized a functional approach to assert the increase of *S1PR5* and its effects on macrophage phagocytic ability. We used a *S1PR3/5* antagonist suramin (Miron, Jung et al. 2008), and found that phagocytosis was dose dependently increased with a maximum effect at 1 μM suramin. Taken together with our findings of significant associations between the increased expression of *SPHK, S1PR2* and *S1PR5,* and alveolar macrophage phagocytic ability in COPD, these data support a functional effect of the

increased gene expression of *S1PR5*. *S1PR5* has been reported to interact with Rho-$G_{12/13}$ protein coupled Rho/ROCK signalling pathway in oligodendrocyte precursor cells (OPCs) and appears to mediate their migration (Novgorodov, El-Alwani et al. 2007). Although we did not find significantly increased expression of *S1PR1* in alveolar macrophages from either smokers or COPD subjects, this receptor was found to be strongly correlated with *S1PR5*. Taken together with our findings of a negative correlation with lung function and phagocytosis of apoptotic cells, these data suggest that both receptors may be associated with COPD progression and defective macrophage function in COPD either by a link between the two or common downstream effectors. Consistent with this, a previous study showed that both *S1PR1* and *S1PR5* enhanced microgliosis (Miron, Ludwin et al. 2010). It is thus likely that the defective efferocytosis in COPD that persists despite smoke cessation could be mediated by *S1PR5*, possibly in association with *S1PR1*. Interestingly, in our previous study the relative mRNA for *S1PR5* was significantly lower in whole lung tissue of patients with COPD compared to controls without COPD (Cordts, Pitson et al. 2011).

In the present study, however, alveolar macrophage-specific *S1PR5* expression was increased when comparing COPD vs. controls. This counterintuitive finding further highlights the finely tuned balance and the tissue specificity of the sphingosine family, and stresses the need to investigate not only whole lung tissue, but individual cell types of interest.

None of the controls in this study suffered from lung cancer whereas 60% of the COPD patients did. We therefore correlated S1P system mRNA expression levels with the presence of lung cancer. The only significant correlation however was for *S1PR3* that showed a positive correlation with the presence of cancer independent of COPD status, suggesting that the findings in COPD subjects in our study were not influenced by the

presence of lung cancer. A potential limitation of the study is that we did not measure S1P levels in BALF as this would have provided an additional understanding of smoke- and/or COPD-related changes in S1P signalling.

S1P degradation enzymes, *SGPP1, SGPP2* and *SGPL1* are enzymes that metabolize S1P in the cell and their importance comes from their role in controlling the levels of S1P in the cell, excessive concentrations of which are detrimental to cell fate and functions (Morales, Lee et al. 2007, Blaho and Hla 2011) as well as drug resistance and cancer (Oskouian and Saba 2010).

In the present study, *SGPL1* was found to be higher in alveolar macrophages from COPD subjects while *SGPP2* was higher in current-smoker COPD subjects compared to healthy controls, with a trend towards a significant correlation between *SGPP2* and lung function. In parallel, we also found that cigarette smoke failed to increase mRNA levels of *SGPP2* and *SGPL1* in our in vitro cell line experiments. Recently, SNP variants on *SGPP2* in lung epithelial cells from adult smoker subjects found to be associated with vitamin D regulations as well as and FEV_1 and FEV_1/FVC ratio (Reardon, Hansen et al. 2013). Furthermore, vitamin D-binding protein (DBP) levels were found to be directly responsible for alveolar macrophage activation as well as related to FEV_1 (Wood, Bassford et al. 2011). We also noted a non-significant trend for an increase in *SGPL1*, the other S1P degradation enzyme, in healthy smokers and both and current-and ex-smoker COPD subjects as well as a positive correlation with *SGPP2*, suggesting a link between these two enzymes in alveolar macrophages in COPD. *SGPP2* converts S1P to sphingosine which can be converted back to ceramide and *SGPL1* degrades S1P, which indirectly can favour an increase in ceramide over S1P, so the increase in both degradation enzymes is likely to impact on the increasing ceramide levels and subsequent defect in alveolar macrophage efferocytosis ability. In addition, the established link between COPD and vitamin D and alveolar macrophage function

(Chishimba, Thickett et al. 2010, Parekh, Thickett et al. 2013, Romme, Smeenk et al. 2013), and the role of these enzymes in degradation of S1P (Spiegel and Milstien 2003) suggests a potential important indirect role of *SGPP2* and/or *SGPL1* in the alveolar macrophage defect in COPD. Interestingly, a correlation between *SGPP1* and *SGPL1* expression by alveolar macrophages was found in the present study, further supporting a link between these two enzymes.

In conclusion, our data strongly support the role of the S1P signalling pathway in the defective alveolar macrophage function and resultant chronic inflammation that is present in the airways of patients with COPD. In particular, we identified *S1PR5* and S1P degradation enzymes as mediators that are independently increased in alveolar macrophages in COPD, independent of the presence of cigarette smoke and, importantly, a significant correlation between *S1PR5* mRNA expression and lung function. These mediators are thus worthy of their further investigation as macrophage-target therapeutic strategies for COPD and other chronic inflammatory lung diseases.

Refereance

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Author Contributions By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

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CHAPTER 3

Pro-phagocytosis and anti-apoptotic effects of thymoquinone in the airways in chronic obstructive pulmonary disease: modulation of the sphingosine-1-phosphate signalling system

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Abstract

Oxidative stress, inflammation, increased bronchial epithelial cell apoptosis and deficient phagocytic clearance of these cells (efferocytosis) by alveolar macrophages are present in chronic obstructive pulmonary disease (COPD). We previously showed that the macrophage dysfunction is associated with changes to the sphingosine 1-phosphate (S1P) signalling system.

We hypothesized that the antioxidant/anti-inflammatory agent, thymoquinone, would improve macrophage phagocytosis via modulation of the S1P system and protect bronchial epithelial cells from cigarette smoke or lipopolysaccharide (LPS)-induced apoptosis. Phagocytosis was assessed using flow cytometry, S1P mediators by Real-Time PCR, and apoptosis of 16HBE bronchial epithelial cells using flow cytometry and immunohistochemistry. Cigarette smoke and LPS decreased phagocytosis and increased S1P receptor (S1PR)-5 mRNA in THP-1 macrophages. Thymoquinone enhanced efferocytic/phagocytic ability, antagonized the effects of cigarette smoke extract and LPS on phagocytosis and S1PR5, and protected bronchial epithelial cells from cigarette smoke-induced apoptosis. Thymoquinone is worth further investigating as a potential therapeutic therapy for COPD.

Key words: Chronic obstructive pulmonary disease, COPD, macrophage, apoptosis, phagocytosis, sphingosine 1 phosphate, thymoquinone

Introduction

Chronic obstructive pulmonary disease (COPD) is an incurable lung disease, strongly associated with cigarette smoking. Smoking cessation is thus an essential step in disease management; however, despite smoking cessation encouragement campaigns, the

incidence of smoking is still increasing, especially in developing countries, and COPD is predicted to become a leading cause of death within the next decade. COPD is a heterogeneous chronic inflammatory lung disease, comprising chronic bronchitis and emphysema, and available treatments are largely ineffective for full disease management (Pauwels, Lofdahl et al. 1999, Global Strategy for the Diagnosis 2010). There is thus an urgent need for new therapeutic innovations.

In a normal lung, cell turnover, repair of injured epithelial cells and resolution of inflammation occur in a tightly controlled process that includes phagocytosis of unwanted apoptotic cells by alveolar macrophages, a process termed 'efferocytosis' (Hodge, Hodge et al. 2003, Henson, Cosgrove et al. 2006, Morimoto, Janssen et al. 2006, Morimoto, Janssen et al. 2006, Vandivier, Henson et al. 2006, Hodge, Hodge et al. 2007, Hodge, Hodge et al. 2008, Hodge, Matthews et al. 2010, Hodge, Matthews et al. 2011). If apoptotic material remains uncleared, secondary necrosis, a stage where cells release their toxic contents, can promote inflammation (Hart, Haslett et al. 1996, Knapp, Leemans et al. 2003, Vandivier, Henson et al. 2006). An increase in apoptotic cells in the airways of COPD patients has been reported (Segura-Valdez, Pardo et al. 2000, Yokohori, Aoshiba et al. 2004, Hodge, Hodge et al. 2005, Imai, Mercer et al. 2005). We showed that a potential cause of this phenomenon is a defect in the efferocytosis ability of alveolar macrophages from both COPD patients and current smokers (Hodge, Hodge et al. 2003, Hodge, Hodge et al. 2007, Hodge, Hodge et al. 2008, Hodge, Matthews et al. 2010, Hodge, Matthews et al. 2011). We, and others, (Taylor, Finney-Hayward et al. 2010) demonstrated that both alveolar macrophages and monocyte-derived macrophages from COPD patients are impaired in their ability to phagocytose bacteria, which may contribute to bacterial colonisation in COPD patients (Taylor, Finney-Hayward et al. 2010). Cigarette smoke significantly inhibited the efferocytosis ability of alveolar macrophages *in vitro*, potentially by changes to efferocytosis recognition

molecules including mannose receptor (Hodge, Hodge et al. 2007, Hodge, Hodge et al. 2008, Hodge, Matthews et al. 2011). Despite these findings however, a complete understanding of the mechanisms leading to defective macrophage phagocytic function in COPD remains unknown.

Sphingolipid molecules regulate vital cell function such as apoptosis, survival, migration and stress response (Futerman and Hannun 2004, Taha, Argraves et al. 2004, Hannun and Obeid 2008). Ceramide, sphingosine, and sphingosine-1-phosphate (S1P) are among the most important and well-studied sphingolipid metabolites. Ceramide, the precursor of S1P, is formed via the acetylation of sphingosine by ceramidase, while the phosphorylation of sphingosine results in the production of S1P by sphingosine kinases (SPHK). Structural modulations of these molecules have been associated with cellular dysfunction (Uhlig and Gulbins 2008) and their dysregulation has been demonstrated in inflammatory lung diseases (Yang and Uhlig 2011). We have previously shown that several genes involved in sphingolipid signalling (including S1P receptors (*S1PRs*)-2 and 5, and the S1P-degrading enzyme, sphingosine-1-phosphate lyase 1 (*SGPL1*) are upregulated in alveolar macrophages from the airways of COPD patients (Barnawi, Tran et al. 2015). Importantly, significant associations were noted between S1PR5 and both phagocytosis and COPD severity (FEV_1). Consistent with a role for the sphingosine pathway in the defective macrophage function in COPD, it has been shown that cigarette smoke-induced ceramides impair alveolar macrophage efferocytosis in COPD (Petrusca, Gu et al. 2010), and that cigarette smoke-induced sphingolipids are involved in COPD pathophysiology (Petrache and Petrusca 2013). Based on this evidence, the present study focused on identifying agents that improve alveolar macrophage phagocytosis by modulating the S1P system, as potential anti-inflammatory therapeutic strategies for COPD.

Natural compounds with low toxicity are attractive therapeutic options. Thymoquinone, an aromatic compound found in *Nigella Sativa* essential oil extract, has been widely studied and reported to have anti-oxidant (Nagi, Alam et al. 1999, Sayed-Ahmed, Aleisa et al. 2010) and anti-inflammatory (Hajhashemi, Ghannadi et al. 2004) properties, with therapeutic potential for inflammatory diseases and cancer (Woo, Kumar et al. 2012). Thymoquinone also has therapeutic potential for the treatment of inflammatory lung diseases. In animal models of asthma, thymoquinone acted as a preventive and/or alleviating agent (Kalemci, Cilaker Micili et al. 2013, Keyhanmanesh, Pejman et al. 2014), and as a suppressor of inflammatory airway mediators (El Gazzar, El Mezayen et al. 2006, El Gazzar, El Mezayen et al. 2006, El Mezayen, El Gazzar et al. 2006), and decreased cigarette smoke-induced lung injury (Kanter 2009, Kanter 2011, El-Khouly, El-Bakly et al. 2012, Suddek, Ashry et al. 2013). Positive effects of thymoquinone on inflammatory responses in animal peritoneal macrophages and RAW 264.7 macrophages have also been reported (El-Mahmoudy, Shimizu et al. 2005, Wilkins, Tucci et al. 2010, Wilkins, Tucci et al. 2011). Taken together, these studies suggest that thymoquinone may be a potential 'multi-faceted' adjunct treatment for COPD, via increased alveolar macrophage phagocytic function, and the protection of airway epithelial cell integrity. There have been no studies addressing the effect of thymoquinone on efferocytosis in the presence and absence of cigarette smoke, or its effect on the expression of S1P-related molecules within the COPD disease context. We therefore investigated the effects of thymoquinone on 1) efferocytosis and sphingosine signalling molecules in response to cigarette smoke extract or *E. coli* lipopolysaccharide (LPS), 2) phagocytosis of non-typeable *Haemophilus influenzae* (NTHi), 3) apoptosis of cigarette smoke extract- exposed bronchial epithelial cells.

Material and methods:

1.1 **Cell culture and stimulation**

THP-1 macrophages

THP-1 macrophages were maintained and differentiated with PMA as previously described (Barnawi, Tran et al. 2015). Cigarette smoke extract was prepared as previously described (Hodge, Hodge et al. 2004, Hodge, Hodge et al. 2006, Hodge, Hodge et al. 2007, Hodge, Hodge et al. 2008). THP-1 macrophages were exposed to 5 µM thymoquinone (Sigma Aldrich, Sydney, Australia) +/- 10 µM lipopolysaccharide (LPS, Sigma, Sydney, Australia) or 10% cigarette smoke extract for 24 h.

1.2 **Bronchial epithelial cells**

Cultured 16HBE bronchial epithelial cells were maintained as previously reported (Hodge, Hodge et al. 2003), with minor modifications. Briefly, cells were cultured in RPMI 1640 medium (Gibco, Karlsruhe, Germany), supplemented with 10% foetal calf serum (Gibco) and 1% penicillin and gentamicin at 37°C with 5% CO_2. Experiments were performed on cells between passages 10 and 30 that had been cultured with 10% cigarette smoke extract +/- thymoquinone for 24 h.

1.3 Cell Viability

1.3.1 **Apoptosis**

The viability of THP-1 macrophages, and of a normal bronchial epithelial cell line (16HBE) following treatment, were measured using flow cytometry and staining with Annexin V (FITC; Bender Med Systems, Vienna, Austria) as we have reported (Hodge,

Hodge et al. 2005, Hodge, Hodge et al. 2006). Stimulated cells were harvested using 500 µL of cold phosphate buffered saline (PBS) for 5 min then lifted from the wells with gentle pipetting. 16HBE cells were lifted from the plate using trypsin treatment before the addition of fresh media for trypsin neutralisation. Cells were transferred to FACS tubes before the addition of 3 mL of Annexin V FITC, further incubation for 10 min, and washing with 2 mL of Annexin V wash buffer (Hepes buffer: 10 mmol/L HEPES (Sigma)/NaOH, pH 7.4, 150 mmol/L NaCl, 1 mmol/L MgCl2, 1.8 mmol/L CaCl). Supernatant was discarded, cells resuspended, and 100,000 total events collected using a FACS Canto II Flow Cytometer.

1.3.2 **Immunofluorescence of cleaved caspase-3**

Preparation of 16HBE cells and their immunofluorescence in chamber slides was carried out as previously described (Tran, Lewis et al. 2012). The primary antibody to cleaved caspase-3 was a rabbit polyclonal IgG (Sapphire Bioscience, Waterloo, NSW, Australia). The F (ab') 2 IgG fragment secondary antibody was a donkey anti-rabbit IgG conjugated to AF594 (Abcam, Sapphire Bioscience). Imaging was implemented on an Olympus IX73 fluorescence microscope (Olympus, Notting Hill, VIC, Australia). Quantitative analysis of immunofluorescence using the ImageJ software (NIH, Bethesda, MD, USA) was carried out as previously described (Tran, Ahern et al. 2014).

1.4 **Phagocytosis of apoptotic bronchial epithelial cells (efferocytosis)**

Efferocytosis was measured as previously reported (Hodge, Hodge et al. 2003, Hodge, Hodge et al. 2007, Tran, Ahern et al. 2014). Briefly, apoptosis was induced in cultured 16HBE bronchial epithelial cells using UV. The apoptotic cells were then labelled with sytox orange (PE, Molecular Probes, Oregon, USA) and incubated with macrophages at

a ratio of 10:1 for 1.5 h. Macrophages were identified based on autofluorescence properties and staining with CD13 phycoerythrin cyanine-7 (PE-Cy7) (BD Biosciences). Positive staining with CD13 and sytox orange identified macrophages that had ingested apoptotic cells.

1.5 **Phagocytosis of NTHi by THP-1 macrophages**

Phagocytosis of NTHi by THP-1 macrophages was measured as previously reported (Tran, Ahern et al. 2014). Briefly, NTHi at 1.36×10^8 CFU were washed then treated with 70% ethanol at room temperature for 30 min. The washed bacteria were then resuspended in 0.85% saline followed by staining with 5 µM sytox green (Molecular Probes, Oregon, USA). For the phagocytosis assay, 2×10^7 CFU of sytox-green-stained NTHi were incubated with THP-1 macrophages at a ratio of 50:1 (bacteria: macrophages) for 1.5 h, then treated as described for efferocytosis above.

1.6 **The effect of thymoquinone on efferocytosis/phagocytosis recognition molecules**

Differentiated THP-1 macrophages were stained with monoclonal antibodies to Colec-12 unconjugated (R&D Systems, Minneapolis, MN, USA), detected with anti-goat IgG allophycocyanin (APC, R&D Systems, Minneapolis, MN, USA), mannose receptor-phycoerythrin (PE, Immunotech/Coulter, Marseille, France) and CD36 (FITC, Immunotech/Coulter, Marseille, France). Staining was measured using flow cytometry as previously reported (Hodge, Hodge et al. 2007, Hodge, Hodge et al. 2008, Hodge, Matthews et al. 2011).

1.7 **Quantitative real-time PCR (qPCR)**

Alveolar macrophages were incubated for 24 h in the presence and absence of 5 µM thymoquinone (Sigma Aldrich). Cells were harvested as previously described (Hodge, Hodge et al. 2003), and frozen immediately at -80°C for subsequent gene expression analysis. The total RNA was extracted and qPCR analyses of the samples were performed as previously reported (Barnawi, Tran et al. 2015). Briefly, total RNA was extracted from shock-frozen THP-1 macrophages using the RNeasy Mini Kit (Qiagen, Doncaster, Australia) according to the manufacturer's instructions. The concentration of the total RNA was determined using standard photospectometry. cDNA was synthesised using a RT² HT First Strand Kit (Qiagen). Synthesised cDNA was stored at −20°C. qPCR analyses were performed using the Custom RT^2 Profiler PCR Array system (Qiagen) and an ABI sequence detection system, a 25µL reaction mixture into 96 well plates coated with primer pairs directed against components of the S1P components system S1P receptors (*S1PR1, S1PR2, S1PR3, S1PR4, S1PR5*) and the S1P-degrading enzymes *SGPP1, SGPP2 and SGPL1*. Glucose 6-phosphate dehydrogenase (*G6PD*), hypoxanthine phosphoribosyl transferase 1 (*HPRT1*) and ribosomal protein L13a (*RPL13A*) genes were used as reference (housekeeping) genes for qPCR results normalization. Threshold cycle (C_T) value was used to determine gene expression using ΔC_T method, $\Delta C_T = C_T$ (target gene) – C_T (average of the reference genes). ΔC_T Values were subtracted from 20, so the higher C_T value, the higher gene expression. Internal controls, reverse-transcription control (RTC), positive PCR control (PPC) and genomic DNA control (HGDC) were used to validate qPCR reaction efficiency and reliability.

1.8 **Statistical analysis**

Data were analysed using SPSS software (SPSS Inc. IBM Chicago, USA). Results are reported as median (q1,q3) unless otherwise indicated. Analyses were performed using the Mann Whitney U-test or Kruskall-Wallis test for non-normally distributed data with *post hoc* testing using Mann Whitney. All results were reported significant when $p<0.05$.

Results:

1.1 **Low dose thymoquinone increases THP-1 macrophage efferocytosis**

Incubation of THP-1 macrophages with thymoquinone for 24 h did not significantly affect macrophage viability (<5% Annexin staining).We performed a dose response to determine the effects of different concentrations of thymoquinone on THP-1 efferocytosis (5, 10, 20, 40 and 80 µM thymoquinone, for 24 h). At lower doses (5, 10 and 20 µM), thymoquinone significantly increased the efferocytosis of THP-1 macrophages. Higher concentrations (40 and 80 µM) had no significant effect (Figure 1). The lowest significantly effective dose of 5 µM was used in all subsequent experiments.

1.2 Low-dose thymoquinone improves efferocytosis under oxidative conditions

We next investigated whether the pro-phagocytic effects of thymoquinone would remain under oxidative stress or inflammatory conditions. The effects of thymoquinone were assessed in a cigarette smoke (‘oxidative’) model. Consistent with previous reports (Hodge, Hodge et al. 2007, Hodge, Matthews et al. 2011), cigarette smoke significantly decreased the efferocytosis ability of THP-1 macrophages (18.4 % decrease). Treatment of THP-1 macrophages with 5 µM thymoquinone during exposure to cigarette smoke

extract for 24 h significantly increased efferocytosis with an increase of more than 44% compared to cigarette smoke-treated cells and equivalent to cells treated with thymoquinone alone (Figure 2A). LPS treatment ('inflammatory model') had no significant effects on efferocytosis; and no additive effect on efferocytosis in THP-1 macrophages treated with thymoquinone (Figure 2B).

1.3 **Low dose thymoquinone improves phagocytosis of NTHi under oxidative conditions**

It has been shown that phagocytosis of bacteria by macrophages occurs by mechanisms that can be independent of those involved in efferocytosis (Taylor, Finney-Hayward et al. 2010). Given the common rates of lower airway colonisation with NTHi in COPD subjects, we investigated the effect of cigarette smoke extract and LPS on the phagocytosis of NTHi in the presence and absence of thymoquinone. We noted no significant effect of thymoquinone treatment *per se* in the ability of THP-1 macrophages to phagocytose NTHi. However, thymoquinone significantly restored phagocytic function to near-normal level (Figure 2C) in cigarette smoke extract-treated macrophages. LPS treatment significantly decreased phagocytosis of NTHi. Co-treatment of THP-1 macrophages with LPS and thymoquinone did not significantly improve phagocytosis of NTHi compared to cells stimulated with LPS alone (Figure 2D).

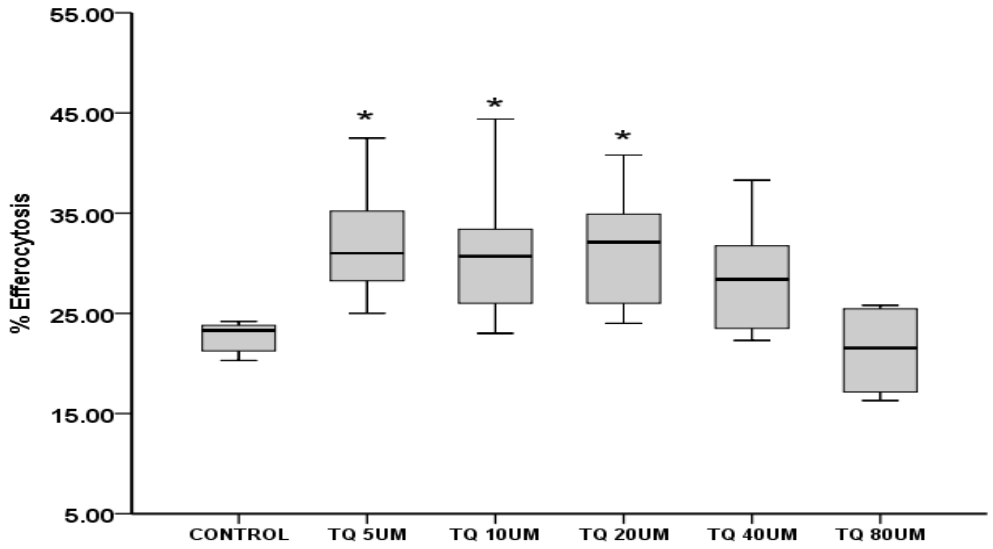


Figure 1: Effects of varying concentrations of thymoquinone on the phagocytosis of apoptotic bronchial epithelial cells by THP-1 macrophages.

Thymoquinone (TQ; 0–80 µM) was added to THP-1 macrophages for 24 h prior to phagocytosis assay. Box plots present median ± 25th and 75th percentiles (solid box) with the 10th and 90th percentiles shown by whiskers outside the box.*significantly ($p<0.05$) decreased expression compared to controls (N=8 experiments, performed in triplicate).

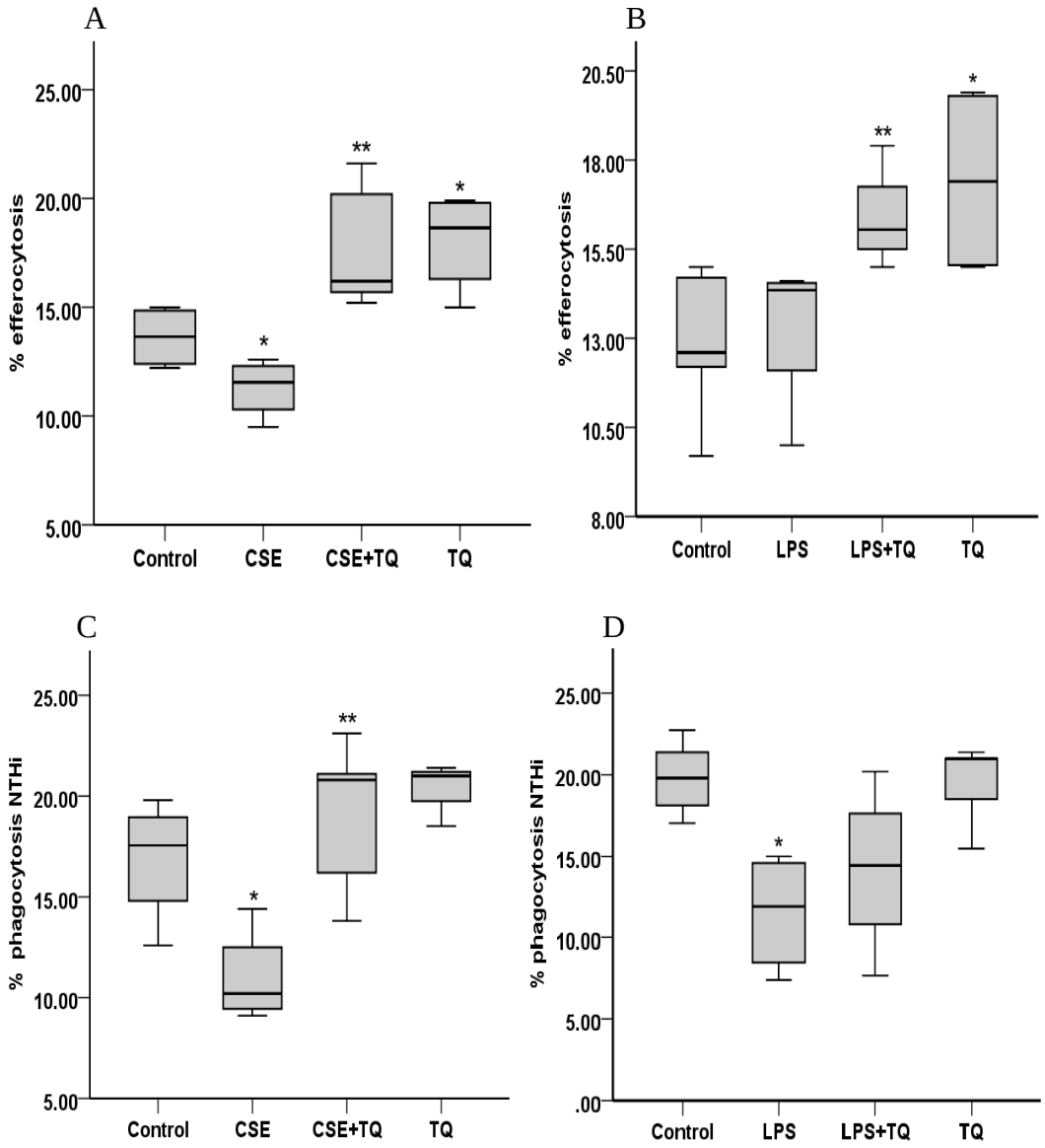


Figure 2: Effects of thymoquinone on the phagocytosis of apoptotic cells or NTHi by THP-1 macrophages in the presence and absence of cigarette smoke and LPS.

(A) thymoquinone (TQ; 5 μM) significantly antagonized the effect of (A) cigarette smoke extract (CSE); and (B) LPS on phagocytosis of apoptotic bronchial epithelial cells. Thymoquinone significantly antagonized the effect of (C) cigarette smoke extract, but not (D) LPS on phagocytosis of NTHi. Data presented as box plots as described in Figure 1. *significantly ($p<0.05$) increased expression compared to controls, **significantly ($p<0.05$) increased expression compared to cigarette smoke extract or LPS (N=5 experiments performed in triplicate).

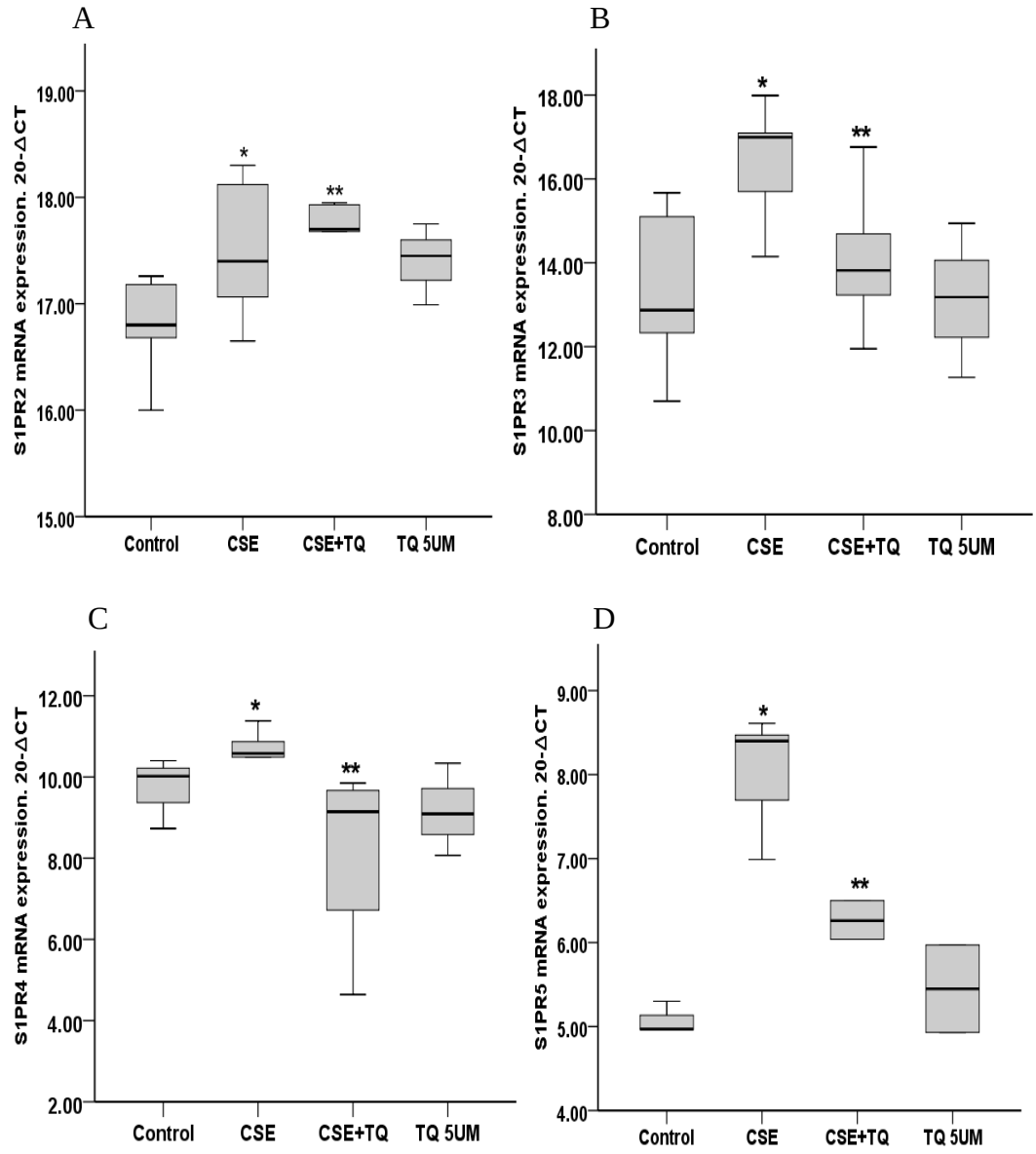


Figure 3: Relative mRNA expression of various components of the S1P signalling system in cigarette smoke extract- exposed THP-1 macrophages in the presence and absence of thymoquinone.Significant increase in mRNA expression levels of (A) S1PR2; (B) S1PR3; (C) S1PR4; (D) S1PR5 in response to 10% cigarette smoke extract (CSE). Thymoquinone (TQ) significantly antagonized the effect of cigarette smoke extract on these receptors Data presented as box plots as described in Figure 1. *$p<0.05$ vs control, **$p<0.05$ vs cigarette smoke extract, N=7 experiments, performed in triplicate. Ct values were subtracted from 20 so that higher values represent higher mRNA expression levels.

1.4 **Thymoquinone decreases the expression of S1PRs 2 and 5 in cigarette smoke or LPS-exposed THP-1 macrophages**

We investigated the effect of thymoquinone on the expression of *S1PRs* (1-5) and S1P-degrading enzymes (*SGPP1, SGPP2, and SGPL1*) in the presence and absence of cigarette smoke extract or LPS.

Cigarette smoke extract significantly increased mRNA levels of *S1PRs* 2, 3, 4 and 5 (Figure 3). Thymoquinone significantly restored gene expression levels of all receptors (Figure 3). Consistent with the effects of cigarette smoke extract, LPS significantly increased mRNA expression levels of *S1PRs* 2, 3 and 5 (Figure 4). Thymoquinone significantly restored gene expression levels of *S1PRs* 2, 3 and 5 in LPS stimulated THP-1 macrophages (Figure 4).

There were no significant changes in the relative mRNA expression levels of *SGPP1*, *SGPP2* and *SGPL1* in THP-1 macrophages treated with thymoquinone, cigarette smoke extract or LPS compared to untreated THP-1 macrophages (data not shown).

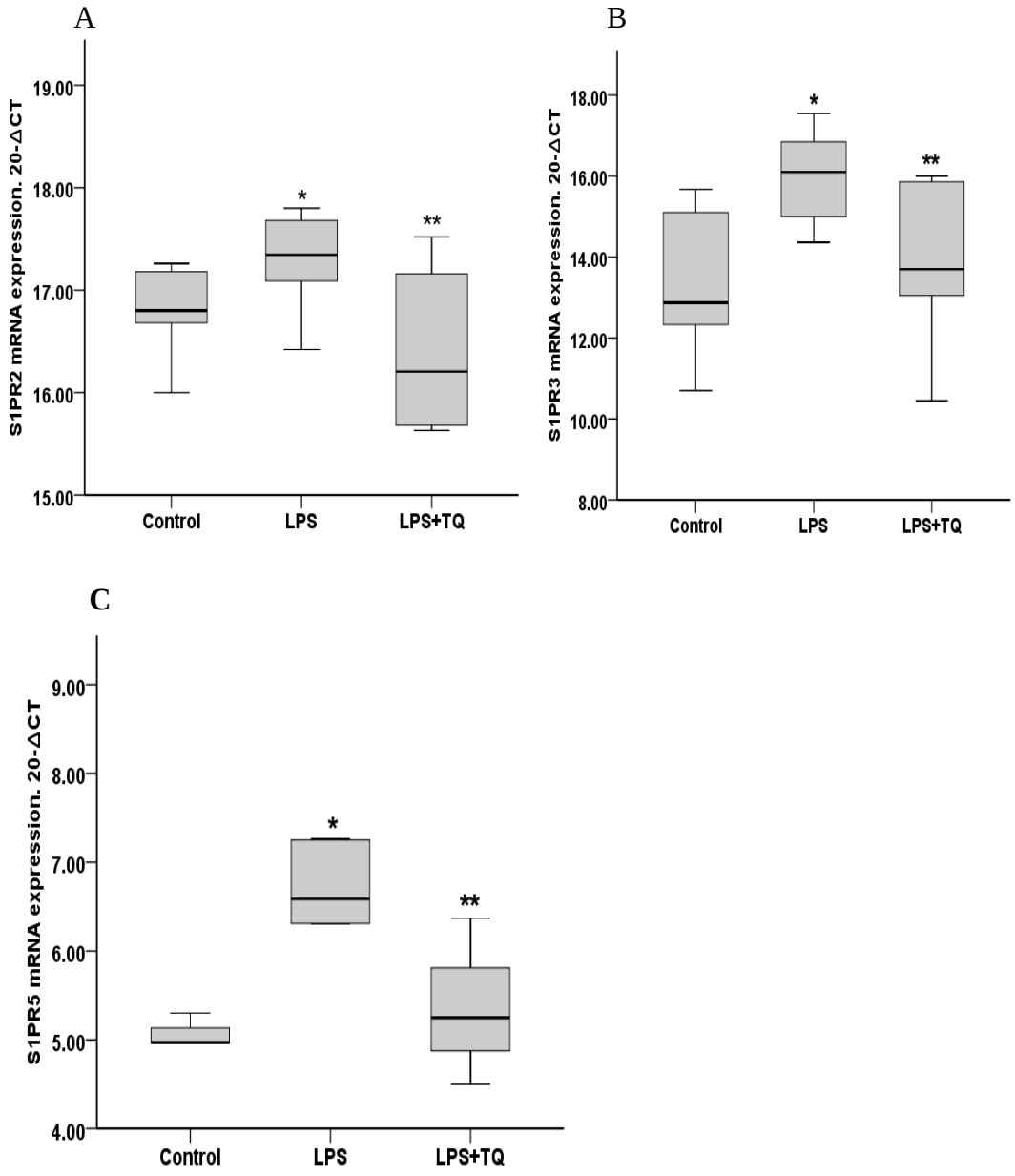


Figure 4: Relative mRNA expression of various components of the S1P signalling system in LPS- stimulated THP-1 macrophages in the presence and absence of thymoquinone. LPS significantly increased mRNA expression level of (A) *S1PR2*; (B) *S1PR3*; (C) *S1PR5*. Thymoquinone (TQ) significantly antagonized the effect of LPS on these receptors. Data presented as box plots as described in Figure 1. *$p<0.05$ vs control, N=5 experiments, performed in triplicate. Ct values were subtracted from 20 so that higher values represent higher mRNA expression levels.

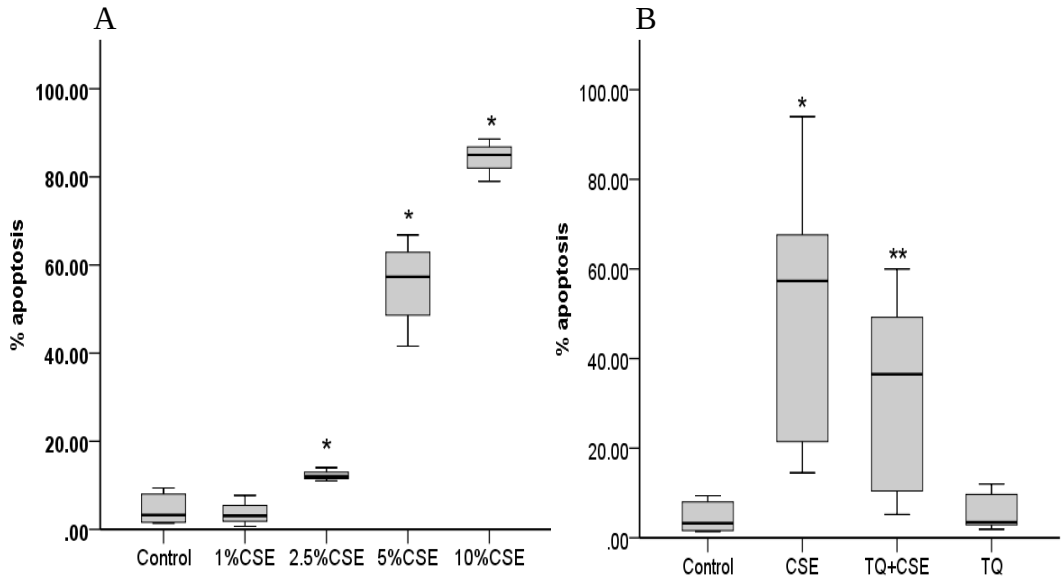


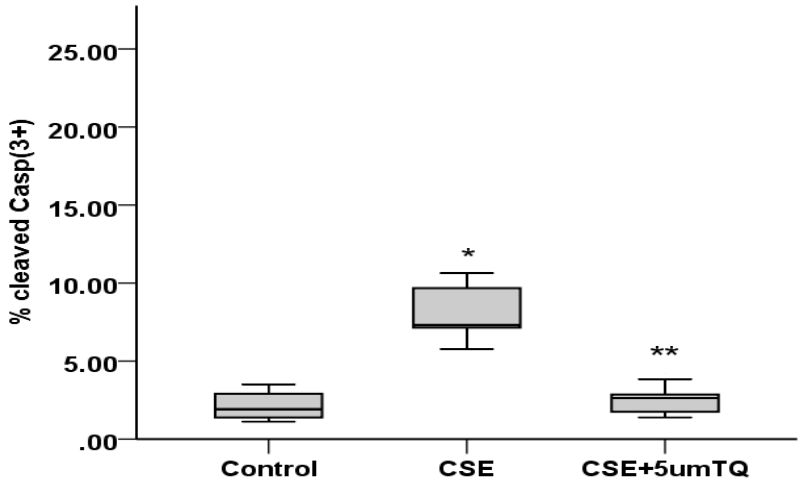
Figure 5: Effect of varying concentrations of cigarette smoke extract on 16HBE bronchial epithelial cell viability in the presence or absence of thymoquinone.

(A) varying concentrations of cigarette smoke extract (CSE) were applied to 16HBE bronchial epithelial cells and viability assessed using Annexin V. (B) The effect of cigarette smoke extract on 16HBE viability was assessed in the presence or absence of thymoquinone (TQ). Cigarette smoke extract significantly increased apoptosis at concentration of 2.5%, 5% and 10%, and thymoquinone (5 µM) antagonized the effect of cigarette smoke extract on apoptosis of bronchial epithelial cells. Data presented as box plots as described in Figure 1. *$p<0.05$ vs control, **$p<0.05$ vs cigarette smoke extract, N=10 experiments, performed in triplicate.

1.5 **Thymoquinone reduces apoptosis in cigarette smoke-exposed bronchial epithelial cells**

In apoptotic 16HBE cells exposed to various concentrations of cigarette smoke extract (1%, 2.5%, 5% and 10%) for 24 h, apoptosis was dose-dependent with significant increases at 2.5%, 5% and 10% cigarette smoke extract (Figure 5). 5% cigarette smoke extract was used in subsequent experiments. Thymoquinone (5 µM) treatment significantly reduced the number of apoptotic cells in the cigarette smoke -exposed 16HBE cells compared to cells exposed to cigarette smoke extract alone (Figure 5). These findings were confirmed by immunofluorescent staining of cleavage of caspase-3 in 16HBE cells exposed to cigarette smoke extract and/or thymoquinone for 24 h (Figure 6).

A



B

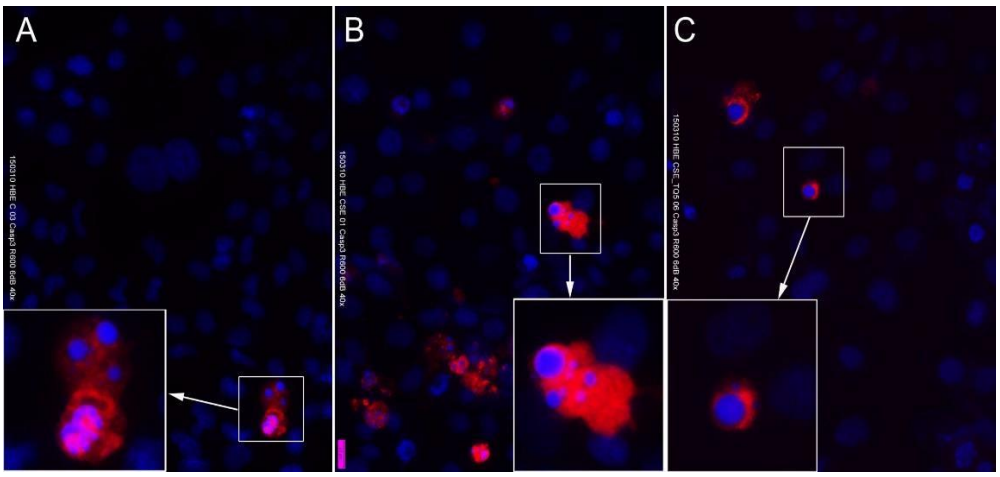


Figure 6: Effect of cigarette smoke extract on 16HBE bronchial epithelial cell viability (cleaved caspase-3) in the presence or absence of thymoquinone. (A) cigarette smoke extract (CSE) significantly increased cleaved caspase-3 and thymoquinone (TQ, 5 µM) antagonized the effect of cigarette smoke extract on cleaved caspase-3 of bronchial epithelial cells (B) Representative immunofluorescence images of cleaved caspase-3 In culture of 16HBE, control (A), treated with 10% cigarette smoke extract (B), and 10% cigarette smoke extract plus 5 µM thymoquinone (C). Blue: DAPI. Scale bar = 20 µM for the main microphotos, and 8 µM for the insets (*$p<0.05$ vs control, **$p<0.05$ vs cigarette smoke extract, N=3).

Discussion:

Increased bronchial epithelial cell apoptosis and defective phagocytic clearance of excess apoptotic cells in the airway linked to airway inflammation has been demonstrated in COPD (Hodge, Hodge et al. 2003, Hodge, Hodge et al. 2005). COPD is further characterized by increased oxidative stress in the airway, associated with a decrease in available glutathione that is required for effective macrophage function (Rahman and MacNee 1998, Moodie, Marwick et al. 2004, Rahman 2008). Inhaled cigarette smoke initiates inflammation and lung injury by interacting with bronchial epithelial cells (Barnes 2004, Barnes 2004), increasing reactive oxygen species (ROS) generation (Bazzini, Rossetti et al. 2013, Bondi, Ferraro et al. 2014) and inducing bronchial epithelial cell apoptosis (D'Agostini, Balansky et al. 2001, Dong, Fu et al. 2015, Zhang, Guo et al. 2015). Given the importance of oxidative stress in COPD development and progression (Rahman 2008, Yao and Rahman 2011) antioxidant treatments that target macrophage function and/or decrease airways structural cell apoptosis would have potential therapeutic relevance for COPD. In this regard, we have previously shown that an anti-oxidant procysteine, a glutathione precursor, normalized the phagocytic ability of both alveolar and tissue-associated macrophages from smoke-exposed mice (Hodge, Matthews et al. 2011).

In the present study, we investigated the effects of a further anti-oxidant, thymoquinone, on the phagocytic ability of THP-1 macrophages exposed to both oxidative (cigarette smoke extract) and inflammatory (LPS) stimulation. Thymoquinone at low dose has been shown to have both antioxidant and anti-inflammatory properties (Mansour, Nagi et al. 2002, Woo, Kumar et al. 2012), although its effects on macrophage phagocytic function are unknown. We firstly showed a significant decrease in the ability of cigarette

smoke-exposed THP-1 macrophages to phagocytose apoptotic cells and bacteria, consistent with previous reports (Hodge, Hodge et al. 2007, Taylor, Finney-Hayward et al. 2010, Hodge, Matthews et al. 2011, Noda, Matsumoto et al. 2013, Pang, Liu et al. 2014). Thymoquinone enhanced macrophage efferocytic/phagocytic ability and antagonized the effects of cigarette smoke on phagocytosis. These effects were noted at low thymoquinone doses of 5, 10 and 20 μM, with no negative effects on macrophage viability.

We next investigated the effects of thymoquinone on apoptosis of bronchial epithelial cells in response to cigarette smoke. Smoke extract exposure significantly increased bronchial epithelial cell apoptosis at concentrations of 2.5%-10%, consistent with previous findings in bronchial epithelial cells (Dong, Fu et al. 2015, Zhang, Guo et al. 2015). Addition of thymoquinone significantly reduced apoptosis in the smoke extract-exposed bronchial epithelial cells, possibly by restoring the oxidant/anti-oxidant balance in the cells, thus preventing DNA damage and induction of apoptosis.

Studies have shown that thymoquinone has low toxicity. In *in vitro* studies, doses ranging from 0.1 to 10 μM had anti-inflammatory, anti-oxidant and cytoprotective effects with minimal toxicity (Ali and Blunden 2003, El-Mahmoudy, Shimizu et al. 2005, Wilkins, Tucci et al. 2009, Woo, Kumar et al. 2012, Zafeer, Waseem et al. 2012, Ismail, Ismail et al. 2013, Acharya, Chatterjee et al. 2014). In *in vivo* animal studies, doses ranging between 5 and 12.5 mg/kg body weight were also shown have to anti-cancer, anti-inflammatory, and anti-oxidant effects with low toxicity. (Houghton, Zarka et al. 1995, Badary, Nagi et al. 1997, Nagi, Alam et al. 1999, Mansour, Ginawi et al. 2001). In human clinical trial, thymoquinone was safe, well tolerated, and efficacious, with minimal toxicity at doses ranging from 1 mg/kg to 10 mg/kg body (A.M. Al-Amri 2009, Akhondian, Kianifar et al. 2011). Furthermore, in a clinical trial of patients with

solid tumours or haematological malignancies, it was shown that the adult human body can tolerate thymoquinone doses up to 2600 mg/day (A.M. Al-Amri 2009).

We also explored the effects of thymoquinone on THP-1 macrophages exposed to an 'inflammatory' stimulus, LPS. LPS exposure significantly decreased the ability of the macrophages to phagocytose NTHi, as reported by others previously (Wonderling, Ghaffar et al. 1996, Feng, Deng et al. 2011). In contrast to other reports (Vogel, Marshall et al. 1979, Wu, Chen et al. 2009, Fuentes, Millis et al. 2014), LPS had no significant effect on efferocytosis, possibly a result of the different time-course and/or the dosage used (Islam, Cinar et al. 2012, Uddin, Nuro-Gyina et al. 2012). For example, inhibition of phagocytosis by LPS occurred as early as 8 h (Feng, Deng et al. 2011), while other reported LPS inhibition after 96 h (Michlewska, Dransfield et al. 2009). Moreover, the differences in cells type used might have led to different responses to LPS as well as the differences in phagocytosis mechanisms between bacteria and apoptotic cells (Taylor, Finney-Hayward et al. 2010).

We then investigated potential cellular molecules or pathways that might be involved in the pro-phagocytic effect of thymoquinone. Oxidants mediate inflammatory responses through the activation of numerous mediators, signal transduction, epigenetic modification and gene expression of inflammatory mediators; however, the pathways involved in the defective phagocytic function are not fully understood (Rahman and MacNee 1998, Janssen-Heininger, Persinger et al. 2002, Moodie, Marwick et al. 2004, Rahman, Marwick et al. 2004). Our study revealed that thymoquinone had no significant effect on the expression of mannose receptor or Colec12; molecules that may be involved in the recognition of either apoptotic cells or bacteria, suggesting that changes

to these macrophage recognition molecules are not involved in the pro-phagocytic effects of thymoquinone.

Recently, there has been accumulating evidence for the role of sphingolipids, particularly S1P-system components and ceramides respectively (Petrusca, Gu et al. 2010, Barnawi, Tran et al. 2015) in the defective phagocytic ability of alveolar macrophages in COPD. We therefore measured S1P system mRNA levels in THP-1 macrophages exposed to thymoquinone in the presence /absence of cigarette smoke extract and LPS. S1P receptors have been linked to macrophage functions (Rivera, Proia et al. 2008) and in our previous study (Barnawi, Tran et al. 2015) we found an increase in S1PRs 2 and 5 in alveolar macrophages from smokers with or without COPD. Consistent with these data, the present study showed a significant increase in mRNA levels of *S1PRs* 2, 3, 4 and 5 in THP-1 macrophages in response to cigarette smoke extract, and significantly increased *S1PRs* 2, 3 and 5 mRNA in response to LPS. The increased expression of *S1PRs* 2, 3, 4 and 5 is likely to contribute to macrophage dysfunction, given their roles in trafficking and migration (Rivera, Proia et al. 2008) and our previous findings of an association between *S1PR5* and the reduced phagocytic ability in alveolar macrophages from COPD subjects (Barnawi, Tran et al. 2015). Treatment of macrophages with thymoquinone significantly antagonised the effect of cigarette smoke extract on all receptors tested. Thymoquinone was also able to antagonise the effect of LPS on a limited number of receptors (S1PRs 2, 3 and 5), suggesting that thymoquinone has a more potent antagonistic effect against the effects of cigarette smoke than LPS.

Inhaled cigarette smoke initiates inflammation and lung injuries by first interacting with bronchial epithelial cells (Barnes 2004, Barnes 2004). It has been reported that cigarette

smoke increases reactive oxygen species (ROS) generation in 16HBE cells (Bazzini, Rossetti et al. 2013, Bondi, Ferraro et al. 2014) and induction of apoptosis in bronchial epithelial cells including HBEpC (Zhang, Guo et al. 2015), and BEAS-2B cells (Dong, Fu et al. 2015). Thus, as thymoquinone has antioxidant/anti-inflammatory, and a free-radical scavenger property, we investigated whether thymoquinone could rescue 16HBE cells from cigarette smoke extract-induced apoptosis. We found that cigarette smoke extract exposure significantly increased bronchial epithelial cell apoptosis at concentrations of 2.5%-10%, which is consistent with previous findings in bronchial epithelial cells (Dong, Fu et al. 2015, Zhang, Guo et al. 2015). Addition of thymoquinone significantly reduced apoptosis in the cigarette smoke-extract- exposed epithelial cells. A mechanism for this effect could be a restoration of oxidant/anti-oxidant balance in these cells, thus preventing DNA damage and induction of apoptosis.

In conclusion, our data demonstrated that thymoquinone promotes macrophage phagocytosis of bacteria and apoptotic cells, possibly by modulation of the S1P signalling system, and protects bronchial epithelial cells from cigarette smoke- or LPS-induced apoptosis. Interestingly, the pro-survival effects of thymoquinone on bronchial epithelial cells occurred at the same low dose that results in the pro-phagocytic effect on macrophages. In this regard, thymoquinone may be worth pursuing as a therapeutic option for inflammatory lung diseases that are associated with defects in airway macrophage phagocytic ability and excess airway cell apoptosis.

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Author Contributions By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

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CHAPTER 4

Differential DNA methylation pattern of sphingosine-1 phosphate receptor 5 in lung macrophages in COPD with/without lung cancer: a potential link to failed efferocytosis

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Abstract

We have previously shown that alveolar macrophages (AM) from COPD patients are defective in their ability to phagocytose apoptotic cells (defective 'efferocytosis'), and that this defect is potentially linked to the S1P system, in particular *S1PR5*. We found that genes encoding several S1P system components are upregulated at the mRNA level in smokers and COPD patients. However, it us unknown whether these changes in mRNA levels are dependend on epigenetically modulation by DNA methylation or whether DNA methylation regulates macrophage function.

Methods: We investigated the effect of the DNA methyltransferase inhibitor, 5-Azacytidine on the ability of THP-1 macrophages to phagocytose apoptotic cells using flow cytometry. Bisulfite Sequencing was used to assess DNA methylation levels at CpG islands associated with genes encoding selected S1P system components, specifically *SPHK1*, *S1PR1* and *S1PR5*, in AM from patients (20 COPD, 7 healthy smokers, and 10 healthy controls). We further determined DNA methylation levels at CpG islands associated with S1P system genes in lung tissue macrophages from patients (15 COPD without lung cancer, 15 COPD with lung cancer, 8 lung cancer without COPD, 10 non-cancer non COPD controls) by Methyl Quantitative Real-Time PCR (Methyl qPCR).

Results: DNA demethylation by 5- Azacytidine increased the efferocytosis capacity of THP-1 macrophages and dose-dependently rescued the cells from the decrease in efferocytosis capacity induced by cigarette smoke. DNA methylation levels at *S1PR5* gene CpG island 1 were lower in AM from COPD patients than controls and negatively correlated with gene expression. DNA methylation levels at *S1PR5* CpG island 1 in lung tissue macrophages from COPD patients showed higher DNA methylation levels

compared to AM, while AM and lung tissue macrophages from controls had similar DNA methylation levels.

Conclusions: DNA methylation is associated with the regulation of *S1PR5* gene expression, as well as efferocytosis function in alveolar macrophages. Our data thus identifies *S1PR5* gene regulation in macrophages as a potential therapeutic target in COPD.

Introduction

Chronic obstructive pulmonary disease (COPD/emphysema) has a high impact on society in terms of human suffering and cost. COPD patients are at increased risk for developing lung cancer and there is strong evidence that pulmonary macrophage dysfunction plays an important role in the pathogenesis of both diseases. There are no effective treatments for COPD and identification of novel pathways associated with the macrophage dysfunction are needed to enable new therapeutic approaches as a matter of urgency.

Our group has shown that pulmonary macrophages in both COPD and lung cancer are defective in their capacity to phagocytose apoptotic airway epithelial cells (defective efferocytosis) (Hodge, Hodge et al. 2003, Hodge, Hodge et al. 2007, Hodge, Hodge et al. 2008, Hodge, Matthews et al. 2010, Hodge, Matthews et al. 2011, Dehle, Mukaro et al. 2013), which contributes to the excess apoptotic material seen in the airways of these patients. This uncleared material can then undergo secondary necrosis and perpetuate the inflammatory response. Chronic inflammation is known to promote malignancy; moreover, impaired phagocytosis of viable cancer cells suggest reduced tumour surveillance. Thus, these data suggest a link between macrophage dysfunction and the increased risk of lung cancer in COPD. The sphingolipid signalling pathway involving

sphingosine kinase (*SPHK*) and its product sphingosine-1-phosphate (S1P) is required for effective macrophage function by promoting maturation and phagocytosis (Kummer, Fink et al. 1998, Xia, Gamble et al. 2000, Pfaff, Powaga et al. 2005).

Recently, we identified dysregulation of the S1P signalling system as a potential cause of defective efferocytosis in alveolar macrophages in COPD (Barnawi, Tran et al. 2015). We have shown that relative mRNA expression levels of several S1P system genes are upregulated in alveolar macrophages in COPD patients, as well as current smokers compared to healthy non-smokers. Sphingosine-1 phosphate receptor 5 (*S1PR5*) expression was shown to be upregulated in alveolar macrophages of both current and ex-smoker COPD patients (Barnawi, Tran et al. 2015). Interestingly, we also reported decreased *S1PR5* in lung tissue from COPD patients (Cordts, Pitson et al. 2011), the discrepancy suggests differential regulation of this gene in alveolar macrophages as compared to the mixed cell types found in lung tissue. Nevertheless, these findings highlight the potential importance of *S1PR5* in COPD and a possible link between smoking and *S1PR5* regulation.

Epigenetic modifications are somatically heritable changes in genomic DNA and chromatin that do not alter the DNA sequence. Environmental stimuli such as cigarette smoke can induce epigenetic modifications, which in turn modulate gene expression. DNA methylation is one of the most important epigenetic modifications of DNA. It is carried out by a family of DNA methyltransferases (DNMTs), which add methyl groups to cytosine (C) nucleotides, in particular at regions enriched in CpG dinucleotides called CpG islands. CpG islands are typically several hundred nucleotides long and are defined by at least 50% CG content and a ratio of observed CpG to expected CpG frequency greater than 0.6 (Han, Su et al. 2008, Rakyan, Down et al. 2011). CpG islands are often found near gene promoters and act as gene regulatory elements. Highly methylated, CpG islands induce formation of repressive chromatin structures that hinder binding of

transcription factors and repress gene transcription (Portela and Esteller 2010, Deaton and Bird 2011, Shukla, Kavak et al. 2011).

Although genetic predisposition to COPD has been tested, many of these studies have been controversial and have produced inconsistent and limited conclusive results (Hobbs and Hersh 2014). Thus, other approaches, such as investigation of epigenetic changes (Mortaz, Masjedi et al. 2011, Yao and Rahman 2011), may be useful in understanding disease mechanisms in COPD (Mortaz, Masjedi et al. 2011).

COPD patients are at increased risk for lung cancer. This is especially alarming as lung cancer is the leading cause of cancer-related deaths and responsible for more cancer-related deaths than colon, breast and prostate cancers combined (Herbst, Heymach et al. 2008). We have shown that alveolar macrophages from patients with lung cancer have a phagocytic defect that is consistent with our findings in COPD (Dehle, Mukaro et al. 2013).

Studying epigenetic modifications in lung macrophages in COPD and lung cancer might therefore be useful in determining the common disease mechanism(s), given these reports of defective efferocytosis in both diseases. To our knowledge, no studies have addressed potential epigenetic modification in human alveolar macrophages, in COPD and/or lung cancer (Cosio, Tsaprouni et al. 2004, Yang, Wang et al. 2014). Here we investigated the effect of the DNA methyltransferase inhibitor, 5-Azacytidine on THP-1 macrophage efferocytosis in the presence and absence of cigarette smoke extract. We have previously shown that *SPHK1* and *S1PR5* genes are upregulated in alveolar macrophages in COPD, and their expression is negatively correlated with alveolar macrophage efferocytosis (Barnawi, Tran et al. 2015). Furthermore, we also reported that relative expression of *S1PR1* is negatively correlated with alveolar macrophage efferocytosis. In the present study we therefore investigated the DNA methylation patterns of CpG islands proximal to the *S1PR1*, *S1PR5*, and *SPHK1* genes in alveolar

macrophages from COPD patients and control subjects, as well as the DNA methylation profiles of several other S1P system genes including sphingosine kinases (*SPHK1*/2), S1P receptors (*S1PR1*-5), and S1P-degrading enzymes (*SGPP1*, *SGPP2*, *SGPL1*) in lung tissue macrophages from COPD patients with/without lung cancer.

Material and methods:

Categorisation of patients

This study was approved by the Royal Adelaide Hospital Ethics Committee (Adelaide, Australia), and informed written consent was obtained from each subject in this study. COPD was diagnosed according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) standards. Gender, smoking status, age, and the presence of lung cancer and lung function were recorded (Table 1). Lung cancer was diagnosed as previously described (Cordts, Pitson et al. 2011, Soriano, Mukaro et al. 2012).

Bronchoalveolar lavage (BAL) and isolation of alveolar macrophages

Bronchoscopy was performed according to the American Thoracic Society's recommendations for the performance of bronchoscopy for investigative purposes, as previously described (Hodge, Hodge et al. 2005). BAL was collected then centrifuged at 1600 RPM for 5 min. Macrophages were isolated by adhering to plastic as previously described, and the concentration adjusted to 4×105 cells/ml (Hodge, Hodge et al. 2003) then snap-frozen immediately and stored at -80°C for later use.

Table 1: Patient demographics (BAL cohort).

	Controls	Control Cur-Smoker	COPD
Number (n)	10	7	20
Age (yr)	56.5(26-71)	36(28-46)	66.5(42-86)
Smoking (cur/ex/no)	0/6/4	/00/7	10/10/0
Gender (M/F)	6/4	2/5	14/6
Lung Cancer (y/n)	0/10	0/7	10/10
Type of Lung Cancer (NSCLC/SCLC)	0/0	0/0	10/0
Pky	4(0-28)	17.5(4-35)	40(10-75)*
FEV_1	106(71-120)	97(82-107)	76.5(47-113)*
FEV_1/FVC	80(70-88)	84(72-87)	63(49-69)*

Data are presented as number, or median and data range. COPD: chronic obstructive pulmonary disease; Smoking history: cur: current, ex: ex-smokers, no: never smoked; Lung Cancer: presence of lung cancer; NSCLC: Non-small cell lung cancer; SCLC: Small cell lung cancer; Pky: smoking pack years; FEV_1: forced expiratory volume in one second, % pred: percentage of predicted; FVC: forced vital capacity. *: significant difference from control group, $p<0.05$. Note that one COPD patient was classified as 'mild COPD', with a FEV_1/FVC ratio of 67.

Human lung tissue

Lung samples were obtained from patients undergoing curative-intent lobectomy for lung cancer or bronchiectasis (two patients) at the Royal Adelaide Hospital as previously described (Cordts, Pitson et al. 2011, Soriano, Mukaro et al. 2012). Informed consent was obtained from each patient. Patients were categorized based on gender, smoking status, age, the presence of lung cancer, cancer type, and lung function (Table 2). Samples of both cancer and non-cancer (control) tissue were obtained by an experienced pathologist using a core biopsy needle. Non-cancer tissue was obtained from lung periphery as distal to the cancer site as possible. Samples were stored in RPMI 1640 media (Gibco BRL, Berlin, Germany), supplemented with 10 % foetal calf serum, 1 % weight per volume L-glutamine and penicillin/streptomycin (culture medium) at 4°C until processing. Tissue processing was carried as previously described (Hodge, Matthews et al. 2010, Cordts, Pitson et al. 2011). Briefly, tissue was cut in to 5 mm x 5 mm sections followed by mechanical disaggregation using a pre-wetted 'Medicon' (BD Biosciences, San Jose CA USA) in a 'Medimachine' tissue disaggregator (BD Biosciences) for approximately 3 min, and resuspended in culture medium to achieve a single cell suspension. Macrophages were obtained by adherence to plastic as described, concentration adjusted to $4x10^5$ cells/ml and snap freezing as described previously for BAL (Hodge, Hodge et al. 2003).

Table 2: Patient demographic (lung tissue cohort).

	Non cancer non COPD tissue	COPD non cancer tissue	COPD with Cancer tissue	Cancer non COPD tissue
Number (n)	10	15	15	8
Age (yr)	71.5(20-88)	71(50-82)	71(50-82)	70(63-88)
Smoking (cur/ex/no)	0/8/2	0/10/5	0/10/5	0/6/2
Gender (M/F)	76/3	9/6	9/6	4/4
Lung Cancer (y/n)	8/2	15/0	15/0	8/0
Type of Lung Cancer (NSCLC/SCLC)	8/0	15/0	15/0	8/0
Type of NSCLC (adeno /squa/ large)	2/6	11/3/0	11/3/0	6/2/0
Pky	45(20-150)	40(10-50)	40(10-50)	85(20-150)
FEV_1	80(66-120)	66(50-87)	66(50-87)	89(66-105)
FEV_1/FVC ratio	75.5(68-89)	62.5(39-73)	62.5(39-73)	76(65-81)

Data are presented as number, or median and data range. COPD: chronic obstructive pulmonary disease; Smoking history: cur: current, ex: ex-smokers, no: never smoked; Lung Cancer: presence of lung cancer; NSCLC: Non-small cell lung cancer; SCLC: Small cell lung cancer; Type of NSCLC: adeno: adenocarcinoma, squa: squamous cell

carcinoma, large: large cell carcinoma; Pky: smoking pack years; FEV_1: forced expiratory volume in one second, % pred: percentage of predicted; FVC: forced vital capacity. *: significant difference from control group, $p<0.05$.

THP-1 culture and stimulation

THP-1 cells are a monocyte-like cell line obtained from a 1-year-old male with acute monocytic leukemia (ATCC, Manassas, VA, USA). These cells are phagocytic and lack surface and cytoplasmic immunoglobulin (Tsuchiya, Yamabe et al. 1980). THP-1 cells were cultured in culture medium as described (Dehle, Mukaro et al. 2013). Cell cultures were maintained in a humidified atmosphere of 5% CO_2 in air at 37°C, and experiments were carried out between passages 6 and 20. Differentiation of THP-1 monocytes into macrophages was carried out by seeding the cells at a density of 4×10^5 cells/mL in 24-well plastic plates, then stimulating them with 100 nM phorbol 12-myristate 13-acetate (Herbst, Heymach et al.) in RPMI 1640 medium at 37°C with 5% CO_2 for 72 h as described (Dehle, Mukaro et al. 2013). The cells were rested in fresh RPMI 1640 medium at 37°C with 5% CO_2 for 24 h before being used in the experiments.

Cell stimulation

The preparation of cigarette smoke extract was carried out as previously described (Hodge, Hodge et al. 2004, Hodge, Hodge et al. 2006, Hodge, Hodge et al. 2007, Hodge, Hodge et al. 2008). THP-1 macrophages were exposed to varying concentration of 5-Azacytidine (Sigma-Aldrich, NSW, Australia) with or without cigarette smoke extract for 72 h (stimulation throughout the differentiation process). Media and stimuli were refreshed every 24 h.

Phagocytosis of apoptotic bronchial epithelial cells (efferocytosis) assay

Phagocytosis of apoptotic cells was measured as previously reported (Hodge, Hodge et al. 2003, Hodge, Hodge et al. 2007, Tran, Ahern et al. 2014). Briefly, stimulated/unstimulated macrophages were incubated with UV exposed 16HBE bronchial epithelial cells (apoptosis induction) at a ratio of 10:1 for 1.5 h. Then supernatant was removed prior to 5 min incubation with cold phosphate-buffered saline (PBS) and then macrophages were gently lifted by pipetting. Lifted macrophages were then stained with CD13 phycoerythrin cyanine-7 (PE-Cy7) (BD Biosciences, San Jose CA, USA). Trypan blue was used as auto-fluorescence quenching agent as reported (Hodge, Hodge et al. 2003, Hodge, Hodge et al. 2007, Tran, Ahern et al. 2014) and up to 30,000 events were acquired immediately using a FACSCanto II Flow Cytometer (BD Biosciences). The percentage of macrophages that had ingested apoptotic cells was recorded.

Methyl quantitative real-time PCR (Methyl qPCR)

Genomic DNA was extracted from lung tissue macrophages using a DNA extraction kit (Qiagen) according to the manufacturer's instructions. The concentration of the isolated DNA was determined using standard photospectometry. Extracted genomic DNA was stored at −20°C for later use. The methyl qPCR analyses of the samples were performed using Custom EpiTect Methyl qPCR Arrays (Qiagen) in the ViiA™ 7 Real-Time PCR System (Life Technologies), following the manufacturer's protocol (Qiagen). Briefly, a reaction mixture of 0.5 µg DNA in nuclease-free water was prepared for each sample and then divided into four digestion reactions, first with a DNA methylation-sensitive enzyme, second with methylation-dependent restriction enzyme digestion, third with both DNA methylation-sensitive and DNA methylation-dependent restriction enzyme

digestion, and fourth with no enzymes. After digestion, each reaction was combined with the PCR master mix. Then a 10 μL reaction mixture was put into 384-well plates coated with ten different primer pairs predesigned to amplify CpG islands flanking the genes encoding the sphingosine kinases (*SPHK1*/2), S1P receptors (*S1PR1*-5), and S1P-degrading enzymes (*SGPP1*, *SGPP2* and *SGPL1*) along with a reference gene. The crossing threshold (C_T) value was determined for each treatment condition and then the percentage methylation for each gene-associated CpG island was calculated using the EpiTect Methyl qPCR Arrays data analysis program (Qiagen).

Bisulfite sequencing

Genomic DNA was extracted from BAL alveolar macrophages and modified by sodium bisulfite treatment using the EZ DNA Methylation-Direct™ Kit, (Zymo Research, USA) according to the manufacturer's instructions. Bisulfite-treated DNA was used as template to amplify CpG islands in the promoter region of S1P system genes (*SPHK1*, *S1PR1* and *S1PR5*) by bisulfite specific PCR (BSP) using the ZymoTaq™ Premix master mix. Bisulfite specific PCR primer sequences are listed in (Table 3). The PCR was carried out under the following conditions: initial denaturation at 95ºC for 10 min, followed by 35 cycles of 95ºC for 1 min, 55ºC for 30 sec, and 72ºC for 30 sec, with a final extension of 72ºC for 7 min and rapid cooling to 4 ºC for at least 5 min. The PCR product was purified using QIAquick PCR Purification Kit (Qiagen) before sequencing. Purified DNA was sequenced according to Flinders University (Adelaide, South Australia) and Macrogen Sequencing facility protocols. The percent methylation for total and individual CpG islands was calculated using the bisulfite sequencing analysis tool QUMA (quantification tool for methylation analysis) (Kumaki, Oda et al. 2008).

Table 3: Primer characteristics. Tm = melting temperature, bp=base pair.

Gene	Primer	Sequence (5′ to 3′)	Products. (bp)	Tm (°C)
SphK1 CPG 1	Forward	GTTTGATTTTTTAAGTGTTGGGAAA	268	55
	Reverse	ACCCATTAATAACCTACCCAAATAAA		
S1PR1 CPG 1	Forward	GGATTGGTTATTGGAGTGTTT	263	55
	Reverse	CATATTTTCTAAATTTTTATTTACCTC		
S1PR5 CPG 1	Forward	GTGAGTTGGTTATGGTTAGTTTTAGG	300	55
	Reverse	CCCCCTCTTCCTACTACTATTACTC		
S1PR5 CpG2	Forward	GAGAATTTTTTGTAAGGAGTGGTG	104	55
	Reverse	AACTAACAACCATAAACTAAATACCC		

Sequence analysis

CpG islands were predicted using MethPrimer software (Li and Dahiya 2002). Prediction of putative transcription factor binding sites (TFBS) in the *S1PR5* CpG island was performed using the TRANSFAC database version 8.3 and PROMO software version 3.0.2 (Farre, Roset et al. 2003).

Statistical analysis

Statistical analysis was performed using SPSS statistic software version 22.0 (SPSS Inc. IBM Chicago, USA). Analysis was performed using the two-sample Wilcoxon rank-sum test, and the Kruskal-Wallis test for more than two groups. Correlations were determined by Pearson's correlation coefficient with significance set at $p<0.05$.

Results

Macrophage phagocytic function is regulated by DNA methylation

The regulation of macrophage efferocytosis by DNA methylation has not been investigated, so we first assessed whether DNA methylation might be involved in macrophage efferocytosis by measuring the ability of THP-1 macrophages to phagocytose apoptotic bronchial epithelial cells when treated with varying concentrations of the DNA methyltransferase inhibitor 5-Azacytidine (0.3, 1 and 3 μM). When compared to non-treated cells, THP-1 cells treated with 5-Azacytidine (0.3, 1, and 3 μM) showed a dose-dependent increase in phagocytosis of apoptotic bronchial epithelial cells (Figure 1). Cigarette smoke is a well-known cause of aberrant methylation in cancer (Smith, Mydlarz et al. 2007, Furniss, Marsit et al. 2008) and non-cancerous smokers (Zochbauer-Muller, Lam et al. 2003), and importantly, defective

macrophage efferocytosis (Hodge, Hodge et al. 2003, Hodge, Hodge et al. 2007, Hodge, Hodge et al. 2008, Hodge, Matthews et al. 2010, Hodge, Matthews et al. 2011). We therefore investigated the phagocytic ability of THP-1 macrophages in the presence/absence of 10% cigarette smoke extract and varying concentrations of 5-Azacytidine (0.3, 1 and 3 μM). Cigarette smoke significantly decreased THP-1 macrophage phagocytic ability, and this effect was inhibited by 5-Azacytidine treatment at concentrations of 1 and 3 μM (Figure 1).

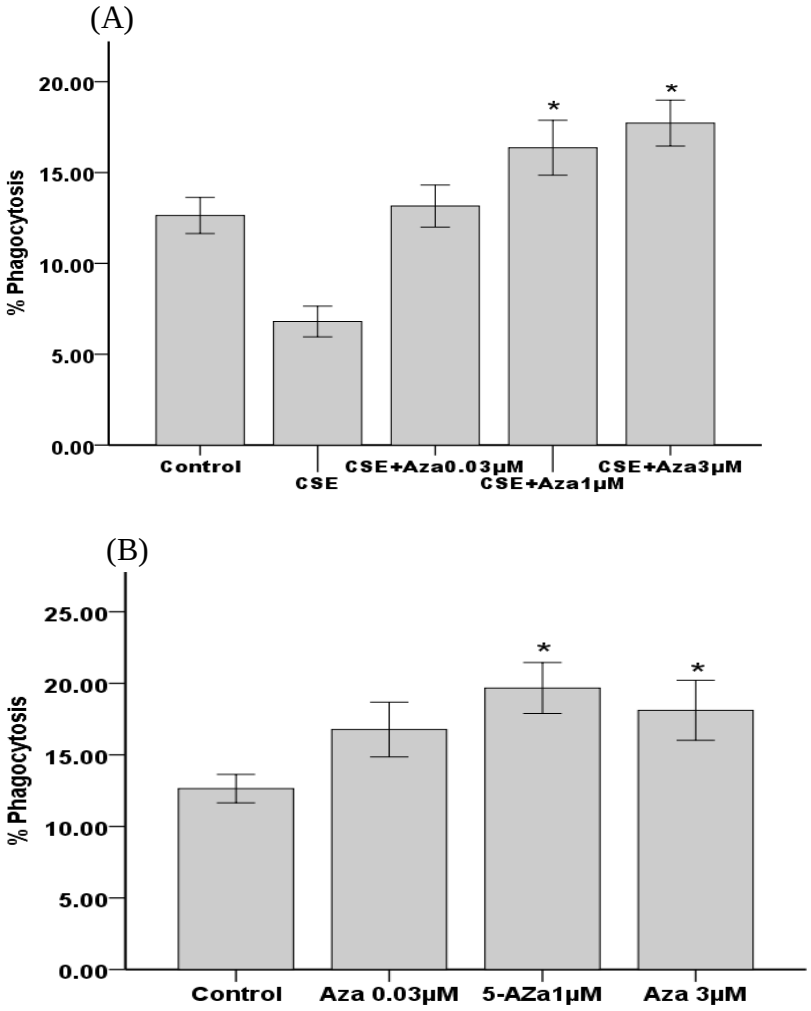

Figure 1: THP-1 macrophage phagocytosis of apoptotic bronchial cells assessed by flow cytometry. (A) THP-1 macrophages were exposed to varying concentrations of 5-Azacytidine +/- cigarette smoke extract Methylation levels of designated S1P pathway gene CpG islands in control lung tissue. (B) THP-1 macrophages were exposed to increasing concentrations of 5-Azacytidine. (*$p<0.05$ vs control; **$p<0.05$ vs cigarette smoke extract). Data expressed as mean ± SEM.

A CpG island in the sphingosine-1 phosphate receptor 5 (*S1PR5*) gene promoter is hypomethylated in alveolar macrophages in COPD and methylation status is negatively correlated with gene expression

No significant differences in DNA methylation levels were observed at the *SPHK1* or *S1PR1* CpG islands between alveolar macrophages from COPD patients and healthy control subjects. For the *S1PR5* gene, two CpG islands were analysed: *S1PR5* CpG island 1 located over the promoter region and first exon, and *S1PR5* CpG island 2 located over the second exon. *S1PR5* CpG island 1 showed significantly decreased DNA methylation levels in alveolar macrophages from COPD subjects compared to macrophages from healthy control subjects (mean±SEM, COPD 8.60±2.53% vs controls 27.85±5.48% $p<0.05$, Figure 2). *S1PR5* CpG Island 1 contains 26 CpG dinucleotides (determined by MethPrimer analysis): these 26 CpG sites were compared between healthy control subjects, healthy current smokers, and COPD patients. We found significantly lower DNA methylation levels of the CpG site at -530, in COPD patients compared to healthy control subjects (Figure 3). No differences in macrophage DNA methylation levels of *S1PR5* CpG island 2 were found between COPD patients and healthy control subjects. Correlation analysis between *S1PR5* gene expression (Barnawi, Tran et al. 2015) and DNA methylation levels of CpG island 1 revealed a significant negative correlation between gene expression and DNA methylation levels ($r = 0.65$, $p<0.05$). Using site-specific correlation we identified four CpG sites, -663, -530, -535, and -469, to be negatively correlated with *S1PR5* gene expression (-663, $r = –0.42$, $p<0.05$; -535, $r = –45$, $p<0.05$; -530, $r = –0.48$, $p<0.05$;-489, $r = –56$, $p<0.05$). This negative correlation is consistent with the known repressive effect of promoter methylation on gene expression.

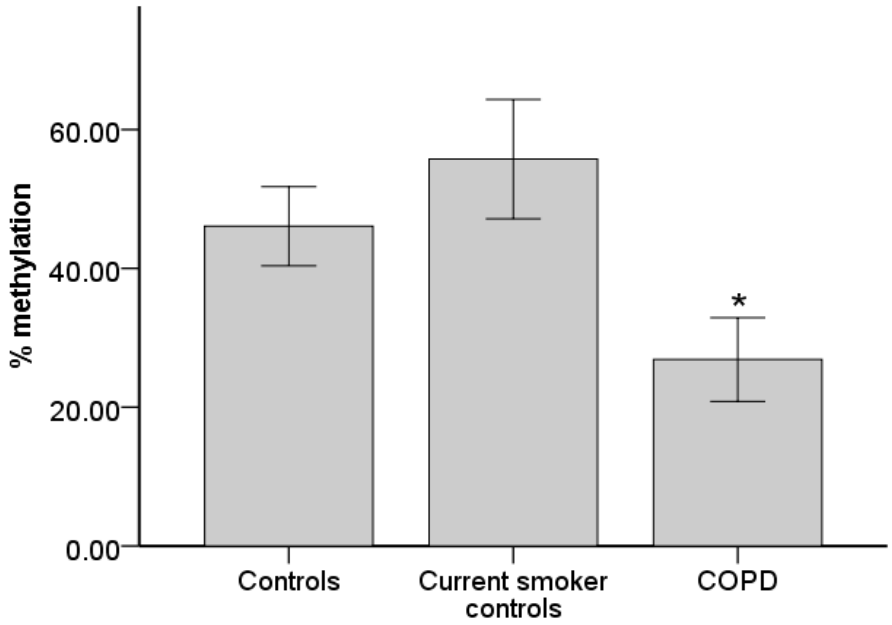

Figure 2: Comparison of the DNA methylation status of overall *S1PR5* CpG island 1 in alveolar macrophages between groups. Significantly lower DNA methylation levels were found in COPD patients relative to healthy controls (*p<0.05 vs control). Non-significant difference in DNA methylation levels between healthy controls subjects and healthy current smokers (P= 0.07). Data expressed as mean ± SEM.

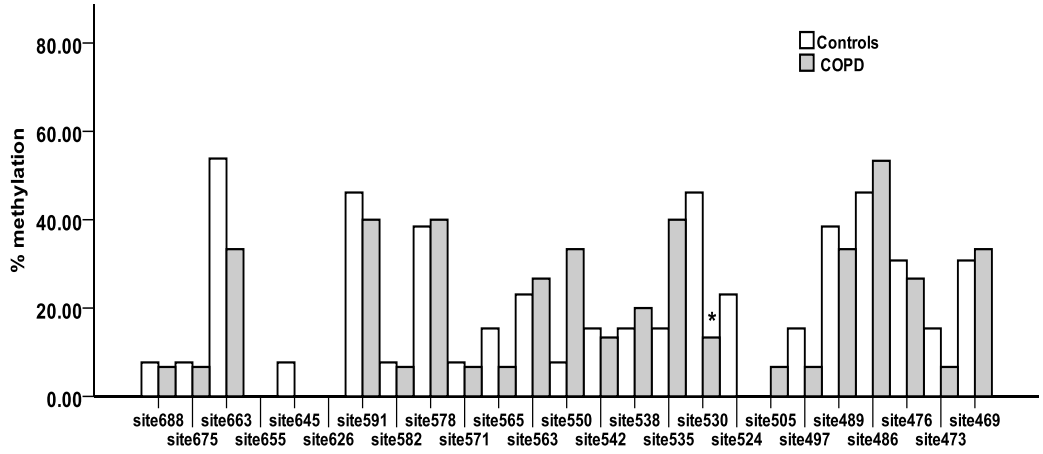

Figure 3: Comparison of the DNA methylation status of 26 CpG sites in *S1PR5* gene island 1 in alveolar macrophages from healthy controls and COPD subjects. Significantly lower DNA methylation levels at site -530 were found in COPD subjects relative to healthy controls (*$p<0.05$ vs control).

Association between *S1PR5* CpG island 1 promoter methylation and FEV_1, FEV_1/FVC ratio, smoking status, pack years, age, gender, and presence of cancer

There were positive associations between *S1PR5* CpG island 1 DNA methylation levels and COPD severity (FEV_1/FVC ratio: $r = 0.32$, $p<0.05$; FEV_1: $r = 0.30$, $p<0.05$). No associations were found between *S1PR5* CpG island 1 DNA methylation levels and smoking status, pack years, age, gender, or presence of cancer.

Bioinformatics and sequence analysis of *S1PR5* CpG island 1

S1PR5 CpG island 1 spans the promoter and first exon of the *S1PR5* gene and contains 26 CpG sites (Figure 4). Identification of the putative transcription factor binding sites (TFBS) within *S1PR5* gene CpG island 1 was performed using the PROMO software

suite that sets the matrix dissimilarity threshold of less than or equal to 15% (Quandt, Frech et al. 1995, Farre, Roset et al. 2003). We identified 31 putative transcription factor binding sites (TFBS) within *S1PR5* CpG island 1. Among them, 12 transcription factor consensus binding sites (TFBS, EBF, ENKTF-1, and TFII-I, ETF, Pax-5, p53, Sp1, GR-alpha, AP-2alphaA, E2F-1, TCF-4E, C/EBPbeta, and RXR-alpha) were found to contain CpG motifs. As mentioned above, CpG site -530 showed significantly different methylation levels in COPD patients and healthy controls, and hence may be particularly important in *S1PR5* gene regulation in alveolar macrophages in COPD. Three transcription factor binding motifs overlapped this CpG site: TFII-I, EBF, and E2F-1 (Figure 4).

(A)

CGCCTTTTTAGACCGGGAAAGTGGCCGCCAGGGCGACCCCCTCCGGGTCA
GGCCCAGCCCAGCGCCCCCAAAGGTCCAGTGCAGTCCACTCACTCTGCGC
CCTGGTCGTGCGCCACCCGCAGTCGCGCTGCCTTGAACCGAGTGAGCGGA
CGCCGCTC**CG**GGGGCGGGGCTGGAGCTGGCAGGCGAGGGGGCGGGCCAG
C
GACGCAAAGGGCCGGCGGTCGCCAGCAGTTGCCCCCTCCCCTTGGGTCCC
GGGCACTCAGCCCATGGCTGCCAGCCTGTGCCACTCGCCGCGGCACCGGA
GCGCGCCCCGGCGGCGACTGGCACAGGCGAGCCCTCAGCTGCCCCCTCCG
GGCCTGGGCTGTCCTCCCAGGGACGCTCCATCGCGGTGGGAGGACTCGGA

(B) TFII-I

CGCCGCTCC**G**GGGGCGGGGCTGGAGCTGGCAGGCGAGGGGGCGGGCCAG
C

ETF

CGCCGCTCC**G**GGGGCGGGGCTGGAGCTGGCAGGCGAGGGGGCGGGCCAG
C

E2F-1

GACGCCGCTC**CG**GGGGCGGGGCTGGAGCTGGCAGGCGAGGGGGCGGGCC
AGC

Figure 4: Sequence and analysis of *S1PR5* gene CpG island 1. (A) Sequence of *S1PR5* gene CpG island 1 individual CpG sites in the region are highlighted in yellow colour and CpG sites -530 are boldfaced and underlined. (B) Putative transcription factor binding in the region that have binding sites within or near CpG sites -530, according to PROMO software are shown in highlighted colours (red, green and purple).

Epigenetic profile of the sphingosine-1 phosphate system in lung tissue macrophages in lung cancer patients with/without COPD

It is still unknown whether the phagocytic defects in COPD and lung cancer share common mechanisms or pathways and whether the diverse macrophage classes in the alveolar and lung compartments have differential epigenetic regulation. We therefore

investigated whether DNA methylation levels of S1P signalling genes in lung tissue macrophages differed between control tissue and cancer and/or COPD tissue, and importantly, whether DNA methylation levels in lung tissue macrophages are similar to that of alveolar macrophages.

First, we examined methylation at CpG islands located within the promoters of several S1P system genes in normal control lung tissue macrophages using a custom PCR methylation array. In lung tissue macrophages from controls, the DNA methylation levels at each of these genes were variable, ranging from low to intermediate DNA methylation levels (0%–60%) (Figure 5). The CpG island of the S1P synthesizing enzyme *SPHK1* showed substantially lower DNA methylation levels compared to *SPHK2*. The CpG islands of the S1P degradation enzymes, *SGPP1*, *SGPP2*, and *SGPL1*, showed great variability, with *SGPP2* the highest and *SGPP1* the lowest methylation level. Among the S1P receptors, *S1PR5* showed the highest, while *S1PR1* and *S1PR3* showed the lowest DNA methylation levels. The order of DNA methylation levels for the S1P receptor subtypes from highest to lowest was *S1PR5>S1PR2>S1PR4>S1PR3* &*S1PR1*.

Next, we compared CpG methylation levels between lung tissue macrophages from controls, COPD subjects with/without lung cancer and lung cancer patients without COPD. No significant differences in methylation were found for the *SPHK1* and *SPHK2* genes between groups. However, significantly higher DNA methylation levels of CpG islands at the *S1PR5* gene were found in COPD patients both with and without lung cancer compared to the control group (mean±SEM, COPD 87.38±4.39% COPD with lung cancer 76.05±7.13% vs controls 41.41±12.57% $p<0.05$, Figure 6). No significant differences were found between groups for other S1P receptors tested. Similarly, no significant differences were found between groups for the S1P degrading enzymes, *SGPP1*, *SGPP2*, or *SGPL1*. Therefore, again we find that COPD is characterised by

differences in CpG methylation levels at the *S1P5* receptor gene CpG island 1, although intriguingly, lung tissue macrophages in COPD show elevated methylation whilst AM in COPD seem to show decreased methylation at the gene. This suggests that *S1PR5* is regulated differently AM and tissue macrophages.

To evaluate if we can find a methylation signature in macrophages in COPD patients independent of comorbidities such as cancer, we compared the DNA methylation levels between alveolar macrophages and lung tissue macrophages in controls and the combined cohort of COPD patients with and without lung cancer. No significant differences in DNA methylation levels were found for any gene between controls and COPD, regardless of the macrophage subpopulation (Laskin, Weinberger et al. 2001, Schneberger, Aharonson-Raz et al. 2011, Ivashkiv 2013, Cai, Sugimoto et al. 2014, Labonte, Tosello-Trampont et al. 2014, Yang, Wang et al. 2014). Nevertheless, we noted a significant difference in methylation levels between alveolar macrophages and lung tissue macrophages from COPD patients (mean±SEM, alveolar macrophages COPD 29.17±6.13% vs lung tissue macrophages COPD 77.22±4.1% $p<0.05$, Figure 6). Again, this is consistent with the notion of differential gene regulation in these two cell types, which is consistent with the many functional differences between these types of macrophages.

Association between sphingosine-1 phosphate system genes, CpG island methylation and FEV_1, FEV_1/FVC ratio, smoking status, pack years, age, gender, and presence of cancer

No significant correlation was found between lung function (FEV_1) and methylation patterns at most of the S1P signalling system genes; the exception was *S1PR5*, for which we observed a significant correlation between FEV_1/FVC ratio and methylation levels

(r = –0.47, p<0.05). There was significant correlation of CpG methylation levels with smoking status for three genes (*S1PR1* r = 0.54, p<0.05; *S1PR5* r = 0.56, p<0.05; *SGPP2* r = 0.49, p<0.05) and with Pky for three genes (*SPHK1* r = 0.65, p<0.05; *SPHK2* r =0.52, p<0.05; *SGPL1* r =0.65, p<0.05). No associations were found between DNA methylation levels and the other variables tested

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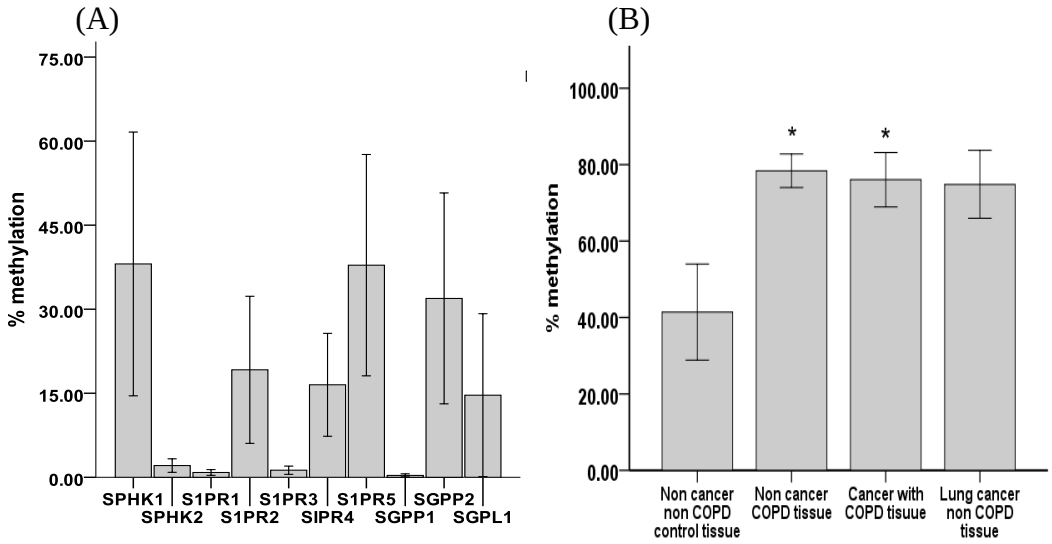

Figure 5: DNA methylation levels in lung tissue macrophages. (A) Methylation levels of designated S1P pathway gene CpG islands in control lung tissue. (B) Significantly higher DNA methylation levels were found at *S1PR5* CpG island 1 in macrophages from COPD patients with/without lung cancer relative to controls. n= (10 for control group, 15 for the COPD groups and 8 for the cancer without COPD group) (*p<0.05 vs control). Data expressed as mean ± SEM.

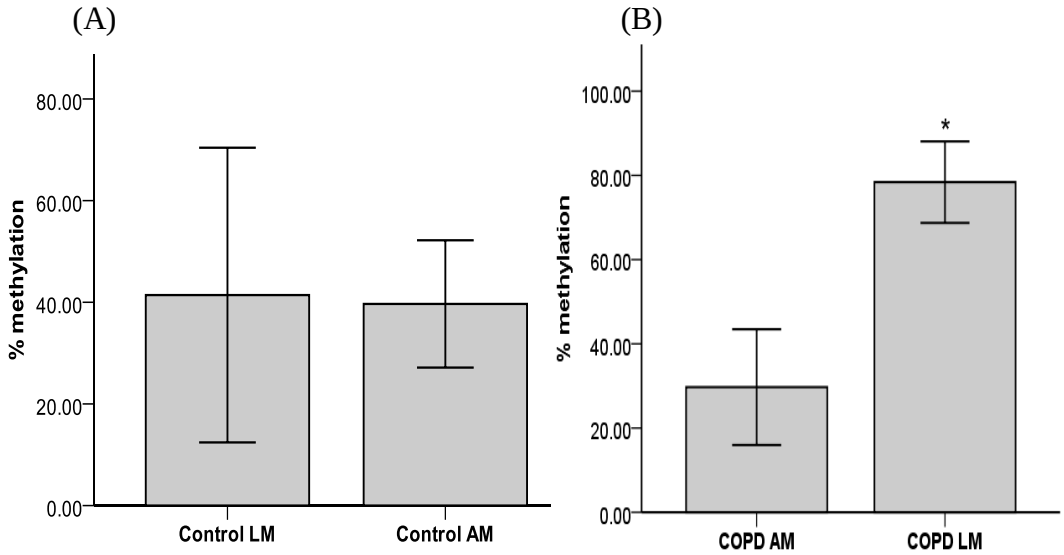

Figure 6: Comparison of DNA methylation at *S1PR5* CpG island 1 in alveolar macrophages and lung tissue macrophages in normal controls and COPD patients. LM: Lung tissue macrophages (n=15); AM: alveolar macrophages (n=20). (*$p<0.05$). Data expressed as mean ± SEM.

Discussion

We have previously shown that there is an increase in the number of apoptotic cells in the airways of COPD patients (Hodge, Hodge et al. 2005). Radiotherapy for lung cancer induces apoptosis in these cells, which require efficient macrophage clearance before they undergo secondary necrosis (Lauber, Ernst et al. 2012). Thus, both effective lung cancer treatment and treatment options for COPD require efficient macrophage phagocytic function. Importantly, we have shown defects in this process in both COPD (Hodge, Hodge et al. 2003, Hodge, Hodge et al. 2007, Hodge, Hodge et al. 2008, Hodge, Matthews et al. 2010, Hodge, Matthews et al. 2011, Dehle, Mukaro et al. 2013) and lung cancer (Dehle, Mukaro et al. 2013). In addition, Pouniotis et al. reported altered macrophage phagocytic function in lung cancer (Pouniotis, Plebanski et al. 2006). Identification of common mechanism(s)/pathways(s) that are involved in the deficient phagocytic function in both COPD and lung cancer would potentially be an important

strategy for the development of macrophage-targeted therapies for both diseases.

Epigenetic modification is one of the important gene expression regulatory mechanisms that has been shown to be modified in COPD and lung cancer (Mortaz, Masjedi et al. 2011). We have previously provided evidence for a potential link between the sphingosine signalling system, in particular *S1PR5* gene expression, and defective alveolar macrophage efferocytosis in COPD (Barnawi, Tran et al. 2015). Furthermore, we have shown this mediator to be potentially important in COPD pathogenesis in lung tissue (Cordts, Pitson et al. 2011). In the present study, we investigated the potential epigenetic regulation of this system in COPD and lung cancer. We found that a *S1PR5* gene-associated CpG island is differentially methylated in alveolar macrophages and in lung tissue macrophages from patients with COPD or lung cancer as compared to control samples. We also show that methylation levels at this CpG island negatively correlate with *S1PR5* expression in alveolar macrophages (specifically, hypomethylation of *S1PR5* correlates with elevated expression (Barnawi, Tran et al. 2015)), and that modulation of DNA methylation in macrophages can potentially regulate phagocytic function.

Epigenetic modifications, such as histone modification, have been reported in COPD and lung cancer in several studies (Mortaz, Masjedi et al. 2011). For example, histone deacetylase (HDAC) activity has been reported to be decreased in alveolar macrophages and COPD lung tissue (Ito, Lim et al. 2001, Ito, Ito et al. 2005). Furthermore, decreased HDAC activity in response to cigarette smoke and a decrease in the ability of murine alveolar macrophages to phagocytose of apoptotic neutrophils have been reported (Noda, Matsumoto et al. 2013). However, the study did not address phagocytosis of apoptotic cells and did not investigate human macrophages, or the role of DNA methylation in efferocytosis. In the present study, we found that inhibition of DNA

methylation by 5-Azacytidine significantly increased the efferocytosis capacity of THP-1 macrophages, suggesting a role for DNA methylation in efferocytosis. Consistent with this finding, specific DNA demethylation has been shown to favour M2 macrophages (Mantovani, Garlanda et al. 2009), which are anti-inflammatory/phagocytic macrophages (Mills, Kincaid et al. 2000).

Given that cigarette smoke is the major cause of COPD and lung cancer, that it has been found to be associated with aberrant methylation patterns (Monick, Beach et al. 2012), and that it suppresses phagocytosis (Hodge, Hodge et al. 2003, Hodge, Hodge et al. 2007, Hodge, Hodge et al. 2008, Hodge, Matthews et al. 2010, Hodge, Matthews et al. 2011, Dehle, Mukaro et al. 2013), it is reasonable to propose that aberrant DNA methylation caused by cigarette smoke plays a role in defective efferocytosis. Consistent with this proposal, we noted that DNA de-methylation by 5-Azacytidine in THP-1 macrophages, rescued these cells from suppressed efferocytosis in response to cigarette smoke. Others have also reported a link between cigarette smoke, defective efferocytosis, and epigenetic modification in alveolar macrophages. Noda et al showed that cigarette smoke decreased HDAC activity and macrophage efferocytosis via inhibition of HDAC/Rac/CD9 pathway (Noda, Matsumoto et al. 2013), Taken together, these data suggest a potential link between cigarette smoking, epigenetic modification, and the defective macrophage efferocytosis capacity in COPD and/or lung cancer.

DNA methylation is a somatically-heritable gene repression mechanism (Yoder, Walsh et al. 1997, Jaenisch and Bird 2003, Fazzari and Greally 2004). We hypothesized that differential DNA methylation of promoter regions could be a mechanism underlying the increase in mRNA levels of *SPHK1* and *S1PR5* that we previously observed in alveolar macrophages from COPD patients. Consistent with our hypothesis, we found that *S1PR5* CpG island 1 was significantly hypo-methylated in alveolar macrophages from COPD

patients. We also found a negative correlation between DNA methylation levels and *S1PR5* gene expression (i.e. CpG island 1 hypo-methylation correlated with increased *S1PR5* levels), suggesting epigenetic control of gene expression of *S1PR5* in these patients. The extent to which elevated *S1PR5* expression due to promoter hypo-methylation negatively influences the function of alveolar macrophages in COPD patients remains to be explicitly tested. Interestingly, our finding that *global* DNA de-methylation by 5-Azacytidine increases efferocytosis in cultured THP-1 cells is not immediately consistent with the hypothesis that hypo-methylation of specific genes such as *S1PR5* in COPD might lead to defective alveolar macrophage function. However, this discrepancy may be explained by the fact that alveolar macrophage efferocytosis is controlled by numerous factors beyond S1P signalling, and that these may be differentially regulated in the event of *global* de-methylation.

Several reports have provided evidence of gene expression regulation by a few or even a single CpG within a CpG island (Nile, Read et al. 2008, Furst, Kliem et al. 2012, Mamrut, Harony et al. 2013). Therefore we further analysed each CpG within *S1PR5* CpG island 1. We found that methylation levels at only a subset of CpG sites correlated with *S1PR5* expression, consistent with previous findings that CpG sites within a single island act heterogeneously (Nile, Read et al. 2008, Furst, Kliem et al. 2012, Mamrut, Harony et al. 2013) . Despite our identification of four CpG sites whose methylation level correlated with *S1PR5* expression, only one CpG site, -530, was found to have significantly different DNA methylation levels in COPD compared to controls. Future work could investigate the roles of potential transcription factor binding sites in CpG island 1, and in particular overlapping CpG -530, in regulation of *S1PR5* expression and potentially alveolar macrophage function.

Studies in lung tissue macrophages presented a more complex picture than those in alveolar macrophages. Lung tissue macrophages from COPD patients with/without lung cancer, and lung cancer patients without COPD exhibited higher DNA methylation levels at the *S1PR5* CpG islands as compared to control macrophages, suggesting that alveolar and lung tissue macrophages undergo different epigenetic regulation events.

There are three classes of macrophages in lung tissue and these macrophages are diverse in terms of functionality, anatomical localization, and response to stimuli (Laskin, Weinberger et al. 2001, Schneberger, Aharonson-Raz et al. 2011, Ivashkiv 2013, Cai, Sugimoto et al. 2014, Labonte, Tosello-Trampont et al. 2014, Yang, Wang et al. 2014). It is possible that the different lung tissue macrophage classes exhibit different modes of epigenetic regulation in COPD, hence presenting a more complex picture than that seen in alveolar macrophages.

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CHAPTER 5

Summary and future direction

Defective macrophages efferocytosis plays a major role in COPD pathogenesis. This defect may attribute to the increased number of apoptotic cells in COPD lungs, which subsequently may undergo secondary necrosis, release their toxic contents and further promote airway inflammation. The process of efferocytosis consists of three major steps; it is initiated by sensing of apoptotic cells by neighbouring macrophages through signalling molecules presented on apoptotic cells, followed by recognition of these signals by macrophages via extrinsic receptors that activate macrophages and finally by engulfment and destruction of apoptotic cells. Although numerous molecules have been identified as potential contributors to the defective macrophage efferocytosis function in COPD, the underlying signalling events are still unknown. The majority of studies either investigated single or several unrelated molecules but did not look at complete signalling systems. Current evidence indicates that defective efferocytosis is potentially caused by changes in a network of molecules that interact with each other, rather than in single molecule or in several unrelated molecules. However, no study has addressed the defective human macrophage efferocytosis in COPD in a 'pathway focused' approach that address closely related intrinsic and extrinsic molecules simultaneously.

The second chapter addressed this gap of knowledge, and examined the potential contribution of the S1P signalling system to the defective ability of alveolar macrophages to phagocytose apoptotic cells in COPD. Data illustrated in Chapter 2 demonstrate that components of the S1P system are potentially linked to the defective alveolar macrophage phagocytic function in COPD. Relative expression levels normally reflect protein expression and the levels for the S1P synthesizing enzymes *SPHK1*, *SPHK2*, and the receptor subtype *S1PR2* were increased in macrophages in response to

cigarette smoke but independent of COPD, as demonstrated by their increased expression in cells isolated from smokers with and without COPD. These data were confirmed by *in vitro* experiments using cigarette smoke extract-exposed THP-1 macrophages. From the five S1P receptor subtypes, only the expression of the *S1PR5* was related to COPD independent of present smoking history, as demonstrated by its increase expression in ex- and current-smoker COPD subjects. The possible link between COPD, *S1PR5*, and defective efferocytosis was further supported using a *S1PR5*-specific antagonist, which increased THP-1 macrophage efferocytosis. This study provided data about novel mediators that play a role in COPD pathology, which might be part of new strategies towards the development of effective therapies for the treatment of COPD.

Oxidative stress and inflammation are hallmarks of COPD and have been associated with increased number of apoptotic cells and defective macrophage function in COPD. They also play a role in activation of a variety of different signalling pathways including but not limited to the S1P system. Antioxidant treatments have shown potential in COPD treatment. However, only a limited number of antioxidants have been used in clinical trials so far, due to their potential toxicity in humans. Therefore, there is an increasing interest in natural antioxidants, which provide limited toxicity and might be a promising tool in the treatment of diseases such as COPD. Experiments presented in Chapter 3 addressed this area and identified the anti-oxidant thymoquinone (TQ) as an effective modulator of S1P system in different cellular components of the airways such as macrophages and epithelial cells. TQ might therefore be a useful for targeted therapy for COPD. TQ demonstrated a pro-phagocytic and pro-survival effect on macrophages and bronchial epithelial cells, respectively. TQ was more efficient in the rescue of efferocytosis capacity of macrophages that were exposed to cigarette smoke than it was

in the rescuing the macrophage function in response to the TLR4 receptor agonist LPS. In addition, TQ abolished the changes in S1P system mRNA expression in response to both cigarette smoke and LPS.

Importantly, TQ was able to significantly decrease mRNA expression levels for *SGPL1, S1PR2, S1PR5, SPHK1&2* that were increased in smokers with and without COPD (Chapter 2). This demonstrates the potential of TQ to control both the S1P system expression and alveolar macrophage phagocytosis in smokers with/without COPD. Cigarette smoke has been shown to play a critical role in increasing the number of apoptotic cells in COPD. Data presented in Chapter 3 demonstrate that cigarette smoke induced apoptosis in bronchial epithelial cell lines *in vitro*, an effect antagonized by TQ treatment in dose-dependent manner. Taken together, these data demonstrate that this clinically approved agent might be a promising novel dual-action therapy for COPD.

Macrophages are defective in their phagocytic ability in COPD and lung cancer, and this defect contributes to the pathology of both diseases. Therefore, it can be speculated that both diseases might share common mechanism(s) or pathway(s) for their alveolar macrophages defects. Epigenetic modification has been linked to COPD and lung cancer pathology. However, little is known about the role of DNA methylation in macrophage efferocytosis. S1P system, in particular *S1PR5* mRNA expression is upregulated in COPD (Chapter 2), In addition S1P system mRNA expression differences have been reported in lung tissue; however, it is unknown whether these changes in S1P system are regulated by epigenetic modification such as DNA methylation or whether DNA methylation is involved in the control of macrophage efferocytosis. Experiments presented in Chapter 4 investigated the question by firstly investigating the effect of the demethylating agent 5-Azacytadine in a macrophage cell line, and on the DNA methylation pattern of *S1PR5* gene in alveolar macrophages from COPD patients. The

experiments also determined the effect of DNA demethylation on the S1P system expression in macrophages isolated from lung cancer patients with/without COPD. Experiments presented in Chapter 4 suggest that DNA methylation regulates *S1PR5* expression in macrophages in COPD and lung cancer. These data provide evidence for the effect of DNA methylation in expression of *S1PR5* and macrophage efferocytosis, which open new prospects for application of new classes of treatments for COPD such as epigenetic therapies.

Recommendation for future directions

The relative mRNA expression of enzymes that synthesize and degrade S1P and of S1P receptors was assessed in human alveolar macrophages in this study and it was shown that these components of the S1P signalling were upregulated in COPD. However, the regulation of the relevant proteins is unknown. Therefore, it will be essential to also determine protein expression in alveolar macrophages from COPD patients and matched controls. *SPHK1 and S1PR5* were shown to be upregulated and negatively correlated with efferocytosis. Inhibition of *SPHK1 or/and S1PR5* expression using robust methods such as small interfering RNA (siRNA) and *SPHK1, SPHK2* and *S1PR5* deficient transgenic mice in a smoker and non-smoker model would be useful to investigate the effect on alveolar macrophage efferocytosis *in vivo* and *in vitro*. This would further extend our understanding of the role of these mediators in macrophage efferocytosis in COPD.

Thymoquinone increased THP-1 macrophage phagocytosis in the presence and absence of cigarette smoke. However, these investigations were limited to cell lines, and using primary human alveolar macrophages *in vitro* would translate these results and further investigate the treatment potential of this agent for the defective phagocytosis in COPD.

Thymoquinone alone did not directly modulate the mRNA expression of *SPHK 1*&2 enzymes and other components of S1P system. However, its direct effects on enzyme activity and/or protein expression of the S1P system was not investigated. Future studies investigating the effect of thymoquinone on proteins and enzymes activity levels will further provide essential information about any potential interaction between this system components and thymoquinone.

The role of DNA methylation in controlling *S1PR5* gene expression in alveolar macrophages and lung tissue macrophages has been well supported by data presented in this thesis. However, this study did not find any association between DNA methylation and expression of *SPHK1*, which is upregulated and negatively correlated with efferocytosis in alveolar macrophages in COPD. There are other epigenetic mechanisms that control gene expression - principally histone modifications, which impact on the expression of almost all known genes. It seems likely that histone modifications also help to control *S1PR5* gene expression, as these processes show crosstalk and many genes are known to be controlled by both mechanisms. Moreover, it would be logical to examine how histone modifications control the expression of *S1PR5* and SPHK1 genes. Three putative transcription binding sites were identified in S1PR5 CpG island 1 in alveolar macrophages from COPD patients. This is significant because DNA methylation and associated chromatin compaction could potentially block access of these transcription factors to these sites. Hence, it would be interesting to further assess the functionality of these transcription factors in controlling S1PR5 gene expression in alveolar macrophages and in other contexts.