Reduced proliferation and increased TSLP expression by lung fibroblasts from COPD patients

By

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Abstract

Chronic obstructive pulmonary disease (COPD) is identified with partially reversible airflow limitation, chronic bronchitis, small airway remodelling, and alveolar destruction. COPD is also progressive in natureTSLP (Thymic stromal lymphopoietin), an Interleukin (IL)-7 like cytokine expressed by structural cells, is a determinant of inflammation. We aimed to characterize human lung fibroblasts (HLF) from human donors with COPD as well as patients without COPD (non-COPD), comparing proliferation and TSLP release. Statins can exert anti-proliferative and anti-inflammatory effects, and their use has been linked to improved lung health, thus we also examined the effect of statins on proliferation and cytokine release by lung fibroblasts from COPD and non-COPD donors. Primary HLF cultures from three COPD diagnosed and non- COPD donors were used. Proliferation was measured using laser scanning cytometry (LSC) counting of H33248-stained cells with 5% fetal bovine along with the addition of simvastatin (0.1μM and 0.5μM). TNFα (tumor necrosis factor α) and/or IL-1β (interleukin 1β) (10ng/mL), and various concentrations of cigarette smoke extract (CSE) were used to stimulate cells. TSLP release, mRNA abundance and transcriptional activity were measured by ELISA, Real Time Polymerase Chain Recation (RT- PCR) and luciferase assay, respectively. RT-PCR was also utilized to profile TNFα and Interleukin 1β receptors. We also ascertained the effect of various stimuli on receptor-mediated signaling pathways using Western blotting. Impact of simvastatin (1-10μM) on TSLP release was determined by ELISA.

During exponential growth phase, HLF from COPD donors proliferated 46.7% slower than from non-COPD. Simvastatin (0.5μM) inhibited proliferation, as indicated by 53%

(P<0.01) and 48% (P< 0.001) fewer COPD and non-COPD donor HLFs at Day 6 culture. At baseline COPD HLFs make approximately 2-fold (P<0.05) more TSLP/cell compared to non-COPD HLFs. TNFα and/or IL-1β (10ng/ml, 48h) induced approximately 1.8 to 2.3-fold (P<0.05) more TSLP release in COPD HLFs. Interestingly, simvastatin had no impact on basal TSLP release, but in the presence of TNFα (5μM, 72h), TSLP release was actually increased approximately 2.19-fold (COPD) and 1.8-fold (non-COPD). TSLP mRNA levels were maximum at 6h in both COPD and non-COPD HLFs and relative TSLP mRNA was approximately 15-fold higher in COPD HLFs compared to non-COPD HLFs (P<0.01). This correlated with human TSLP promoter luciferase reporter assays that showed baseline transcription in COPD HLFs is markedly (approximately 17-fold) higher than non-COPD HLFs. In TNFα-stimulated cultures, TSLP luciferase activity was approximately 10-fold higher in COPD HLFs compared to HLFs from non-COPD. There was no difference in mRNA abundance of receptor subunits for TNFα or IL-1β between patient groups. Phosphorylation of mitogen activated protein kinases, ERK1/2 and p38MAPK, were also comparatively higher in COPD HLFs when stimulated with TNFα suggesting increased response via TNFα receptors. Additionally, in TNFα-stimulated cultures, Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcriptional activity was approximately 2.6-fold higher in COPD HLFs as compared to HLFs from non-COPD donors.

Collectively our data show that proliferation of HLFs from COPD subjects is lower and can be further reduced by simvastatin. COPD HLFs exhibit increased basal and cytokine-stimulated TSLP mRNA expression, transcription and release, suggesting these cells carry stable intrinsic differences in regulatory mechanisms. Differences in TSLP levels

are not due to differential abundance of TNFα and IL-1β receptors although the intracellular responses mediated by TNF α receptor is higher in COPD HLFs. Simvastatin augments TNFα-induced TSLP release in COPD and non-COPD HLFs. These data indicate further investigation of the role of TSLP and its response to therapy, in COPD is warranted.

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Abbreviations

ANOVA	Analysis of variance
AAT	Alpha 1 antitrypsin
ASM	Airway smooth muscle
ATS	American Thoracic Society
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CLE	Centrilobular emphysema
COPD	Chronic Obstructive Pulmonary Disease
CO_2	Carbon dioxide
CSE	Cigarette smoke extract
DAB	3'3'-Diaminobenzidine
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
ECL	Enhanced chemiluminescence
EDTA	Ethylene diaminetetraacetic acid
EGTA	Ethylene glycoltetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
ERS	European Respiratory Society
FBS	Fetal bovine serum

FEV_1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GOLD	Global Initiative for Chronic Obstructive Lung Disease
h	hour
H_2O_2	Hydrogen Peroxide
HCl	Hydrochloric acid
HLF	Human Lung Fibroblast
HMG-CoA	3-hydroxy-3-methylglutaryl Co-enzyme A
HRP	Horse radish peroxidase
ICAM-1	intercellular adhesion molecule-1
ITS	Insulin Transferrin Selenium
IFNγ	Interferon gamma
JAK	Janus Kinase
IL	Interleukin
IL-1β	Interleukin 1 beta
LAAC	Long acting anti-cholinergic drugs
LABA	Long-acting β2-agonists
LSC	Laser scanning cytometry
MA	Mevalonic acid
MAPK	Mitogen-activated protein kinase
MCP	Monocyte Chemoattractant Protein

Min	minutes
mRNA	Messenger ribonucleic acid
MMP	Matrix metalloproteinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NICE	National Institute of Health and Care
OD	Optical density
OCT	Optical cutting temperature
P13-K	Phosphotidylinositol 3-kinase
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PDE4	Phosphodiesterase 4
Pen/Strep	Penicillin Streptomycin
PLE	Panlobular emphysema
RIPA	Radioimmunoprecipitaion Assay
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
STAT	Signal transducer and activator of transcription
TBS	Tris-buffered saline
TEMED	N,N,N',N-tetramethylethylenediamine
TGFβ	Transforming growth factor β

TNFα	Tumor necrosis factor α
Tris	Tris (hydroxymethyl) amino methane
TSLP	Thymic stromal lymphopoietin
Tween-20	Polyxyethylene (20) sorbitan monolaurate
VCAM-1	Vascular cell adhesion molecule-1
VWF	Von Willebrand factor
WHO	World Health Organization

1. Introduction

1.1 COPD

1.1.1 Definition and epidemiology

According to The Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines, chronic obstructive pulmonary disease (COPD) is described as a preventable and treatable disease that is identified with partially reversible airflow limitation, along with an increased inflammatory response to various noxious particles as well as gases and is progressive in nature (www.goldcopd.org) Significant extra pulmonary effects are also related with COPD as the disease progress to later stages [1]. Similarly, the World Health Organisation (WHO) and Canadian Thoracic Society (CTS) define COPD as a respiratory disorder largely caused by smoking, characterized by partially reversible airway obstruction. Airways of those suffering from COPD become permanently obstructed or blocked and gradually lose their ability to function. Moreover, the American Thoracic Society (ATS) and the European Respiratory Society (ERS) define COPD as a preventable and treatable disease that is characterised by airflow limitation which is not fully reversible, is progressive - being associated with an abnormal inflammatory response of the lungs to noxious particles or gases, primarily caused by cigarette smoking - and, that produces significant systemic consequences [2].

COPD is the outcome of additive exposures to various stimuli across years and its existence and morbidity diversify over countries partly due to differences in survey methods, analytic approaches and the criteria for diagnosis. According to The Global Burden of Disease Study (GBD), COPD was ranked fifth as a cause of death in 2002, and by 2030, it is very likely to be the fourth major cause of death [3]. COPD morbidity

augments as the person ages and can be affected by various other chronic conditions that are frequent in COPD patients such as diabetes mellitus, cardiovascular disease and musculoskeletal impairment. COPD is identified with serious economic stress and is a significant determinant of death in Canada and worldwide. According to the Canadian Lung association 2009 report, 1.5 million Canadians alone suffer from COPD and 1.6 million are undiagnosed for the disease even though they display the signs of COPD. There is a direct connection that exists between the expenses of care and severity of COPD and it has been recognized that as the disease advances, the cost of care also increases. The associated expenses with COPD influence negatively the family, healthcare and the society in large due to the abundant loss of productivity. At the same time there is a significant damage to the condition of the patient as even common daily activities such as dressing and walking becomes demanding [4].

1.1.2 Diagnosis and classification

The diagnosis of COPD is considered in each patient who display symptoms of cough along with production of sputum, dyspnea and has been exposed to risk factors related to COPD (eg.smoking) in the past. The diagnosis of COPD is performed by spirometry which is a common pulmonary function test (PFTs) to measure the lung function. A spirometer is utilized to measure forced expiratory volume in one second (FEV_1) (i.e- the maximum volume of air that can be forcibly exhaled out in the first second after a full breath) Spirometer also measures forced vital capacity (FVC) (the maximum volume of air that can be forcibly exhaled after a single whole breath). In normal circumstances, around 70% of the FVC is exhaled out in the first second (i.e. the FEV_1 / FVC would be 70% or more). A FEV_1 / FVC ratio smaller than 70% following the patient has taken a bronchodilator (eg. inhaled β2 receptor agonist) signifies the existence of persistent airflow limitation which is an evidence of COPD. Interestingly, the updated National Institute for Health and Clinical Excellence (NICE) guidelines in 2010 suggested the use of post-bronchodilator spirometry to validate COPD diagnosis [2, 5, 6]. In order to curtail variability in the results, spirometry must be executed after the administration of a sufficient dose of a short-acting inhaled bronchodilator. Although post-bronchodilator spirometry is advised for the diagnosis and judgement of the stages of COPD, the airflow limitation reversibility (e.g., measuring FEV_1 before and after bronchodilator or corticosteroids) is not recommended any further as a criteria for disease severity per se.

A simple four stage classification of COPD severity established on post bronchodilator spirometric parameters has been suggested by GOLD (Table1). At GOLD Stage 1 which

is considered as mild COPD, patients may not be conscious that their lung function is abnormal whereas at GOLD Stage II which is moderate COPD, signs of COPD have usually advanced and would have involved shortness of breath that progresses during exertion. At GOLD Stage III which is considered as severe COPD there is further decline on shortness of breath along with limitation in common day to day activities of the patient, and COPD related exacerbations increases. During very severe COPD which is known as GOLD Stage IV, condition of life further declines and every COPD exacerbations could result in a threat to life condition.

Table 1: GOLD classification.		
Stages/severity	**FEV_1/FVC**	**FEV_1 (% predicted)**
Stage I (Mild COPD)	FEV_1 / FVC < 70%	$FEV_1 \geq$ 80% predicted
Stage II (Moderate COPD)	FEV_1 / FVC < 70% to 50%	FEV_1< 80% predicted
Stage III (Severe COPD)	FEV_1 / FVC < 70% to 30%	FEV_1< 50% predicted
Stage IV (Very severe COPD)	FEV_1 30% + chronic respiratory failure	
Global Initiative for Chronic Obstructive Lung Disease (GOLD). Chronic Obstructive Pulmonary Disease (COPD). Forced expiratory volume (FEV_1) is the volume of air exhaled out during the first second of a full breath. Forced vital capacity (FVC) indicates the amount of air that can be forcibly exhaled out from the lungs after a maximum breath.		

1.1.3 Symptoms and risk factors

The characteristic symptoms of COPD are chronic and persistent cough along with sputum or increased mucus production, gradual dyspnea, chest constriction, wheezing and airflow limitation that leads to patient tiredness. The primary risk factor for COPD is persistent tobacco smoking [7]. Cigarette smoke exposure is determined in pack-years, which is the quantification of cigarette smoking. A pack year is measured by the average number of packages of cigarettes smoked per day multiplied by the number of years the person has been smoking. Apart from tobacco smoking, other identified COPD risk factors are occupational exposures to dust, fumes and some specific chemicals (cadmium) [8]. Exposure to air pollution [9] and vulnerability to airborne particles [10] is also considered as risk factor for COPD. Also, in many developing countries, indoor biomass fuel burning and passive smoking can activate COPD by itself or have a cumulative effect on the impact of tobacco smoking [11]. The definitive role of allergy and airway hyper-responsiveness as risk factors for COPD is poorly established and needs further research. [12].

A strong genetic susceptibility to the disease is also suggested which is evident from the fact that COPD is more prevailing among relatives of COPD patients who presents a history of smoking than unrelated smokers [13]. There are several genetic studies conducted to identify COPD susceptibility genes. There are a total of 192 genes that has been identified with COPD in which *ADRB2*, *TGFB1*, *TNF*, *GSTM1*, *GSTP1*, *SERPINA1*, and *EPHX1* have been widely studied. *TNF* is strongly recommended as a COPD susceptibility gene despite of several studies that found no correlation of TNF gene to

COPD phenotype. *SOD3* gene is usually replicated in COPD although it is not widely established. *SERPINA1* which is responsible for encoding the alpha-1 antitrypsin protein is the most extensively studied COPD susceptibility gene [14]. Alpha 1 antitrypsin (AAT) is a protease inhibitor that is known to protect the lungs against injury done by protease enzymes like elastase and trypsin. These protease enzymes are released as consequence of tobacco smoke created inflammatory response and the inadequacy of these enzymes can result in injury to the lung and sometimes the liver. Patients with severe AAT deficiency, particularly if they smoke are at higher risk for developing COPD [15, 16]**.** It should be also considered that despite the fact that severe AAT deficiency demonstrate only a limited number of cases of COPD, somewhere around 3% but at the same time when connected with smoking, augments the possibility of panlobular emphysema [17].

1.1.4 Chronic Bronchitis and Emphysema

Chronic bronchitis is clinically described by mucus overproduction as a result of glandular structure hypertrophy and metaplasia of goblet cells in the proximal airways. This leads to 3 months of daily cough with sputum per year, for 2 consecutive years [18]. There is higher mucus production, narrowing of the airways that result in cough with sputum. Remodeling of the walls of the small conducting airways that include bronchi and bronchioles less than 2 mm in diameter and airway inflammation are the essential pathological feature of COPD [19]. Also terminal bronchioles are disappeared during chronic bronchitis [20]. The result of these alterations brings about a more-or-less fixed restraint of airflow. A study done on smokers death outside of a hospital first demonstrated that perhaps there are development of structural changes in the small airways prior to the diagnosis of COPD is established [21]. Airway remodeling decline with mucus hypersecretion and diminshed mucociliary clearance leading to aggregation of mucus and inflammatory debris in the lumen. Small airway remodeling is identified by elevated volume of airway wall tissue that includes the epithelium, smooth muscle and lamina propria. There is also increased volume of adventitia that is positioned between the epithelial surfaces and muscle layer. All these results in decline of forced expiratory volume in the first second of expiration (FEV_1) [22, 23].

Emphysema was originally described by Laennec in 1834 during his studies on post mortem human lungs that had been air-dried while inflated [24]. Emphysema is described as “unusual stable expansion of airspaces near the terminal bronchioles along with damage of the air space walls without obvious fibrosis” [25]. In emphysema, presence of

abnormal gas exchange properties is also observed that corroborate in hypercapnia and hypoxemia [12, 26]. Expiration is passive in normal lungs where air is pushed out of alveoli into the proximal airways utilizing the reserved mechanical energy that is generated during inspiration in the stretched alveolar walls. As a result of the damage of the walls of air spaces, during breathing, there is reduced surface area for the exchange of oxygen and carbon dioxide. Lung elasticity is considerably reduced and there is a loss of support for the lung embedded airways due to which the airways are prone to collapse and resulting in further obstruction to airflow [27-30]. There is considerable damage in elastic recoil pressure along with elevated lung compliance, consecutively leading to premature cessation of the small airways that result in lung hyperinflation, gas trapping along with restraint in the expiratory flow [31].

Centrilobular emphysema (CLE) and panlobular emphysema (PLE) are the major types of emphysema [32]. CLE influence the lobules surrounding the central respiratory bronchioles and is considered closely associated with cigarette smoke-induced COPD. CLE is more prominent in the upper lung zones [33-35]. In contrary, PLE is mostly seen in the lower lung zones, mostly related with α-antitrypsin deficiency, and evenly affects the complete secondary lobules of the lung [36, 37]. As compared to the normal surrounding lung, in CLE, there is hyperinflation and alteration of pressure volume relationship of the affected emphysematous lung. There is a decline in the available pressure to force air out of the lung, volume recoil pressure is also decreased which leads to decline in lung elastic recoil and reduced expiratory flow [38-40]. COPD patients mostly display both emphysema and small airway remodeling, but some of them have

prominent prevalence of either emphysema or small airway disease [41]. Figure 1 indicates the distinctive features of chronic bronchitis and emphysema, some of these characteristics coexist in many patients regardless of clear demarcation of emphysema or chronic bronchitis phenotype [42].

Gosselink and coworkers used laser capture micro dissection technique along with differential Taqman qualitative PCR on lung tissue specimens collected from airway or emphysematous regions in COPD patients and proved that emphysema and airway remodeling in COPD display independent familiar aggregation and they established that there is differential gene expression profile between the airway wall and surrounding parenchyma. At the same time their studies suggested that factors which increased tissue growth and proliferation were elevated when there is airway remodeling, whereas the tissue destruction promoting factors were enhanced in the neighboring areas of emphysema [43, 44]

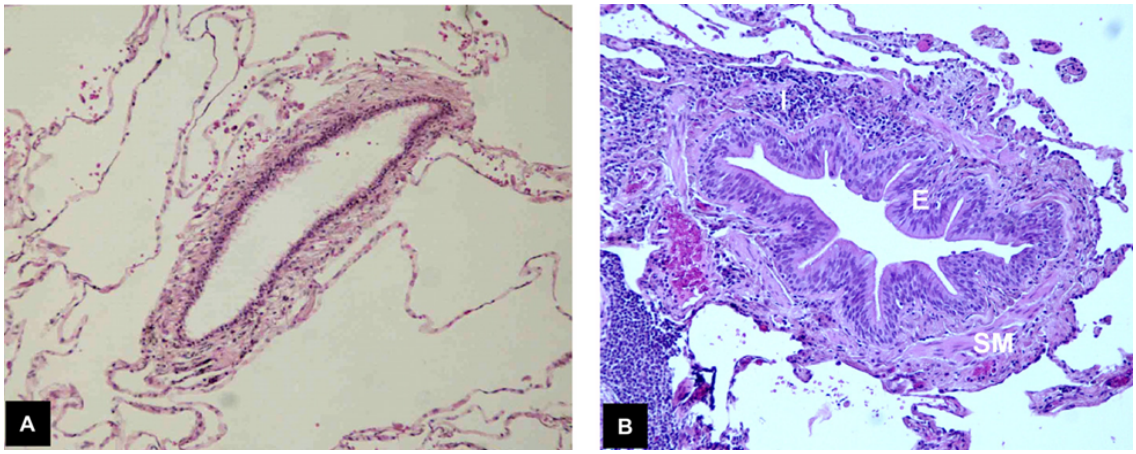

Figure 1: Mechanisms of airflow obstruction in COPD.
(A) Emphysema causes loss of alveolar attachments to the airway wall, predisposing it to expiratory collapse. (B) Small airway remodeling, as evidenced by epithelial thickening (E), smooth muscle hypertrophy (SM), and chronic airway inflammation (Adapted from Kim V, Rogers TJ, Criner GJ.New concepts in the pathobiology of chronic obstructive pulmonary disease, Proc Am Thorac Soc. 2008. Reprinted with copyright permission from American Thoracic Society).

1.1.5 Role of lung fibroblasts

Fibroblasts synthesize collagen and the extracellular matrix that decide the cell phenotype and function. They are the most prevalent cells of connective tissue in animals and they are involved in the initiation and the resolution phases of wound healing. Fibroblasts in the lung are located in the vicinity of the endothelium and epithelium. They participate in the repair process during lung injury though the secretion of proteoglycans and collagens which are the primary components of extracellular matrix and thus contribute to the integrity of the lung. Elastin, which is an important component of the alveolar extracellular matrix and provides elasticity to the lung, is also made my lung fibroblasts [45]. Since lung fibroblasts play a major role in determining the lung matrix, they can play a crucial role in airway remodeling through enhancing matrix deposition and cytokine release is different airway diseases [46, 47].

There is an alteration in the quantity and phenotype of airway and lung fibroblasts in most of the lung disease and these changes in the airway and lung fibroblasts identified with the diseases results in the damage to the usual tissue construction and function of the lung [48]. There is an increase in cell death in the COPD lung cells [49-51]. Prostaglandin E made by lung fibroblasts from COPD patients carry an autocrine and paracrine effect on prostaglandin E receptors (EP2, EP4) that includes the production and signaling of cyclooxygenase-2–dependent reactive oxygen and the activation of p53. All these changes result in inflammation and senescence of lung fibroblasts [52]. Cigarette smoke, being a major risk factor in COPD plays an important role in inducing cellular

senescence by restricting proliferation of normal fibroblasts. This negatively impairs the alveolar repair process in the lung during lung injury [53, 54].

Fibroblasts from COPD patients display reduced decreased repair and maintenance. Studies have shown that there is reduction in proliferation and contraction of extracellular matrix along with altered chemotaxis. The expression of decorin is altered along with a decrease in the elastin mRNA expression [55, 56]. Prostaglandin E prevents the repair function of lung and these changes in the COPD fibroblasts function have been attributed to insensitivity to transforming growth factor (TGFβ) as well as to the increased production of prostaglandin E [48]. Interestingly, microRNA, miR-146a is also associated with the altered function of COPD fibroblasts [57]. Altogether, these studies indicates that the altered maintenance of lung tissue along with lung structural cells and extracellular matrix can be related to modified lung mesenchymal cell populations in COPD.

Moreover, lung fibroblasts express adhesion molecules such as intercellular adhesion molecules (ICAM-1) and vascular cell adhesion molecule 1 **(**VCAM-1) that are associated with leukocyte recruitment into the lung tissues. They also produce many inflammatory cytokines and chemokines and thus plays a major role in regulating the immune and inflammatory responses of the lung. [58]. Upon TNF-α stimulation, lung fibroblasts can release RANTES, eotaxin and some amount of IL-4. There is no release of IFN-γ. Lung fibroblasts can also make monocyte chemoattractant protein (MCP)-3 or MCP-4 mRNA on stimulation with TNF-α or IL-4. Also on stimulation with IL-4 or IL-13, lung fibroblasts can produce IL-6 and MCP-1 [59]. Interestingly, both granulocyte

macrophage colony stimulating factor (GM-CSF) and stem cell factor are made by lung fibroblasts. They can enhance activation, survival as well as differentiation of mast cells and eosinophils. It would be interesting to target mesenchymal cells such as lung fibroblasts to be used therapeutically in lung diseases that have aspects of inflammation and tissue remodeling [58].

1.1.6 Current treatment and drawbacks

COPD can be treated as well as prevented though presently there is no cure for COPD. COPD management procedure is mainly focussed on to eliminate or decrease the risk factors. Also to treat and avoid acute exacerbations, certain methods are implemented. It is important to closely study and analyze the disease, thus controlling stable COPD.

According to Large Lung Health Study, smoking cessation is perhaps the most effective measure to decline the progression of COPD [60]. Nicotine-replacement therapy can also be a great help to patients in quitting smoking [61]. Bupropion, a drug that is used as a noradrenergic antidepressant is also known to decrease the progression of disease but in combination with nicotine patch there was an improved effect [62].

Since, respiratory tract infections are a common cause for COPD exacerbations, many studies are focussed on to control this aspect of COPD. A major study on the benefits of influenza vaccination in COPD was done on elderly patients with a data set of 713,872 individuals. The study suggested that administration of the influenza vaccine had a beneficial effect on these patients evident from the decline of hospitalization rates [63]. Yet in another study, administration of pneumococcal vaccination reduced the rate of hospitalization for pneumonia [64]. Interestingly, when both of these vaccines (influenza and pneumococcal) are combined, there is a cumulative benefit to the patients suffering from COPD so both of these vaccines are advised to COPD patients. Bacterial immunostimulation such as OM-85 BV (Bronchovaxom) is also administered to reduce

exacerbations of COPD though their effects are not clearly established and needs further research [65-68].

Bronchodilators, more specifically the short-acting inhaled beta 2 agonists are commonly approved bronchodilators for the early treatment of COPD exacerbations [1] although there are several studies that have displayed the effectiveness of salmeterol and formoterol which are long-acting β2-agonists (LABA) [69-71]. Salmeterol is mostly advised and when added with fluticasone display beneficial effects in reducing inflammation in COPD patients [72]. Long acting anti-cholinergic drugs (LAAC) can be used as effective bronchodilators and they act by inhibiting parasympathetic driven bronchoconstriction and bronchial hyper secretion. Tiotropium bromide, a LAAC, was administered once-daily for the maintenance treatment of COPD patients. Tiotropium bromide showed reduction in COPD exacerbations, FEV1 decline and fewer symptoms of COPD [73].

In COPD, there is an actively neutrophil-driven inflammation where activated lymphocytes are major contributing factors. The use of inhaled corticosteroids (ICS) by itself or along with β-agonists can reduce airway inflammation and result in better bronchodilatory effects [74], although the decrease in exacerbations is mostly attributed by the LABA factor, role of ICS is not clearly established in the studies [72, 75, 76] where in one study [77], patients taking ICS did not demonstrated any recovery in inflammation markers such as IL-6 and C-reactive protein. Moreover, administration of fluticasone along with salmeterol (LABA), have not displayed impressive impact in culminating inflammation associated with neutrophils [78].

Phosphodiesterase 4 (PDE4) are also associated with COPD as they are expressed by inflammatory cell. Selective phosphodiesterase 4 inhibitors are under investigation as future therapies for airway diseases. PDE4 inhibitors such Cilomilast and roflumilast have the potential to increase cAMP levels thereby reducing inflammation [79]. Some studies also suggested that theophyllines which are nonselective phosphodiesterase inhibitors, can decrease exacerbation frequency although their therapeutic use is poorly established with a constant need to monitor plasma levels and their interaction with other medication can be toxic in nature. [80, 81]. The use of nonselective phosphodiesterase inhibitors needs more research to clearly establish as a therapeutic approach in COPD pathogenesis.

In contrast, administration of long term antibiotics such as macrolides showed some beneficial effects due to their anti-inflammatory and antimicrobial properties. Erythromycin when prescribed twice daily in moderate to severe COPD patients over 12 months decreased the rate of moderate or severe exacerbations and reduced exacerbation length [82] although there is a growing concern of developing antibiotic resistance in these patients. Mucolytic agents that have the ability to clear the mucus from the airways such as carbocysteine, have been reported to decrease exacerbation frequency in selected patients although regular administration of these agents is still not recommended [83].

Phosphatidylinositol-3-kinase (P13-kinase) activity is significantly enhanced in peripheral blood monocytes in COPD patients. This suggests the involvement of reduced corticosteroid sensitivity pathway in these patients [84] which suggests that perhaps addition of P13-kinase inhibitor could have some beneficial effects on these patients.

Interistingly, administration of a P13-kinase inhibitor have demonstrated some positive effects to restore steroid sensitivity [85].

Role of mitogen-activated protein kinase (MAPK) is also utilized to develop newer therapeutic approaches. COPD patients displayed higher p38 MAPK activity in the alveolar macrophages [86] and selective p38 MAPK inhibitors have shown to curtail cytokine production induced by lipopolysaccharide in alveolar macrophages. It was also seen that there were enhanced effects of dexamethasone and cytokine inhibition [87].

Surgery is considered in the later stages of COPD and the lung volume reduction surgery has been reported to enhance morbidity and mortality in a subset of COPD patients. The National Emphysema Treatment Trial reported that there were positive effects of the surgery in COPD patients who had essentially upper-lobe emphysema and low baseline exercise capacity. It was also suggested that lung volume reduction surgery can decrease the rate of COPD exacerbations likely by the decline in dynamic hyperinflation and postoperative improvement in lung function [88, 89]. Long-term oxygen therapy has also demonstrated a decrease in COPD driven death although its role in reducing exacerbations in not clearly known [90, 91]. In COPD patients whose respiratory drive rely on their degree of hypoxia as compared to hypercapnia, administration of oxygen has to be closely monitored and should be prescribed with caution.

Pulmonary rehabilitation programs that includes exercise and education programs can also decrease hospitalization rates in the COPD patients thus improving their overall condition of life along with different maintenance programs [92]. In conclusion, in all the approaches to reduce COPD exacerbations, smoking cessation that can slow the decline

of lung function and long-term oxygen therapy that can increase survival rate to some extent, rest other therapies despite their added benefits did not impose significant increase in survival rates and lung function [5, 93].

1.1.6.1 Statin overview and role in airway disease

Statins are hydroxymethylglutaryl (HMG)-CoA reductase inhibitors and are extensively used as cholesterol lowering drugs. Statins are known to strongly inhibit HMG-CoA reductase (Figure 2), the enzyme that converts HMG-CoA into mevalonic acid (MA) which is the rate limiting step of cholesterol synthesis pathway [94]. Simvastatin, a type of statin has the ability to bind to HMG-CoA reductase with increased affinity, changing enzyme conformation and inhibiting the binding of HMGCoA to the reductase active site. MA is a precursor of cholesterol, is converted to cholesterol through the production of various isoprenoid precursors of cholesterol such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) [95], thus through inhibiting the mevalonate pathway, statins also inhibit the formation of FPP and GGPP isoprenoid intermediates. These isoprenoids are essential for the post-translational modification of the small G proteins. By inhibiting the formation of isoprenoids, statins can also prevent the formation of small GTP-binding proteins such as Ras and Rho isoforms. For the activation of these proteins, both the γ subunit of heterotrimeric G proteins and small GTP-binding proteins of the Ras superfamily need prenylation to translocate from the cytoplasm and bind to the plasma membrane [96]. This leads to the decrease in various inflammatory mediator production. In murine models of allergic asthma, statins administration has shown decrease in the formation of various inflammatory mediators [97, 98].

Various studies have indicated beneficial role of statins on lung health as a result of their anti-inflammatory effect. In a study done by Søyseth et al on 850 hospitalized with acute COPD [99], statin administration resulted in decreased mortality rates where the mortality rate/1000 person was 110 in patients taking statins as compared to 191 in patients who were not taking statins. Yet in another studies on COPD hospitalized patients, statin administration led to decline in the exacerbations and intubation cases as well as a decline in inflammatory cytokines in the blood. There was also an indication of decrease in COPD mortality with statin treatment [100-103].

Statins use in asthma is not very clearly established though statin use has been related with a decline in hospitalization rates that are primarily due to asthma exacerbations [104]. Interistingly, in a study done on smokers with mild to moderate asthma, short term treatment with atorvastatin had some positive impact on the asthma quality although lung function was not improved [105]. Simvastatin administration in eosinophilic asthma patients had marginal progress in lung function [106]. At the same there are studies which suggests that there is no beneficial effect of statins on asthma patients such as in one study, statin administration resulted in decline of lung functions [107] whereas in a literature survey conducted on 18 articles [108] to identify the potential beneficial effect of statin administration in asthma, suggested that out of total 18 studies, only three of the studies mentioned beneficial role of statins in the lung. Other studies showed a gradual reduction in lung function in the elderly, current smokers and ex-smokers [109] [110] suggesting some beneficial role on the decline of lung function. In asthma patients with a background of cigarette smoking, the sensitivity to Inhaled corticosteroids (ICS) is

declined [111], these studies suggested that statins along with the current asthma medication can be used as anti-inflammatory treatment.

Simvastatin was described to enhance lung function by inhibiting helper T cell type 1, 2 and chemokines in a mouse model of asthma, [112]. In allergic bronchial asthma, administration of Lovastatin which is a naturally occurring statin showed some beneficial effect on improving airway hyper responsiveness [113, 114] whereas in mouse allergic asthma, administration of simvastatin demonstrated in a decline in the total number of inflammatory cells, ovalbumin-specific IgE levels. There was also less CD40, CD40L and VCAM-1 expression along with a declined expression of interleukin (IL)-4, IL-13 and tumor necrosis factor (TNF)-alpha. Small G proteins, goblet cells and matrix metalloproteinases (MMPs) were also decreased upon statin administration [98]. Intraperitoneal injection of simvastatin in OVA sensitized mice also resulted in decreased total inflammatory cell infiltrate and eosinophils [97] whereas administration of Pravastatin in an OVA challenged mouse lung resulted in a decline in antigen presentation and IL-17 production [115].

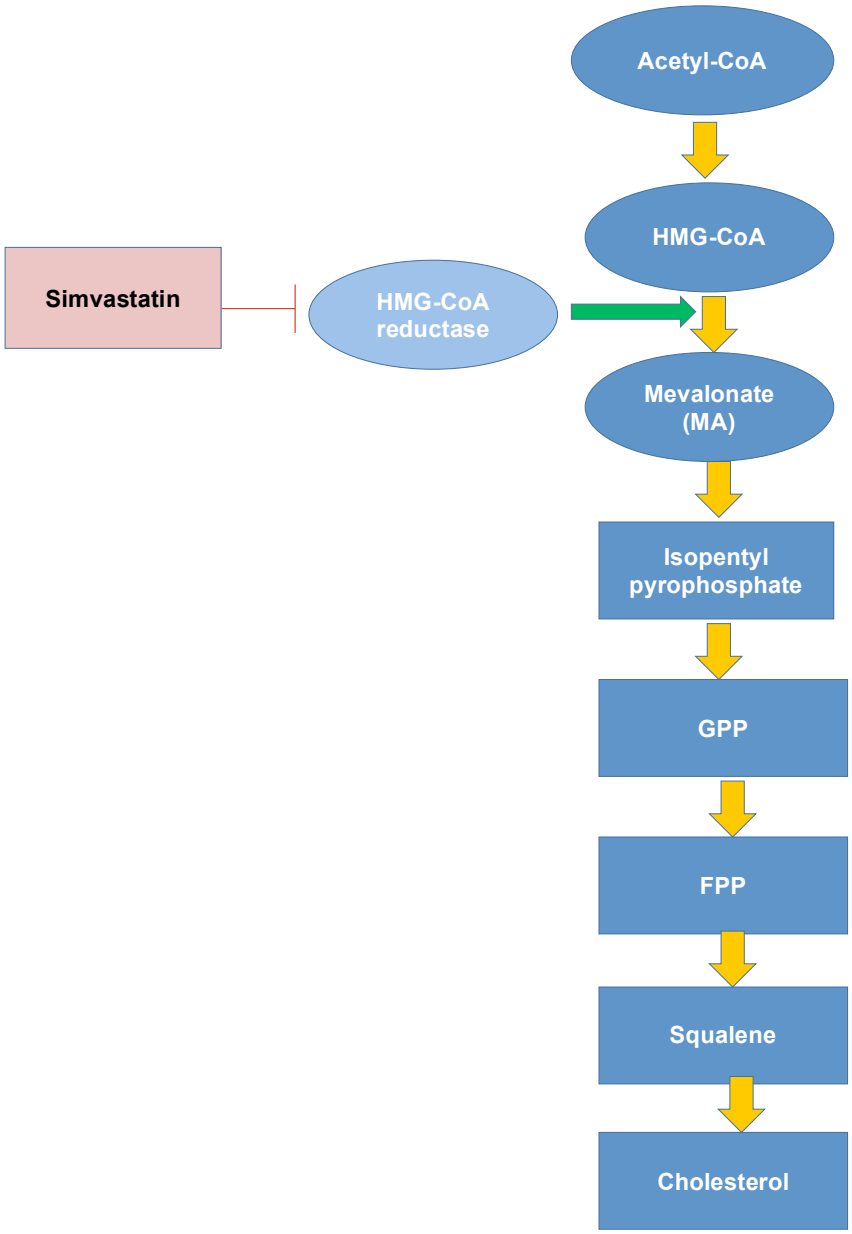

Figure 2: The mevalonate pathway for cholesterol biosynthesis.
Simvastatin inhibit the enzyme hydroxymethylglutaryl (HMG)-CoA reductase which converts HMG-CoA to mevalonate (MA). MA is converted to isopentylpyrophosphate and is further converted into geranyl pyrophosphate (GPP) and farnesyl pyrophosphate (FPP). FPP is converted to squalene and finally to cholesterol.

1.2 Role of inflammatory mediators in COPD

1.2.1 Tumor necrosis factor α

Tumor necrosis factor (TNF) collectively refer to a family of cytokines that can cause cell death and TNFα is the most investigated cytokine member of the family. TNFα is a protein which is made of 185 amino acids. The protein is glycosylated at positions 73 and 172. TNFα protein is synthesized in a precursor form comprised of 212 amino acids and is inactive in nature. Bioactive soluble form of TNFα is made by the cleavage of membrane associated inactive form of TNFα by TNFα converting enzyme (TACE) [116]. TNFα is similar to TNFβ (lymphotoxin), a 25-kDa protein. There is around 30% homology in the amino acid sequence and both TNFα and TNFβ share the common receptors with similar cellular functions [117]. Lipopolysaccharide-stimulated macrophages secretes TNFα and when injected into mice with tumor, it causes necrosis of tumors in vivo [118].

TNFα is secreted by various cell types such as monocytes/macrophages. Mast cells, eosinophils, T cells and epithelial cells are also known to produce TNFα [119]. By T-helper 1 (Th1) lymphocyte activation, mast cells can be associated with neutrophilic inflammation mediated by TNFα [120].

TNFα can binds to receptor TNF-R1 (TNF receptor type 1; CD120a; p55/60) as well as receptor TNF-R2 (TNF receptor type 2; CD120b; p75/80). Both the soluble and membrane bound form of TNF can bind to the receptor. TNF-R1 is ubiquitously present in many tissues. Inflammation is caused by the binding of TNF α ligand to the receptor, and by the activation of various signal transducers that can in turn activate various

downstream signaling pathways (Figure 3). Compare to TNF-R1 which is commonly found in many tissues, TNF-R2 is found only in cells belonging to the immune system. Also, TNF-R2 binds to only membrane-associated TNF. TNF-R2 is mostly engaged in T lymphocyte mediated chronic inflammation, increasing TNFα binding to TNF-R1. Inflammatory responses in different cell types including lipopolysaccharide (LPS) induced shock are mostly mediated by TNF-R1 [121].

Chronic TNFα over expression activates pathological changes similar to emphysema and pulmonary fibrosis in experimental animal (mice) models [122]. Some studies have related TNF α with COPD where one study indicated that TNF α plays a major role in lung inflammation [123] whereas in another study TNF levels in the sputum of COPD patients were considerably higher [124]. Yet in another study, increased neutrophil counts and TNF-R1 were observed in COPD patients although sputum levels of TNFα in healthy smokers and smoking related COPD patients were similar [125] .

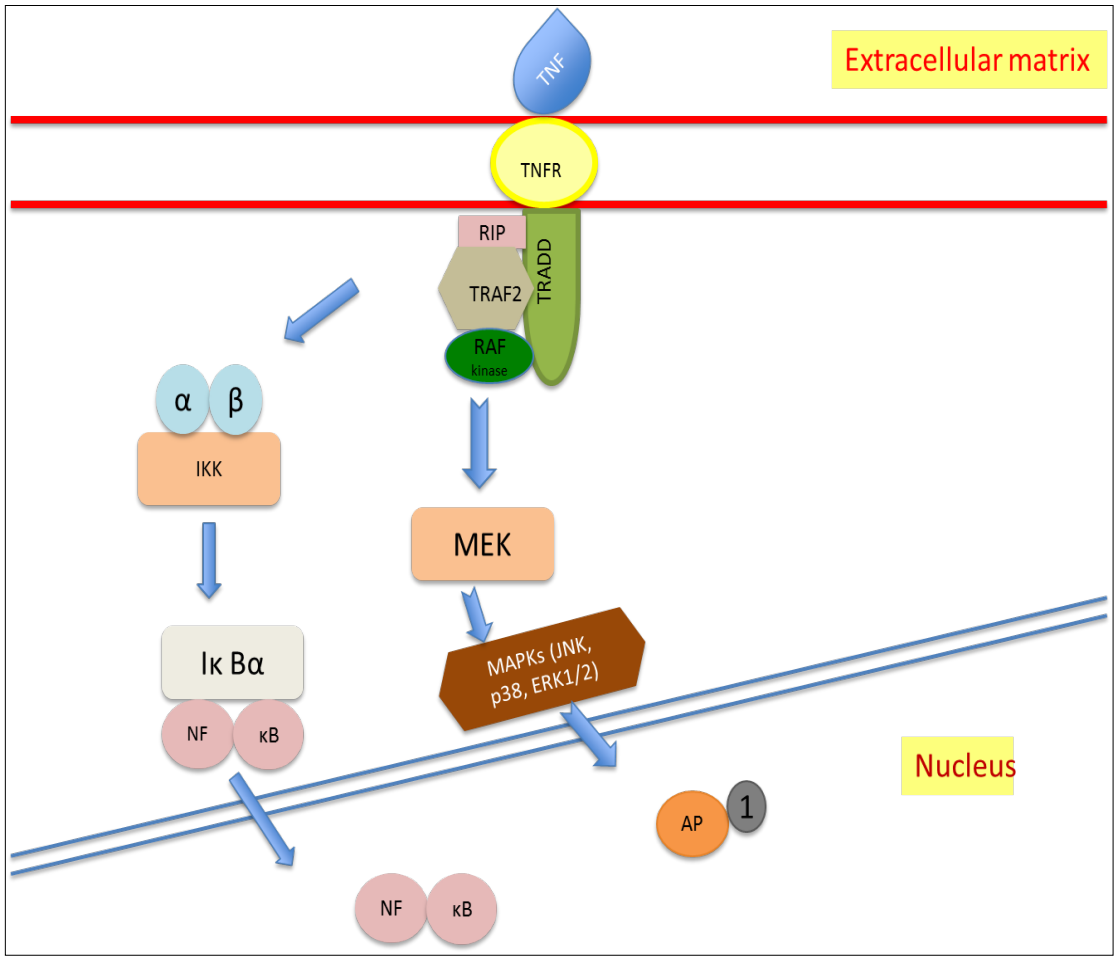

Figure 3: TNF signaling pathway. Tumor necrosis factor (TNFα) binds to TNF receptor, releases the adaptor protein, tumor necrosis factor receptor type 1-associated death domain protein (TRADD) and the binding of further proteins for downstream signaling proteins. TRADD binds to TNF receptor-associated factor 2 (TRAF 2). This association mediates the recruitment of IκB kinase (IKK). Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IκBα), an inhibitor protein is bound to NF-κB, is phosphorylated by IKK and degraded. NF-κB is then released and shifts to the nucleus to facilitate the transcription of various proteins associated with inflammation. TRAF 2 can also activate MEK and downstream signaling induced by mitogen-activated protein kinases (MAPK).

1.2.2 Interleukin 1 beta

Interleukin 1 (IL-1) members form an important part of the inflammatory response of the body against infection. IL-1 family consists of a group of 11 cytokines that plays a major role in regulating the inflammatory process by activating pathways that leads to the production of pro inflammatory cytokines [126]. IL-1 cytokines are also called as endogenous pyrogens due to their ability to increase the body temperature during an infection.

The IL-1 family is composed of three structurally related polypeptides: IL-1α and IL-1β, and IL-1 receptor antagonist (IL-1ra). IL-1α and IL-1β are pro-inflammatory cytokines and IL-1ra is a naturally occurring anti-inflammatory agent. Both IL-1α and IL-1β are synthesized by numerous cell types as a precursor form. Macrophages, mast cells, microglia, astrocytes and neuronal cells and Schwann cells can produce both IL-1α and IL-1β. Also, endothelial cells, smooth muscle cells, keratinocytes and fibroblasts have the ability to produce these pro inflammatory cytokines [127, 128]. Toll like receptors (TLRs) or RIG- like receptors (RLRs) activates cells of the innate immune responses by the formation of inflammasome which are basically nucleotide binding leucine rich repeat containing pyrin receptors (NLRPs) and in turn activates caspase-1. Caspase-1 is also known as IL-1β converting enzyme (ICE) and is necessary for the processing and releasing of active IL-1β. IL-1 type 1 receptor is activated by both IL-1α and IL-1β [129, 130]. IL-1α stays associated with the cell membrane as a paracrine messenger whereas IL-1β is secreted in the extracellular matrix. IL-1ra competes with both IL-1α and IL-1β to bind to the receptor thus acting as a control mechanism of IL-1-mediated

signaling as the harmony between IL-1β and IL-1ra is crucial for the pathophysiologic effects of IL-1β. In various experimental animal models of inflammatory diseases, an imbalance of IL-1 β and IL-1Ra imbalance is seen and it has been observed that inflammation was reduced by the administration of recombinant IL-1ra [131].

IL-1 is linked to COPD pathogenesis. In COPD related exacerbations, IL-1 is one of the crucial pro-inflammatory cytokines that plays a major role in inflammation [132]. Transgenic mice expressing human IL-1β demonstrated that expression of IL-1β in lung epithelium of adult mice causes pulmonary inflammation [133]. COPD phenotype is driven by IL-1β through increasing the secretion of neutrophil chemo-attractants and lymphocytic aggregates in the airways. There is also increased thickening of conducting airways and increased mucin production [133]. In a study done on IL-1β type 1 receptor knockout mice it was seen that the receptor was associated with 27% of the emphysematous changes [134]. Also in COPD smokers, there was increase in matrix metalloproteinase-9 (MMP-9) levels as compared to healthy smokers and non-smokers upon IL-1β stimulation [135]. A positive correlation between Il-1β concentrations and number of neutrophils in BAL fluid was observed in BAL fluid supernatants of smokers as compared to non-smokers after LPS exposure [136].

LPS-stimulated BAL fluid macrophages in smokers exhibited higher IL- 1β activity and interestingly at the same time they also exhibited decline in IL-1Ra inhibitory activity [137]. Yet in another study associated with cigarette smoke exposure, IL-1β was identified to play a crucial role in alveolar macrophage recruitment in the lung [138] Also in COPD patients, infection with *Chlamydia pneumonia* causes an imbalance in the

IL-1β /IL-1Ra ratio where the ratio becomes higher as evident from the altered secretion of IL-1β and IL1Ra from alveolar macrophages and peripheral blood monocytes [139].

1.2.3 Thymic stromal lymphopoietin

Thymic stromal lymphopoietin (TSLP) is an epithelial-derived interleukin 7 (IL-7) like cytokine which was initially isolated in 1994 in the culture supernatant of a mouse thymic stromal cell line and identified as a pre B cell growth factor [140]. Like IL-7, it is a four-helix bundle cytokine sharing some common biologic effects. The mouse TSLP gene is located on chromosome 18 whereas the human TSLP gene is located on chromosome 5q22.1 [141]. TSLP is produced mainly by epithelial cells of lung, skin, gut and keratinocytes. Dendritic cells (DCs), mast cells, smooth muscle cells and lung fibroblasts can also produce TSLP [142, 143]. Human and murine demonstrate similar biological functions although they display poor sequence homology (40%) [142]. Allergen activated basophils can also produce TSLP [144]. TSLP is not produced by many hematopoietic cells such as B cells, T cells, natural killer cells, macrophages and granulocytes [145]. However other studies demonstrated expression of TSLP mRNA in T cells, B cells, mast cells, monocytes and natural killer cells (NKT). TSLP mRNA expression was also observed in skeletal muscle, heart tissue, liver and kidney [146].

TSLP expression can be accomplished by an array of stimuli. Viral infection is a major factor for TSLP production. TSLP expression can be increased in human bronchial epithelial cells (HBECs) by Rhinovirus and respiratory syncytial virus (RSV) infection [147]. In HBECs, pro-inflammatory cytokines such as TNFα IL-1β along with bacterial peptidoglycan, lysophosphatidic acid (LPA), toll like receptor ligands can produce TSLP [148-150]. It was observed that when HBECs were stimulated with a variety of cytokines (IL-4, IL-13, interferon (IFN)-β, TGF β), there was an increase in TSLP mRNA

expression although at protein level there was no significant TSLP production [148]. Mast cells were found to produce, store, and release TSLP upon FceRI aggregation [151] In human skin keratinocytes, IL-1α or TNFα stimulation in combination with Th2 cytokines (IL-4 or IL-13) was able to enhance TSLP production [151].

The TSLP receptor (TSLPR) is a heterodimeric cytokine receptor consisting of the IL-7 receptor alpha chain (IL-7Rα) and a TSLP-specific receptor chain which is similar to the common gamma receptor chain (γc) [152, 153]. TSLP receptor (TSLPR) is part of the hematopoietin receptor family and binds with less affinity. For a high-affinity binding of TSLP to the receptor, a combination of IL-7 receptor alpha chain (IL-7Rα) and TSLPR is essential [146]. Both mouse and human TSLP can bind to TSLPR to initiate their activities [152]. In the absence of Janus family tyrosine kinase 3 (JAK3), TSLPR is described to initiate phosphorylation of signal transducer and activator of transcription 5 (STAT5) [141, 154]. For STAT activation, TSLP requires JAK1 and JAK2 [155]. Also, human TSLP receptor is able to utilize STAT3 activation [154]. In Airway smooth muscle (ASM), TNF α and IL-1β stimulate TSLP expression via mitogen activated protein kinase pathways (MAPK) that includes p38, JNK and ERK1/2 MAPK. TNF α and IL-1β induces phosphorylation of STAT3 but not STAT5 [156, 157]. It was also reported that IgE induces TSLP expression in ASM via transcriptional mechanism involving spleen tyrosine kinase (Syk), nuclear factor-κB (NF-κB), and activator protein 1 (AP-1) [158]. Epithelial cells express TLR 2, 3, 4 and 9 although stimulation of ASM with LPS and cytosine phosphate guanine (CpG) did not activate TSLP release in these cells [159, 160].

TSLP expression in HBECs was also increased with exposure to diesel exhaust particles (DEPs) via an oxidant induced pathway both at mRNA level and protein level [161]. In bronchial mucosa and BAL fluid of COPD patients, oxidative stress also induced TSLP mRNA and TSLP protein [162]. In a BALB/c ovalbumin mouse model of asthma, cigarette smoke extract has been shown to activate TSLP mRNA expression and protein production [163] and a recent study showed that a 1–2% cigarette smoke extract (CSE) exposure enhances the basal TSLP expression in ASM [164].

1.2.4 Cigarette smoke extract

Cigarette smoke is the major risk factor and an important determinant of COPD worldwide. Smoke comprises of thousands of injurious agents [165] so it is increasingly difficult to study each component of cigarette smoke. Tobacco smoke leads to a considerable exposure to oxidants that includes organic free radicals that can cause lipid peroxidation. There is also exposure to α,β-unsaturated aldehydes, N_2O, and nitric oxide [166]. Demonstration of immediate host responses to the inhalation of the toxic and oxidant components is not very well understood. In a span of minutes or hours of exposure with tobacco smoke, airway inflammatory responses are activated in both humans and rodents [167, 168]. There is an alteration in the vascular and airway barrier function along with exertion of lung circulating inflammatory cells [169, 170]. The acute inflammatory response is suggested to be transient in nature and mediated by NF-κB where NF-κB–dependent inflammatory responses pathways are initiated by oxidants present in the cigarette smoke. It is obvious that this pathway is likely to be affected by the factors that inhibit NF-κB pathway [171, 172]. Loss of function of the NF-κB p50 subunit leads to an increase in cigarette smoke induced inflammatory responses suggesting that NF-κB perhaps have a protecting role in the lung [173].

Cigarette smoke is related to alveolar injury. It induces nitric oxide synthase and generates oxidants such as peroxynitrite ($ONOO^-$) [174]. Proline-glycine-proline peptide (PGP) is a collagen degradation product and an endogenous mediator of cell injury and inflammation in early lung responses due to cigarette smoke [175]. Moreover, LPS present in cigarettes can activate NF-κB responses by inducing TLR4-expressing cells in

the lung although the precise role of TLR4 is not very clear where the absence of it can lead to spontaneous emphysema in knockout mice [176, 177]. The emphysematous destruction observed in the lungs of smokers could be attributed to the pro-inflammatory activity and cytotoxic properties of cigarette smoke [178]. Upon CSE exposure, the ECM produced by lung fibroblasts demonstrate pro-proliferative characteristics and the functional properties of ECM is also altered [179].

2. Hypothesis and objective

2.1 Rationale and Hypothesis

TSLP is a key pro-allergic cytokine that has been recently associated to chronic airway diseases, such as COPD and asthma. High levels of TSLP were found in the bronchial mucosa of asthma and COPD patients suggesting TSLP's biological role beyond a signature 'Th2-favoring' or 'pro-allerergic ' cytokine.

Statins have been reported to benefit patients with cardiovascular disease and high cholesterol, recent research shows that statins may provide significant benefits for chronic obstructive pulmonary disease (COPD) patients by suppressing Rho kinase activity.

The primary hypothesis tested was that human lung fibroblasts carry intrinsic difference in viability, proliferation and inflammatory regulatory mechanisms compared to those in humans without COPD. I also tested an ancillary hypothesis that, simvastatin inhibits proliferation and inflammatory cytokine release in human lung fibroblasts, and that lung fibroblasts from human donors with COPD are more sensitive than cells from donors who do not have COPD.

2.2 Research Objectives

To test our hypotheses, I performed studies related to 3 specific research objectives:

1. Develop methods to establish primary cultures of human lung fibroblasts from human donors diagnosed with COPD or without COPD.
2. Compare phenotypic and functional properties of human lung fibroblasts from COPD and non-COPD subjects, including cell viability, proliferation and pro-inflammatory activity - using TSLP release as an index for inflammation.
3. Determine the effect of simvastatin on viability, proliferation and TSLP release by lung fibroblasts from human donors with or without COPD.

3.Materials and methods

3.1 Materials

3.1.1 Chemicals and reagents

Reagents used and their respective suppliers are listed in Appendix I.

3.1.2 Antibodies

Antibodies that were used are listed in Appendix II.

3.1.3 Buffers

Buffers and solutions utilized in the experiments and their composition are listed in Appendix III.

3.2 Experimental Methods

3.2.1 Primary human lung fibroblast culture preparation

Primary human lung fibroblast (HLF) cultures were established using cells isolated from peripheral lung tissue specimens obtained from subjects undergoing lung resection surgery for cancer. In collaboration with Dr. Helmut Unruh (Head, Section of Thoracic Surgery, University of Manitoba), subjects were recruited by a research nurse (Section of Respiratory Disease, University of Manitoba) who obtained informed consent and compiled clinical characteristics and demographic data relevant to COPD and cigarette smoking status (see subsequent sections for summary). Samples were obtained in according with a protocol approved by the Respiratory Ethics Board of the University of Manitoba. Peripheral lung specimens, ~2-4cm^2 and devoid of conducting airways, were isolated in the operating room from regions of lobes that were free of tumour. Tissues were then supplied from the operating room in cold Krebs-Henseliet buffer for immediate transport to the laboratory for processing.

As outlined in Figure 4, each lung tissue specimen was divided in to three equal pieces. One section was subjected to formalin fixation and stored at 4^{o}C until further use. The second section was placed into OCT (optimal cutting temperature) cryopreserved in liquid nitrogen. The third section was utilized for the preparation of lung fibroblast culture. For isolation of primary fibroblasts, pleura were removed from the lung tissue section using sterile dissection scissors, forceps and scalpel blades. The tissue was then equilibrated in Krebs-Henseleit buffer containing antibiotic/antimycotic solution (10,000 units/mL of penicillin, 10,000 µg/ml of streptomycin, and 25 µg/ml of amphotericin B) &

gentamicin (50 mg/mL in deionized water) for 1 h at 4^{o}C. The tissue was then transferred to HBSS containing antibiotic,/antimycotic, gentamicin and incubated for an additional hour at 4^{o}C. Following incubation in HBSS, the tissue was transferred to a 60 mm sterile Corning cell culture dish. The tissue was finely minced with the help of 0.009" sterile single edge razor blades. The minced tissue was transferred to a 50 ml sterile culture tube which contained 25ml of 0.22µm sterile filtered digestion buffer containing antibiotic/antimycotic & gentamicin. The tube was sealed with parafilm and incubated at 37^{o}C for 1 hour in a shaking water bath. Tissue debris was then removed by filtration through sterile gauze and the solution was subjected to centrifugation at 800 × g for 5 min (IEC Centra CL2 centrifuge). After carefully aspirating the supernatant with a Pasteur pipette, the cell/tissue pellet was resuspended in 5ml of 1X RBC lysis buffer and incubated in ice for 4-5 min to eliminate red blood cells and again centrifuged at 800 × g for 5 min at room temperature. Supernatant was discarded by aspirating with sterile Pasteur pipette and the pellet was re-suspended in a sterile filtered cell culture growth medium, Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (50 U/ml streptomycin, 50 µg/ml penicillin). The media was pre warmed in a 37^{o}C water bath before use. Cell number was determined by counting live cells in a hemocytometer. The cells were plated in 100 mm culture plates containing 8ml of sterile filtered DMEM with 10% FBS and antibiotics at a cell density of 500,000 cells per plate. Plates were then placed into humidified culture chambers supplemented with 5% CO_2. The culture was identified as passage 0 (P0). The culture medium was changed 3-4 days later (ensuring maximum cell attachment). Thereafter

culture medium was changed every 48h until the cells reached 90% confluence. The cells were then detached from the plate using 2ml/plate trypsin/EDTA (0.025% trypsin and 0.01% EDTA in Phosphate Buffered Saline), re-suspended in DMEM with 10% FBS culture medium to stop the action of trypsin-EDTA and transferred to new sterile cell culture plates using a 1:4 ratio (i.e cells from one 90% confluent plate equivalent were re-seeded into four plate equivalents). After these first passage (P1) cultures cells were lifted with 2ml/plate trypsin EDTA solution, re-suspended in DMEM with 10%FBS and centrifuged at 800 × g for 5mins at room temperature. Supernatant was discarded with a sterile Pasteur pipette and pellet was re-suspended in freezing medium (10% sterile Dimethyl sulphoxide and 90% FBS). Cells from one cell culture plate were re-suspended in 1ml of freezing medium and stored in cryovials in liquid nitrogen until further use.

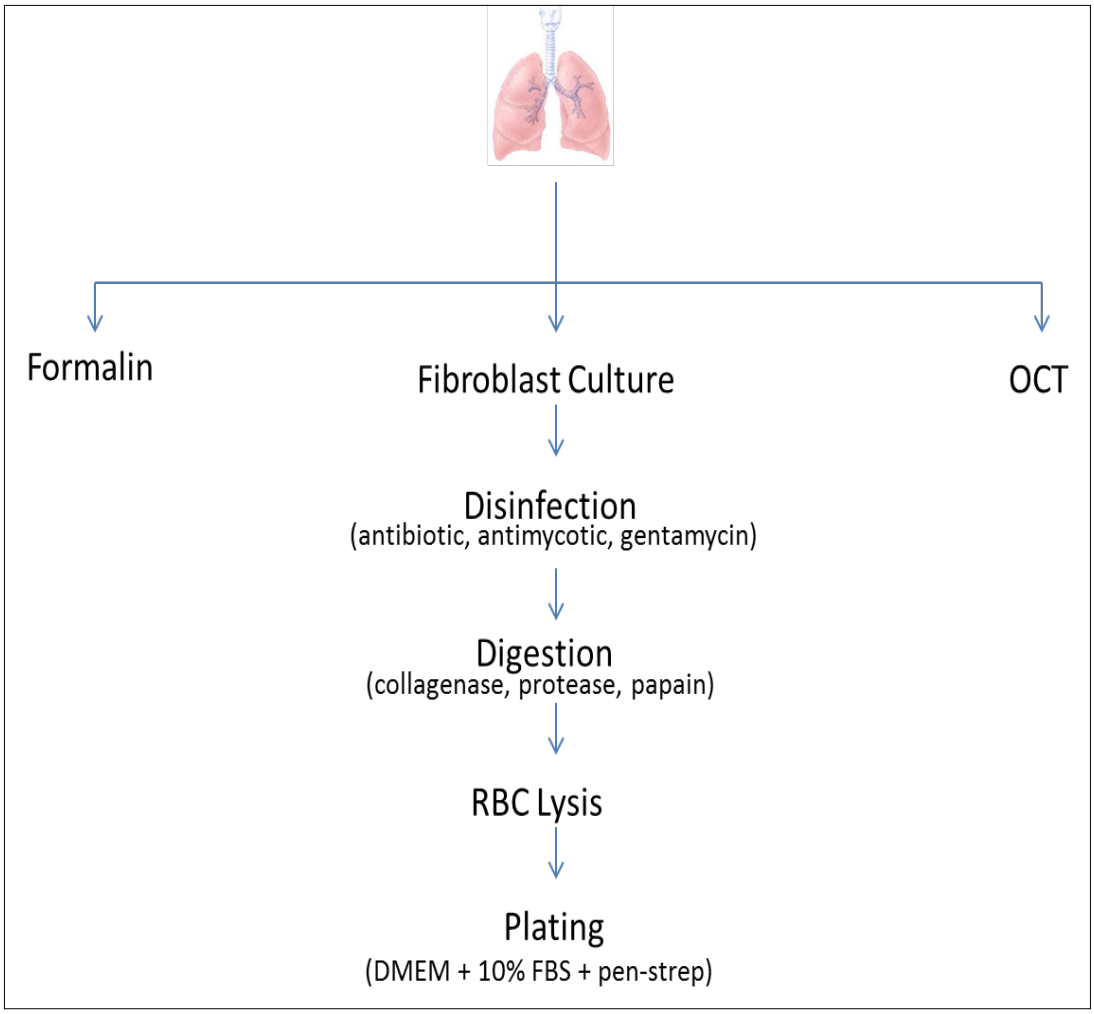

Figure 4: Human lung fibroblast culture protocol.
OCT – optimal cutting temperature, FBS – fetal bovine serum

3.2.2 Classification of the lung fibroblast culture

Primary HLF cultures were classified into mild, moderate and severe COPD (Table 1) according to the 2003 Global Initiative for Chronic Obstructive Lung Disease (GOLD) classification (http://www.goldcopd.org/). The non-COPD criteria was $FEV_1/FVC > 70\%$ and $FEV_1 > 80\%$ predicted, FEV_1 being the forced expiratory volume which indicates the volume of air exhaled out during the first second of a full breath. FVC stands for forced vital capacity which indicates the amount of air which can be forcibly exhaled out from the lungs after a maximum breath.

3.2.3 Hematoxylin and Eosin Staining

Formalin fixed tissues were processed to make paraffin embedded tissue blocks. The tissue was processed in 70% ethanol twice for 4hr each followed by treatment in 80% ethanol for 4hr. The tissue was then placed in 100% ethanol for 2hr followed by ethanol/xylene (50:50) treatment for 20 min followed by treating in xylene for 20 min. After the xylene treatment, the tissue was finally processed in wax twice for 2hr. Paraffin sections of approximately 4-6μm thickness were made using a microtome. The sections were then mounted on slides and the sections were dewaxed by incubating the slides in 58^{o}C oven for 30 min. After melting the wax, the slides was processed in xylene thrice for 5 min and then in 100% ethanol twice for 1 min each, followed by 95% and 70% ethanol treatment for 1 min each. The slides were rinsed in running tap water for 1 min. The slides were stained with hematoxylin (Sigma, Oakville, ON, Canada) for 3 min. Slides were rinsed in running tap water for 10-20 sec and dipped in 3X acid alcohol (acid alcohol-10 ml of concentrated HCl in 1 litre of 70% alcohol). The slides were again rinsed in running tap water for 10 sec followed by dipping in 2X ammonia water (7.5 ml of concentrated ammonia in 1 litre of distilled water). Slides were again rinsed in running tap water for 3 min, rinsed in 70% alcohol for 30 sec for a total of 5 times. The slides were dipped in Eosin Y for 5-10 times followed by rinsing in 70% alcohol 5 times, 30 sec each. Thereafter the slides were dehydraded by placing the slides in 70%, 95% and 100% ethanol for 1 min each, followed by xylene treatment for 2 min. Permount (Fisher, Pittsburgh, PA) was applied after xylene treatment and slides were coverslipped. The slides were allowed to dry before storing them in a box.

3.2.4 Cigarette smoke extract preparation

Cigarette smoke extract was prepared by the method established by Carp and Janoff with few modifications [180]. Briefly, two Kentucky reference research grade cigarettes (Kentucky 3R4F) were purchased from the University of Kentucky. Filters were removed from the cigarettes and were combusted with a modified valved syringe-driven apparatus (Figure 5). The smoke from each cigarette was bubbled through 25ml of DMEM for 2 sec maintaining a gap of 20 sec from each draw in order to imitate the actual cigarette puffing. This process was carried out at room temperature until the entire cigarette was combusted. The resulting suspension was adjusted to pH 7.2 with concentrated NaOH and filtered through a 0.22μm syringe filter to eliminate bacteria and impurities. The solution was considered as 100% CSE and adequate dilutions were made with DMEM and stored at -80^{o}C for further use.

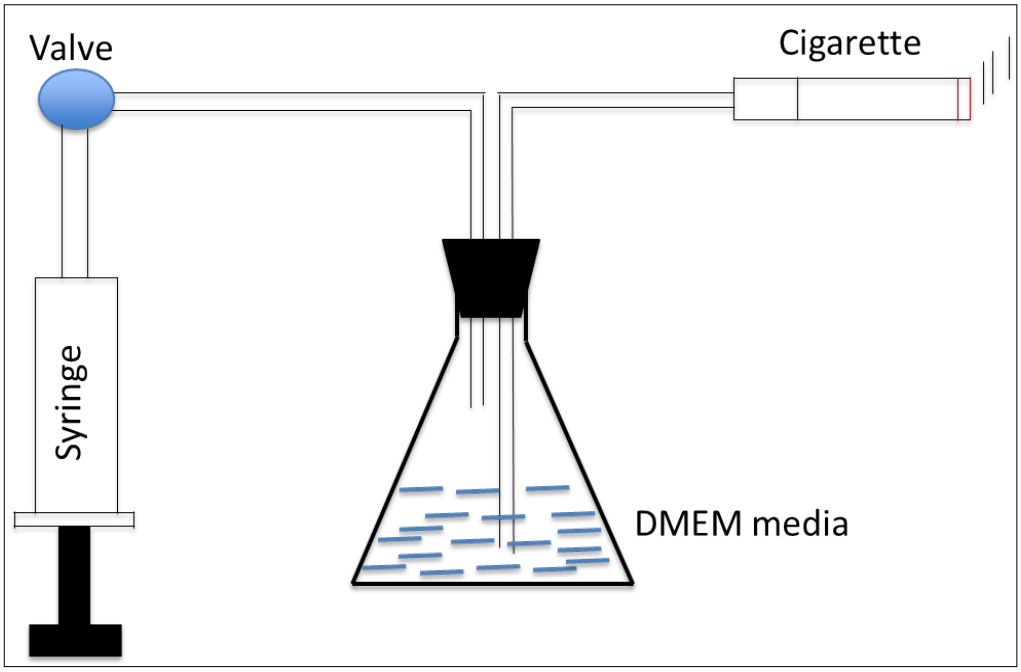

Figure 5: Cigarette smoke extract preparation.

Cigarette smoke extract (CSE) was collected from combusting cigarette and the smoke was bubbled through 25ml of Dulbecco's Modified Eagle's Medium (DMEM) with the help of a 50 ml syringe.

3.2.5 Cell viability assay

Cell viability was determined by the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) assay. HLFs from three moderate COPD and three non – COPD donors were plated at a density of 12,000 cells/ well in 96 well culture dishes using DMEM medium supplemented with 10% FBS and antibiotic (50 U/ml streptomycin, 50 µg/ml penicillin). At 60% confluency, culture medium was changed to serum-free DMEM supplemented with 1% insulin transferrin sodium selenium (ITS) and 1% pen-strep (50 µg/ml penicillin, 50 U/ml streptomycin) for 48h. After 48 h of serum deprivation respective treatments were added with fresh DMEM with 1% ITS and 1% pen-strep. DMEM and treatments were replenished at every 48h. MTT assay was performed at 24, 48, 72 and 96h. To measure cell viability, 20µl of MTT was added to the culture media and incubated for 2 h at 37^{o}C. The media was removed and 200µl of DMSO was added while mixing and OD at 570 nm was measured using an ELISA plate reader (Power Wave X Bio-Tek Instruments).

3.2.6 Proliferation assay

HLFs from three moderate COPD and three non – COPD donors were plated at 96,000 cells/well in 6 well sterile cell culture plates and grown using DMEM medium supplemented with 10% FBS and 1% pen-strep. The cultures were grown to 30 % confluency followed by serum deprivation for 48h in DMEM supplemented with 1% ITS and 1% pen-strep. Thereafter, culture medium was changed to DMEM supplemented with 5% FBS or inhibitor treatment such as different concentrations of simvastatin. As a control some wells were retained in serum-deficient DMEM. Proliferation was assessed at 2, 4 and 6 days after addition of 5% FBS using a cell counting assay that employed an iCys Laser Scanning Cytometry (LSC). Briefly, the DNA dye, Hoechst 33248, was added to culture medium at a final concentration of 10μg/ml and incubated for 10 min (37°C in a CO_2 incubator) to stain the nuclei. Cultures were then fixed with 3% paraformaldehyde (15 min at 4°C) and washed with sterile Cyto-TBS buffer. Cultures were stored in cyto-TBS buffer until counted by Laser Scanning Cytometer (LSC). LSC was used to measure DNA fluorescence of individual nuclei and simultaneously capture images using light scatter. The images can be viewed as scattograms or blue max pixel data. Although the technology utilizes the principle of flow cytometry, it allows analysis of adherent culture cells thus preserving the sample at its actual position in the culture dish. Signals for blue channel fluorescence from contoured cell nuclei and light scatter data for shaded relief images were captured.

3.2.7 Immunoblotting

HLFs from three COPD and non-COPD cultures were plated at a density of 70,000 cells/cm2 in 60mm culture plates in DMEM supplemented with 10% FBS and 1% pen-strep. When the cultures reached 80% confluency, the medium was changed to serum-free DMEM supplemented with 1% ITS and 1% pen-strep for 48h. After 48h of serum deprivation, fresh DMEM with TNFα (10ng/mL) and 1% pen-strep were added at 15, 30 and 45 min. DMEM without TNFα were used as negative controls. After the treatment period, cells were washed with ice cold phosphate-buffered saline (PBS) and thereafter whole cell lysates were made by adding ice cold 1ml of Radioimmunoprecipitation assay (RIPA buffer) containing 1mM of PMSF, protease inhibitor mix and phosphate inhibitor mix per 100mm culture plate. Cell lysates were prepared by scraping the plates that were placed on ice with a cell scraper. The cell lysate was sonicated on ice for 10 sec and centrifuged (800 x g, 5 min, 4°C). The supernatant was then removed and stored at −20°C for further protein analysis.

Protein content in supernatant samples was determined using the Bio-Rad protein assay which utilizes Lowry method of protein estimation and bovine serum albumin (BSA) as a reference (Bio-Rad, Hercules, CA). Proteins were loaded on to SDS- polyacrylamide gels ((SDS-PAGE) room temperature, 100V constant) at a concentration of 20µg of protein per lane. The proteins were then electroblotted to nitrocellulose membranes using a wet transfer method (SDS PAGE transfer buffer, 4^{o}C, 100V constant, 1h). The membrane were then blocked in Tris-buffered saline (TBS) (10 mM Tris·HCl, pH 8.0, 150 mM NaCl) containing 5% (wt/vol) skim milk powder and 0.1% Tween-20. Blocked

membranes were then incubated with primary antibodies (1:1000 dilution in TBS containing 1% (wt/vol) skim milk and (0.1%) Tween-20) overnight at 4^{o}C. The membranes were then incubated in HRP-conjugated secondary antibody (diluted at 1:3000 for anti-mouse and 1:5000 for anti-rabbit in TBS containing 1% (wt/vol) skim milk powder) for 1h at room temperature. Later the membrane was visualized on photographic film using enhanced chemiluminescence (ECL) reagents (Amersham ECL western blotting detection reagent, GE health care life sciences). GAPDH or β-actin were used as loading controls of all the samples.

3.2.8 Real time Polymerase chain reaction

HLF cultures from three moderate COPD and non – COPD subjects were plated at a density of 96,000 cell/well in 6 well dishes in DMEM with 10% FBS and 1% pen-strep. At 70% confluency the cultures were serum deprived for 48 h in DMEM supplemented with 1% ITS and 1% pen-strep. Cultures were then stimulated with 10 ng/ml of TNF α and unstimulated cells were used as time matched controls. Semi-quantitative PCR was performed using various human cytokine primers as listed in Table 2. For real time quantitative PCR experiments, total cellular RNA was isolated at 0, 12, 24, 48 and 72h using the RNeasy Plus Mini Kit (Qiagen, Mississauga, ON) and 1mg was reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen, Mississauga, ON). RNA purity was determined by the ratio of absorbance at 260nm and 280nm where the ratio was close to 1.8. The abundance of TSLP, TNFα receptor and IL-1β receptor mRNA was determined using the Applied Biosystems 7500 Real-Time PCR System thermocycler and the Power SYBR Green PCR Master Mix. Dissociation curves were generated at the end of each PCR reaction to identify that a single product amplification. Oligonucleotide primers were as follows:

Human TSLP (GenBank accession no. NM_033035)

forward 5’TATAGAGTGGGACCAAAAGTACCG 3’;

reverse 5’ GGGATTGAAGGTTAGGCTCTGG3';

Product size: 97 base pairs

Homo sapiens tumor necrosis factor receptor superfamily, member 1A (TNFRSF1A); NCBI Reference Sequence: NM_001065.3

Forward 5' TCAATTGCAGCCTCTGCCTCAAATG3';

Reverse 5'ACAACTTCGTGCACTCCAGGCTTT3';

Product size: 194 base pairs

Homo sapiens interleukin 1 receptor, type I (IL1R1); NCBI Reference Sequence: NM_000877.2

Forward 5' TTACCACAGGGACCACAGTCTGCAA 3'

Reverse 5' TGCAGTTTCTCCTTAGTGGCTGGT 3';

Product size: 220 base pairs

18S rRNA primers

Forward 5'-CGCCGCTAGAGGTGAAATTC-3' and

Reverse 5'-TTGGCAAATGCTTTCGCTC-3' served as the endogenous reference gene.

Product size: 120 base pairs

The relative expression levels of TSLP, TNFα receptor and IL-1β receptor were normalized to 18S rRNA.

Table 2: Human cytokine primers	
Gene	**Expected size (base pairs)**
IL-4	169
IL-5A	95
IL-8	110
IL-10	127
IL-13	115
IL-17A	144
Eotaxin-1	108
TNFα	128
TGF β	135
IFN-gamma	144
GAPDH	122

3.2.9 ELISA analysis

HLF cultures from three moderate COPD and non-COPD subjects were plated at a density of 50,000 cells/well in 24 well cell culture plates in DMEM with 10% FBS and 1% pen-strep. At 70% confluency the cultures were serum deprived for 48h in DMEM supplemented with 1% ITS and 1% pen-strep. Cells were then stimulated in fresh FBS-free DMEM containing graded concentrations (0, 0.1, 1, 10, and 100ng/ml) of human TNF-α, IL-1β, or TNF-α and IL-1β combined. In some experiments, cells were pretreated for 1 h with simvastatin (5μM or 1μM) before stimulation with TNF-α (10ng/ml), IL-1β (10ng/ml), or both at 10ng/ml for 24, 48 and 72h. Supernatants were then collected in sterile cryovials at 24, 48, 72h and centrifuged at 8000 x g for 7 min at 4°C to remove cellular debris, and stored at −80°C until further use. Supernatants were later analysed by Enzyme Linked Immunosorbent Assay (ELISA).

Immunoreactive TSLP was measured in the cell culture supernatant by ELISA with matched TSLP antibodies included in a TSLP ELISA kit (DY1398, R&D Systems). TSLP protein was measured according to the manufacturer's protocol. ELISA reader from PowerWave X Bio-Tek Instruments Incorporated was used to measure the optical density of each well at 450nm. A four parameter logistic curve fit was created using the computer software provided in the instrument for the calculation of final result. The sensitivity limit of the TSLP assay was 3.9pg/ml.

3.2.10 Luciferase reporter assays

The plasmid pGL3-basic vector (Promega, Madison, WI) encoding wild-type human TSLP promoter was kindly provided by Dr. Soussi Gounni (University of Manitoba). HLF cells (4×10^4) were plated into 24-well culture plates in DMEM containing 10% FBS and 1% pen-strep. At 70-80% confluency, cells were transfected with wild-type human TSLP promoter luciferase construct. Transient transfection of HLF cells was done utilizing an in vitro transfection agent: ExGen 500 (MBI Fermentas, Canada). Wild-type TSLP promoter DNA (1.6μg) and Renilla luciferase reporter vector pRL-TK (0.4μg) were co-transfected in the cells and incubated overnight at 37^{o}C. Next day, the medium was changed, and the cells were washed twice with sterile PBS and fresh DMEM along with recombinant TNFα (10ng/ml) was added to the cells. Medium without TNFα were used as controls. Cultures were further incubated at 37^{o}C for 12h. Cells were washed twice with sterile PBS and 100μl of reporter lysis buffer (Promega) were added to each well. Cell lysates were collected with continuous shaking at room temperature for 20 min. Luciferase activity was measured by the dual-luciferase assay system kit (Promega, Madison) and using a luminometer (model LB9501; Berthold, Bad Wildbad, Germany). 20μL of cell lysate was mixed with 25μL of luciferase assay reagent II and firefly luciferase reading was taken. Then, 25μL of Stop-and-Glo reagent were added and Renilla luciferase activity was measured. All the values were normalized to Renilla and expressed as relative luciferase units (RLU) to the transfected DMEM controls.

3.2.11 Immunofluorescence

Formalin fixed lung tissue sections from three COPD and non-COPD donors were paraffin embedded and cryosectioned at a size of 4-6μm thickness and fixed on a glass slide. For immunofluorescence, sections were rehydrated and antigen retrieval was performed by heating in 10mM sodium citrate (pH 6.0) in a boiling water bath for 20 min. 0.1% Sudan black in 70% ethanol were added to the sections and incubated for 20 mins at room temperature to block auto-fluorescence. Slides were then incubated with blocking buffer containing Roche blocking solution, maleic acid buffer, FBS, PBS, Tween -20 for 30 min at room temperature. The sections were incubated in primary antibody with subsequent dilutions made in blocking solution overnight at 4°C. Next day, the slides were washed thrice in PBS containing 0.05% (v/v) Tween-20 (PBST) followed by incubation with secondary antibody which was also diluted in blocking solution for 1h. The sections were then washed thrice and incubated with DAPI (1:5000 dilution in PBS) for 5 min. Later, sections were mounted by applying prolong GOLD. Primary antibody was human TSLP (ProSci incorporated) and secondary antibody was rhodamine-labeled anti-rabbit IgG (Invitrogen).

3.2.12 Immunohistochemistry

Formalin fixed lung tissue sections from three COPD and non-COPD were paraffin embedded and cryosectioned at a size of 4-6μm thickness and fixed on to a glass slide. For immunohistochemistry, tissue sections were rehydrated and antigen retrieval was performed in 10 mM sodium citrate (pH 6.0) while heating in a boiling water bath for 20 min. After washing with 1X PBS, samples were incubated in blocking solution for 30 min at room temperature then washed thrice in PBS. The sections were then incubated in a solution of 3% H_2O_2 and PBS for 10 min. Further, the sections were incubated in Avidin and Biotin blocking solution for 15 min followed by incubation with primary antibody (4°C overnight) and biotinylated secondary antibody at room temperature for 1h. The sections were washed thrice with PBS and finally incubated in ABC solution (Vector Inc) for 30 min. The slides were developed with 3,3-diaminobenzidine (DAB) substrate and nuclei were counter-stained with Mayer's haematoxylin and mounted with prolong GOLD.

3.2.13 Statistical analysis

The results from the MTT assay, proliferation assay, ELISA, Real time PCR, Luciferase promoter assay and Western blotting experiments were expressed as mean ± SEM (standard error of means) and statistical differences were evaluated by one-way or two-way ANOVA (as specified in figure legends) followed by Bonferroni's post hoc test or Bonferroni multiple comparison test using Graph Pad Prism 5. Differences were considered statistically significant at a $P< 0.05$.

4. Results

4.1 Characterization of primary human lung fibroblasts from COPD and non-COPD donors

4.1.1 Cell lines used for experiments

Cell lines that were used in the experiment are listed in Table 3 and Table 4.

Table 3: COPD human lung fibroblast cell lines.

Number	Gender	Age	Smoking Status	GOLD-classification	FEV_1	FEV_1/FVC
HLF015	M	79	Ex:30pyrs/Q38yrs ago	II - moderate COPD	2.28=78%	2.28/3.34=68%
HLF086	M	69	Ex:22pyrs/Q:15yrsago	II - moderate COPD	2.12=56%	2.12/3.24=**65%**
HLF118	M	68	C:100pkyrs/2ppd50yrs	II - moderate COPD	2.69=76%	2.69/3.83=70%
HLF012	M	76	C:60/yrs1ppd/60pyrs	II - moderate COPD	1.86=65%	1.86/2.85=65%
HLF014	F	71	C:46yrs/0.5ppd/23pyrs	II - moderate COPD	1.35=67%	1.35/2.23=60%
HLF0124	M	63	C:50yr./0.8pkd/40pkyrs	II - moderate COPD	1.8=55%	1.8/3.48=52%
HLF013	F	76	C:50yrs/0.6ppd/30pyrs	II - moderate COPD	1.16=53%	1.16/1.68=69%
HLF080	F	72	C:50yrs/0.5ppd/25pyrs	II - moderate COPD	1.15=57%	1.15/1.73=67%
HLF 129	M	58	C:49pkyrs	II - moderate COPD	1.75=51%	1.75/3.49=50%

Table 4: Non-COPD human lung fibroblast cell lines.						
Number	**Gender**	**Age**	**Smoking status**	**COPD classification**	**FEV_1**	**FEV_1/FVC**
HLF017	M	73	Ex:45pyrsQ:25yrs ago	Non-COPD	3.07=99%	3.07/3.71=83%
HLF090	F	56	Ex:41pyrs/4yrs ago	Non-COPD	2.63=111%	2.63/3.49=93%
HLF091	M	65	Ex:45p/yrs/21yrsago	Non-COPD	2.78=86%	2.78/3.86=72%
HLF110	M	66	Ex:25pyrs-Q:20yrs	Non-COPD	2.98=94%	2.98/3.87=77%
HLF117	F	85	Ex:65yrs-ago	Non-COPD	1.80=120%	1.80/2.37=76%
HLF128	F	57	Ex:20.5pkyrs	Non-COPD	2.77=92%	2.77/3.91=71%
HLF137	F	73	Ex:40pk/yrs/Q3.5 ago	Non-COPD	1.83=90%	1.83/2.49=73%
HLF138	M	55	C:21pkyrs	Non-COPD	3.35=98%	3.35/4.48=75%
HLF082	F	78	Never smoked	Non-COPD	2.12=113%	2.12/2.92=73%
HLF103	F	80	Never Smoked	Non-COPD	2.19=104%	2.19/2.74=80%

4.1.2 Primary human lung fibroblast cultures showed fibroblast morphology

Primary HLF cultures were derived from COPD and non-COPD donors using an enzymatic digestion approach. HLFs were plated in DMEM supplemented with 10% FBS and 1% pen-strep, and were termed as P0 (passage 0) at the time of plating (Figure 6A). The media was not changed for the first 3-4 days in order to let the cells attach to the culture dish. Thereafter, the media was changed at every 48h until fibroblasts reached 70-80% confluency (Figure 6B). The cells were then passaged using a 1:4 split ratio to generate P1 (passage 1) primary cultures. At P1, after they reached 90% confluency (Figure 6C) they were either frozen in liquid nitrogen or passaged again and used for further experiments (P2-P4). The fibroblasts appeared to have the typical morphology of fibroblast; with branched cytoplasm surrounding an elliptical, speckled nucleus having one or two nucleoli.

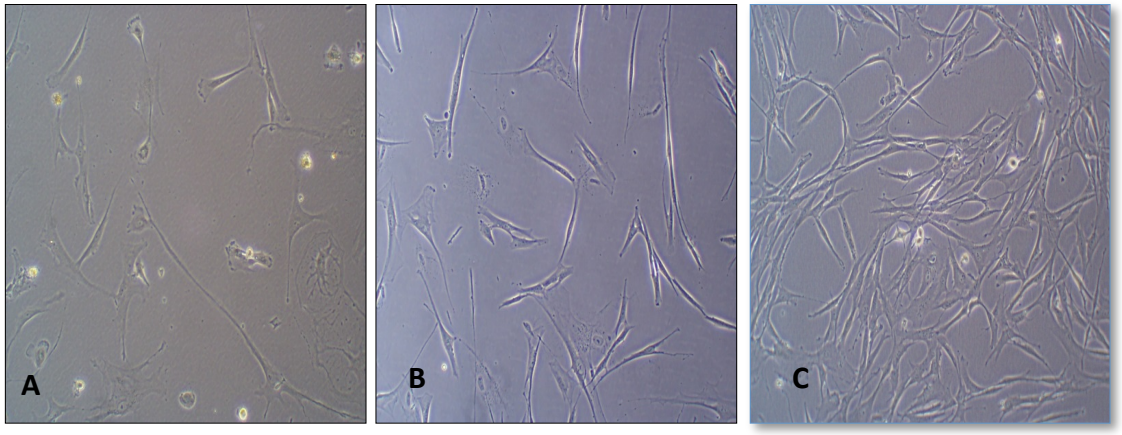

Figure 6: Primary human lung fibroblast (HLF) cultures exhibited typical fibroblast morphology. HLFs were plated in DMEM supplemented with 10% FBS and 1% pen-strep. HLFs were termed as P0 (passage 0) at the time of plating after isolation from lung tissue (A). The media was not changed for the first 3-4 days in order to let the cells attach. When cells reached ~60-70% confluency (B) they were passaged to P1 (C).

4.1.3 Peripheral lung fibroblasts exclusively express vimentin

For all the cultures we ascertained fibroblast phenotype, we characterized them using Western blotting to detect vimentin which is commonly used as a fibroblast marker. In addition, we assessed abundance of E-cadherin, as epithelial marker, and von willebrand factor (VWF) as endothelial marker. Cell lysates were obtained from three COPD and non-COPD HLFs and analyzed for the presence of vimentin, E-cadherin and VWF. Immunoblots showed that all the cultures expressed abundant vimentin, but we could not detect E–cadherin or VWF (Figure 7). This confirmed that our primary lung fibroblast cultures didn't contain any epithelial or endothelial cells and they were purely comprised of cells of mesenchymal origin, i.e. fibroblasts.

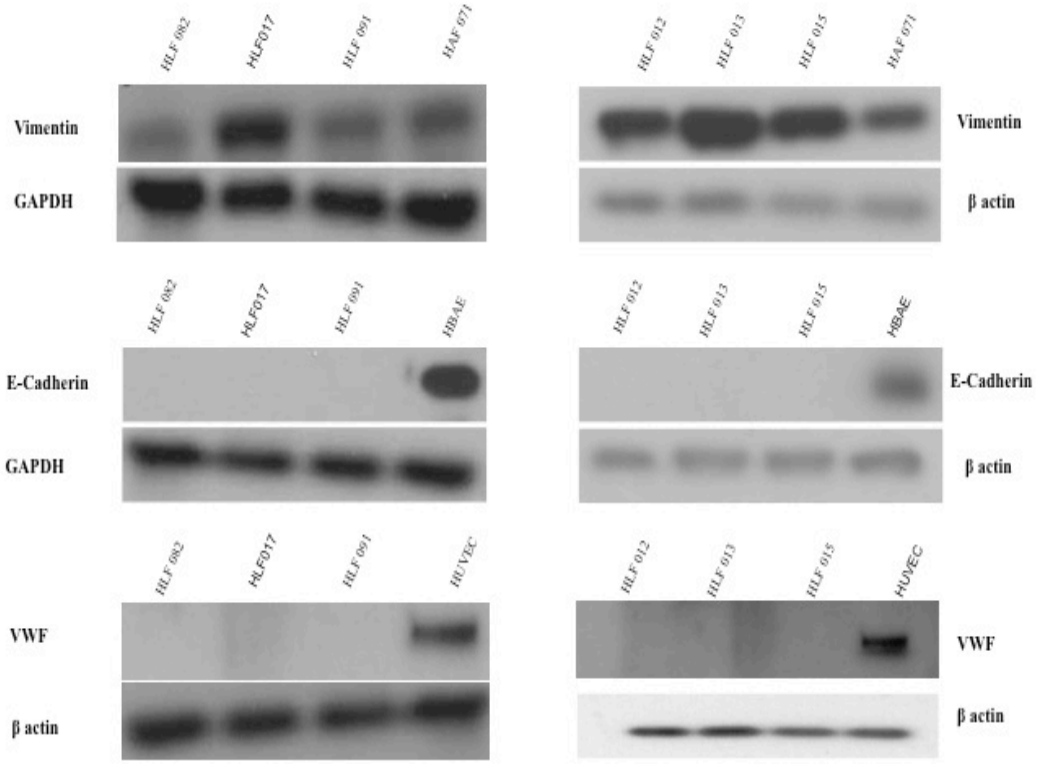

Figure 7: Characterization of human lung fibroblasts. COPD (HLF-082,017,091) and three COPD (HLF-012,013,015) donors were compared to positive controls for fibroblast (HAF – human airway fibroblast), epithelial cell (HBAE – human bronchial airway epithelial cells), endothelial cells (HUVEC – Human umbilical vein endothelial cell). Cell lysates were obtained from all the cultures at P1 (passage 1). Western blot was utilized to analyze cell lysates using vimentin as a marker for fibroblast, E-cadherin as a marker for epithelial cell and VWF (von willibrand factor) as a marker for endothelial cell.

4.1.4 Characteristic destruction of lung tissue in COPD lung sections

Lung damage and inflammation of the air sacs (alveoli) is linked with emphysema, an expansion of the air spaces near to the terminal bronchioles with considerable loss of air space walls. To ascertain lung damage in the tissue specimens from which we derived primary HLF cultures we performed Haematoxylin and Eosin staining on tissue adjacent to that used for primary culture derivation. Non-COPD lung tissue appeared to have normal lung morphology with no evidence of alveolar wall destruction whereas in COPD lung there was destruction of alveolar walls, evident by markedly increased ratio of airspace: alveolar wall (Figure 8).

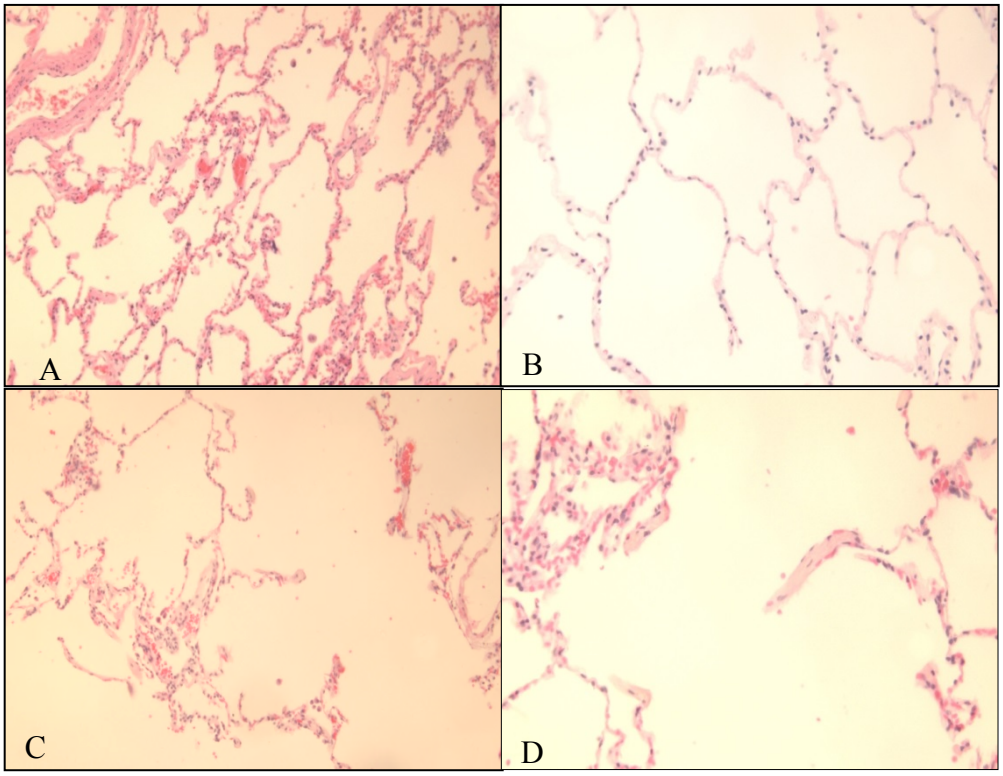

Figure 8: Haematoxylin and Eosin staining of COPD and non-COPD lung.
Absence of alveolar walls, and increased airspace was evident in COPD lungs (C & D) compared to non-COPD donors (A & B) 10X (A and C) and 20X (B and D).

4.1.5 MTT assay - viability of HLF treated with simvastatin

Primary HLFs at Passage 2-3 from COPD and non-COPD donors were grown in DMEM supplemented with 10% FBS and 1% pen-strep. At 60% confluency the cells were serum deprived in DMEM with 1% ITS for 48h. Simvastatin (0.1 to 5μM) was added for up to 96 h, refreshing media and supplements after 48h. MTT assay was performed every 24h to assay cell viability. Data demonstrated that up to 5μM simvastatin is not toxic to the cells over 96h. Indeed, 5μM simvastatin appeared to have a tendency to increase viability in COPD HLFs (Figure 9). We also compared (Figure 10) the raw OD values at 570nm in DMEM controls of COPD and non-COPD HLFs. We observed that non-COPD HLFs were more viable then COPD HLFs at 48, 72 and 96 h ($P < 0.05$).

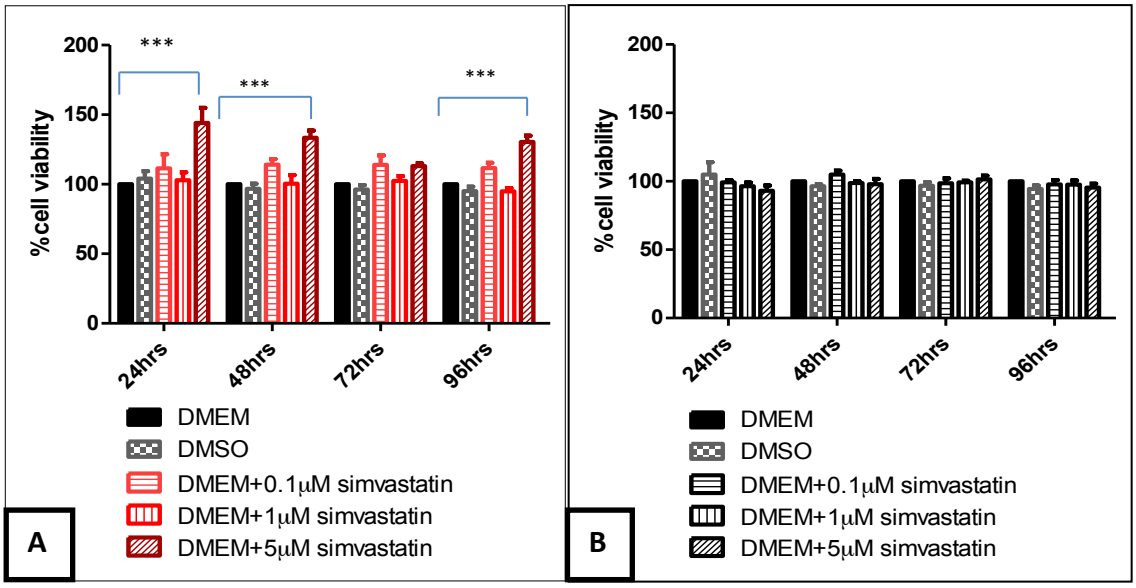

Figure 9: Effect of simvastatin on COPD and non-COPD HLFs viability.
Peripheral HLFs from (A) COPD and (B) non-COPD donors were grown to 60%confluency before serum deprivation of 48 h. Simvastatin was added to the culture medium at a concentration of 0.1μM, 1μM and 5μM. Media and treatments were replaced at 48 h. MTT assay was performed at 24 h to 96 h to analyze cell viability. Data represent the ± SEM (standard error of means) of triplicate values from three independent experiments done with cells from three COPD and non-COPD HLFs. Statistical analysis: Two-way ANOVA, Bonferroni post hoc analysis using Graph Pad Prism version 5. $p<0.05$ was considered significant. *Note:****$p<0.001$

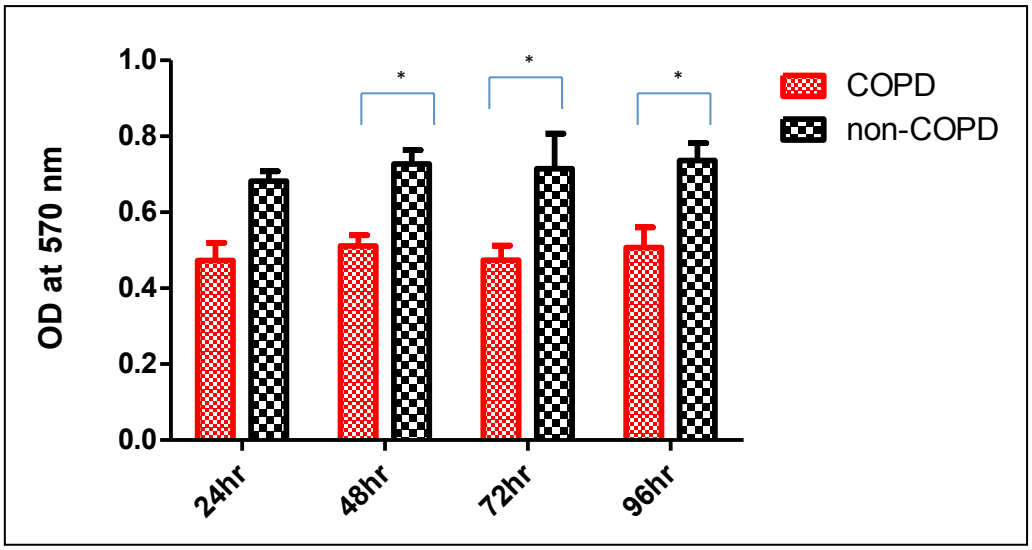

Figure 10: Comparison of cell viability of COPD and non-COPD HLFs DMEM controls. Peripheral HLFs from COPD and non-COPD donors were grown to 60%confluency before Serum deprivation of 48 h in 1% ITS. MTT assay was performed after serum deprivation for 24 h to 96 h to analyze cell viability. Raw OD values at 570 nm were plotted to compare cell viability in DMEM controls. Data represent the ± SEM (standard error of means) of duplicate values from three independent experiments done with cells from three COPD and non-COPD HLFs. Statistical analysis: Two-way ANOVA, Bonferroni post hoc analysis using Graph Pad Prism version 5. $p<0.05$ was considered significant. *Note:**$p<0.05$*

4.1.6 Proliferation of COPD HLFs is slower than Non-COPD HLFs

To compare proliferation of HLFs from COPD and non-COPD donors, we used 5% FBS as the mitogen. Cultures grown in only DMEM served as negative controls. The cells were fixed with 3% paraformaldehyde and the nuclei were stained with the DNA dye, Hoechst 33248, then cell number was determined by counting nuclei using LSC after 2, 4 and 6 days of stimulation. LSC can measure DNA fluorescence of individual nuclei and simultaneously capture images using light scatter. FBS initiated proliferation in both COPD and non-COPD HLFs. Interestingly, proliferation of COPD HLFs was slower as compared to non-COPD HLFs, a result consistent with findings from other groups [181]. After 4 and 6 days of FBS exposure respectively, COPD HLF proliferation was 51.6% (P<0.05) and 42% (P<0.001) less than that of non-COPD HLFs (Figure 11A). We further calculated their proliferation rate during the exponential growth phase and confirmed that COPD HLFs proliferated 46.7% slower than non-COPD HLFs (Figure 11B). We also compared (Figure 12) the proliferation of COPD and non-COPD HLFs DMEM controls and observed that there was more proliferation (P<0.05) in non-COPD HLFs at 2, 4 and 6 days.

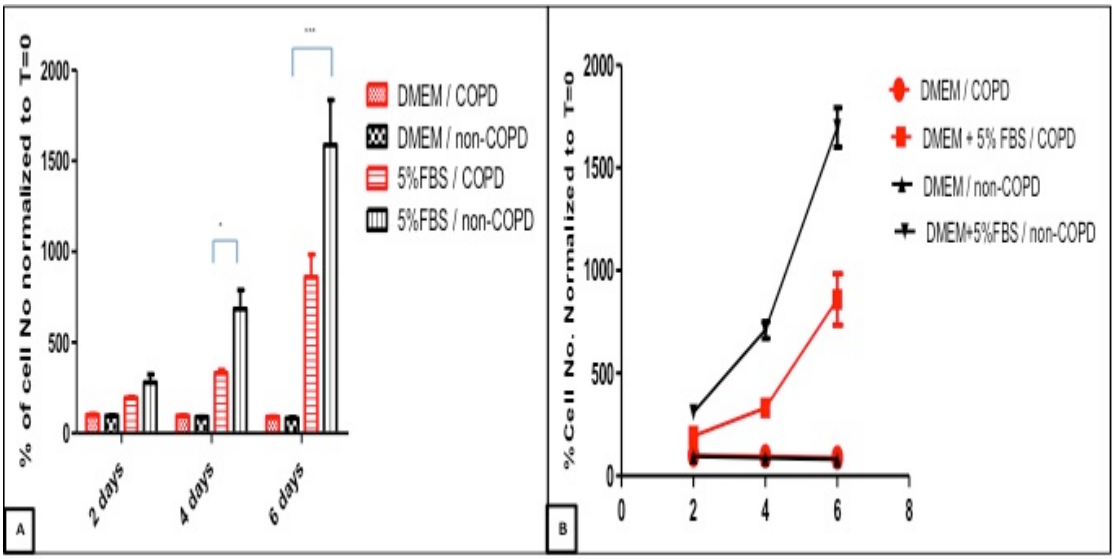

Figure 11: Proliferation of COPD and non-COPD HLFs. Proliferation of HLFs from COPD and non-COPD donors was compared using 5% fetal bovine serum (FBS) as the mitogen. Nuclei were stained with Hoechst 33248 and counted using LSC. Cultures grown in only DMEM were used as controls. Proliferation (A) and exponential growth phase (B) was measured from 2 to 6 days and normalized to cell number at T=0 time point. Data represent the ± SEM (standard error of means) of triplicate values from three independent experiments done with cells from three COPD and non-COPD HLFs. Statistical analysis: Two-way ANOVA, Bonferroni post hoc analysis using Graph Pad Prism version 5. $P<0.05$ was considered significant. *Note:****$P<0.001$;*$P<0.05$

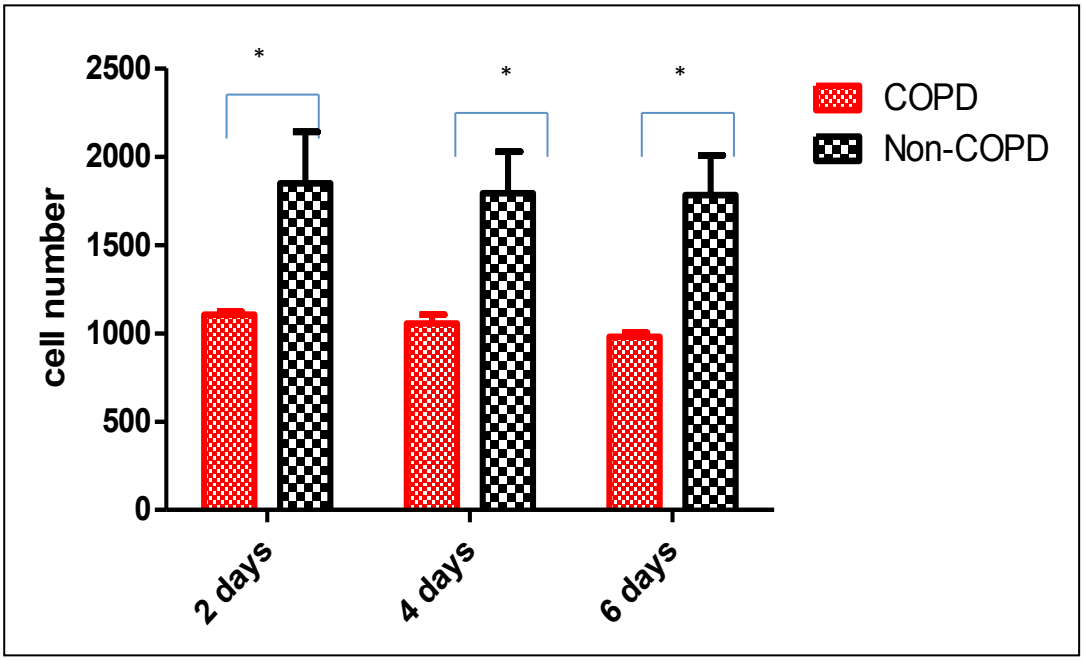

Figure 12: Comparison of proliferation in COPD and non-COPD DMEM controls. Proliferation of HLFs from COPD and non-COPD donors were compared using 5% fetal bovine serum (FBS) as the mitogen. Nuclei were stained with Hoechst 33248 and counted using LSC. Cultures grown in only DMEM were used as controls. Data represent the ± SEM (standard error of means) of triplicate values from three independent experiments done with cells from three COPD and non-COPD HLFs. Statistical analysis: Two-way ANOVA, Bonferroni post hoc analysis using Graph Pad Prism version 5. $P<0.05$ was considered significant. *Note:*$*p<0.05$.

4.1.7 Simvastatin significantly inhibits proliferation of lung fibroblasts

COPD and non-COPD HLFs were cultured in the absence and presence of simvastatin (0.1μM to 0.5μM) for up to 6 days. In cells treated with simvastatin, cultures were pre-incubated for 1hr before adding FBS. The cells were fixed with 3% paraformaldehyde and the nuclei were stained by Hoechst 33248 at 2, 4 and 6 days after the addition of simvastatin. At a concentration of 0.5μM, simvastatin significantly inhibited proliferation of both COPD and non-COPD HLFs. Indeed, at 6 days of culture 0.5μM simvastatin inhibited proliferation of COPD HLFs (Figure 13A) by 47% (P<0.001) and non-COPD HLFs (Figure 13B) by 52% (P<0.001).

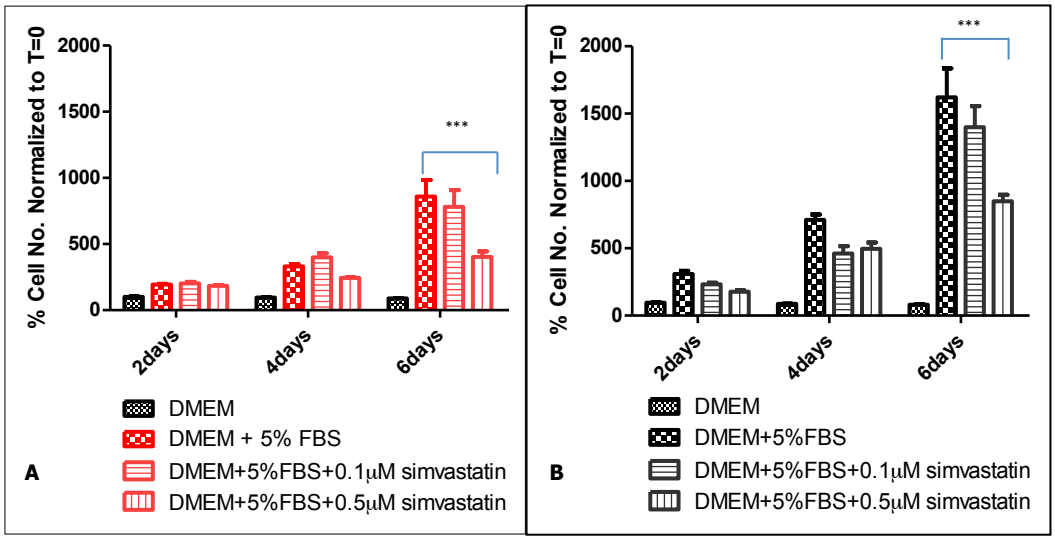

Figure 13: Effect of simvastatin on cell proliferation. Simvastatin was added to the culture medium at a concentration of 0.1μM to 0.5μM. Proliferation was determined by counting Hoechst Stained nuclei by LSC up to 6 days. Data represent the ± SEM of triplicate values from three independent experiments done on three COPD (A) and non-COPD (B) cell lines. Effect of simvastatin was compared with un-treated FBS controls. FBS (fetal bovine serum), SEM (standard error of means). Statistical analysis: Two-way ANOVA, Bonferroni post hoc analysis using Graph Pad Prism version 5. $P<0.05$ was considered significant. *Note:****$P<0.001$

4.1.8 Semi-quantitative PCR analysis indicates TSLP mRNA is induced by TNFα

A number of studies have shown that pro-inflammatory cytokines such as TNFα are elevated in COPD. In order to investigate the cytokine profile induced by TNFα, we performed a pilot experiment in one non-COPD HLF cell culture, stimulating cells with 10ng/ml of TNFα then preparing cell lysates 0, 12, 24, 48 and 72h later for PCR analysis. Semi-quantitative PCR was performed to assess the expression of 11 different cytokines associated with inflammation in COPD: IL-13, IL-5A, IL-4, TNFα, TGFβ1, IL-17A, IL-6, IL-8, IL-10, INFγ, eotaxin-1 (Figure 14) and TSLP (Figure 15). As controls we used cultures that were not exposed to TNFα. Our analysis demonstrated that though HLFs expressed many of these cytokines, TNFα *did not* induce substantive IL-13, IL-5A, IL-4, TNF α, TGFβ1, IL-17A, IL-6, IL-8, IL-10, INF, eotaxin-1 mRNA expression. It was interesting to observe that TNFα induced TNFα mRNA at all the time points. TGFβ, eotaxin-1 and IL-8 mRNA levels were observed both in the DMEM controls and the TNFα induced group. IL-4 mRNA was observed in the TNFα induced group at 24h. A prominent TSLP band was seen only after stimulation with TNFα at 12h. The TSLP band was present at 24, 48 and 72h but due to a smaller product size (97bp), the band was masked by the loading dye and appeared to be a very faint band.

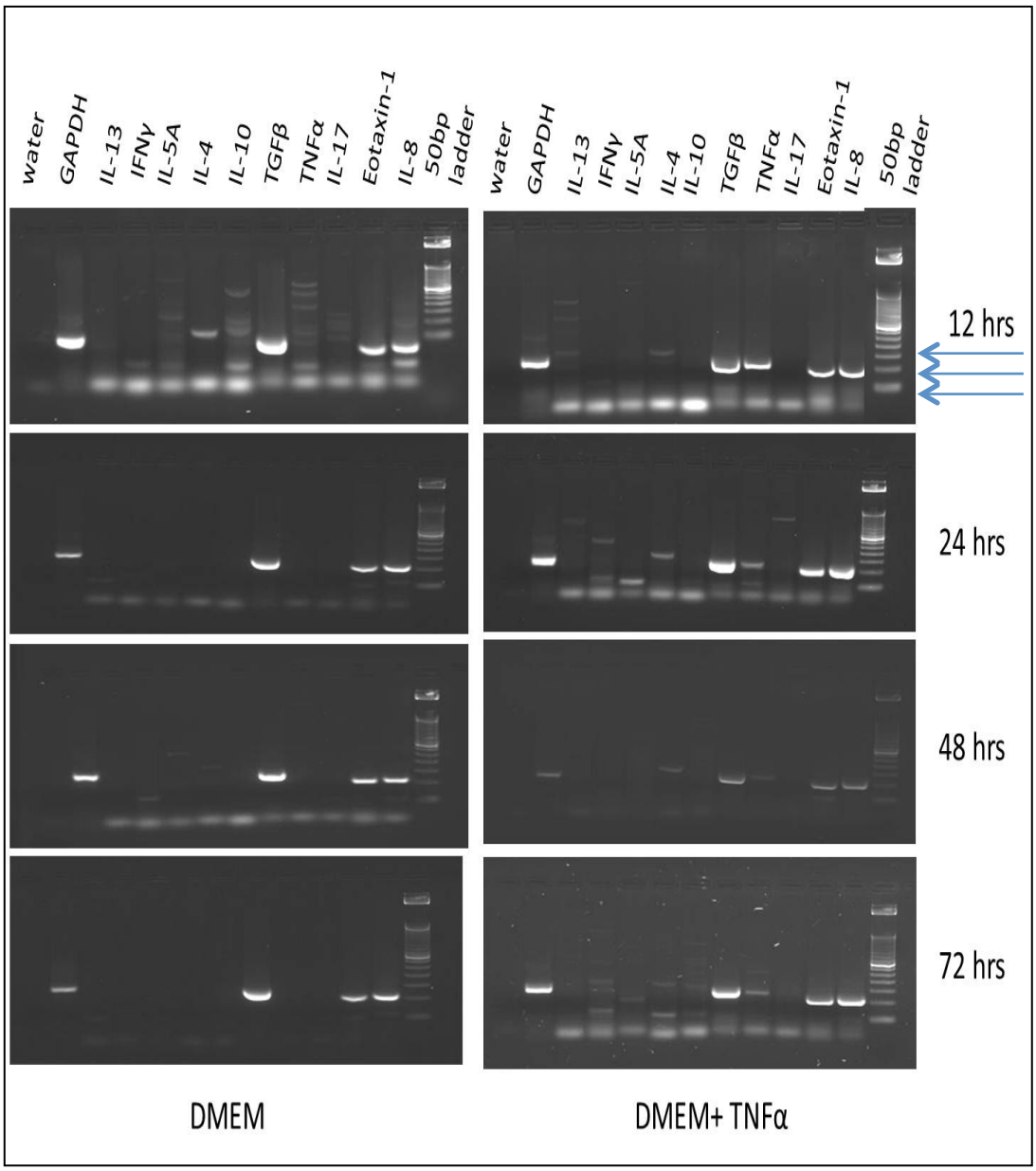

Figure 14: Relative mRNA levels of cytokines induced by TNFα. Semi – quantitative PCR was used to compare relative mRNA levels of various inflammatory cytokines stimulated by TNFα (10ng/ml) in a non-COPD HLF at 12, 24, 48 and 72 h. GAPDH was used as internal control.

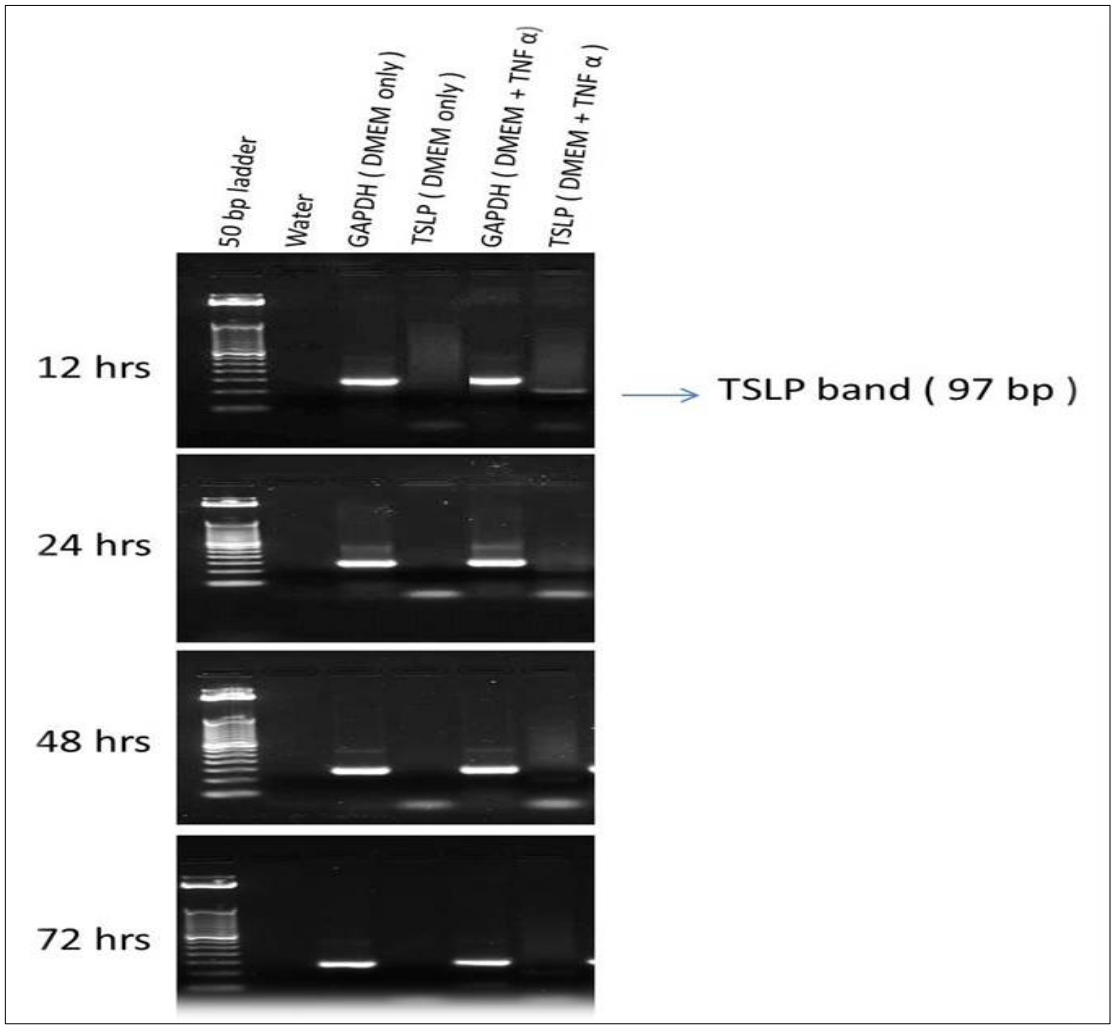

Figure 15: Relative mRNA levels of TSLP induced by TNFα. Semi – quantitative PCR was used to compare TSLP mRNA levels stimulated by TNFα (10ng/ml) in a non-COPD HLF at 12, 24, 48 and 72 h. GAPDH was used as internal control.

4.1.9 TNFα, IL-1β induce TSLP protein release from human lung fibroblasts

We wanted to know whether pro-inflammatory cytokines such as TNFα and IL-1β can induce TSLP protein release, thus we stimulated cells (10ng/mL TNFα and/or IL-1β) then used ELISA to assess TSLP release in the culture media. TNFα and/or IL-1β treatment were added to the culture medium after 48 h of serum deprivation in 1% ITS. The time point when the treatments were added to the culture was considered as T=0 h. Media and treatment were replenished after 48 h. Un-stimulated cultures were used as controls. Cell culture supernatant was collected at 0, 48, 72 and 96h (media and treatment was replenished after collecting the cell culture supernatant at 48h followed by collecting the culture supernatant for the next 24 and 48h respectively, which was considered as 72 and 96 h) and TSLP protein was measured by ELISA. After collecting the supernatant we fixed the cells in the culture plates and nuclei were stained with Hoechst 33248 dye to allow the use of LSC to count nuclei in each well so that ELISA data could be normalized on a per/cell basis. We found that TNFα and IL-1β, alone or in combination, significantly induced TSLP protein release in both COPD HLFs (Figure 16A) and non-COPD HLFs (Figure 16B). TNFα and IL-1β had an additive effect in TSLP release at 48 h in COPD HLFs though there was no additive effect of TNFα and IL-1β was observed in COPD HLFs at 72 h or non-COPD HLFs at 48 and 72 h.

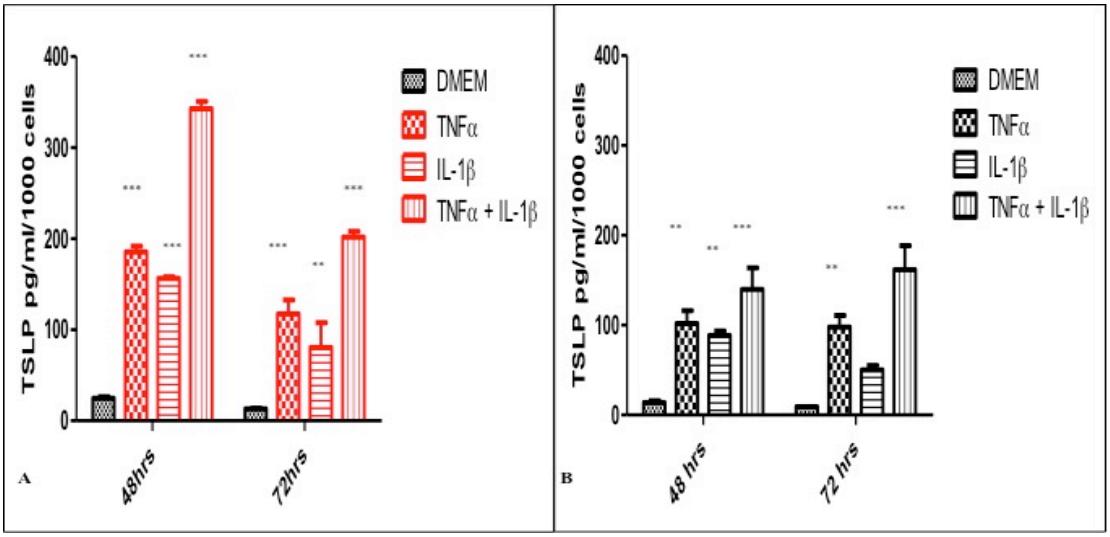

Figure 16: TSLP protein secretion induced by pro inflammatory cytokines. TSLP protein secretion was measured in COPD HLFs (A) and non-COPD HLFs (B) by ELISA through assessing cell culture supernatant. TNFα (10ng/ml), IL-1β (10ng/ml), TNFα & IL-1β (10ng/ml each) were added to the cell cultures after 48 h of serum deprivation. Data represent the ± SEM (standard error of means) of triplicate values of three independent experiments done on three COPD and non-COPD HLFs. TSLP release was compared to DMEM controls. Statistical analysis: Two-way ANOVA, Bonferroni post hoc analysis using Graph Pad Prism version 5. $P<0.05$ was considered significant. Note: ** $p<0.01$;*** $p<0.001$.

4.1.10 Cigarette smoke extract did not induce TSLP protein release

As tobacco smoking is the major cause of COPD, we assessed whether cigarette smoke extract (CSE) was also able to stimulate TSLP protein release. We added increasing concentrations (2%, 5%, 10% and 15%) of CSE to the cell culture medium for 48 and 72 h. Un-stimulated cultures were used as controls. Cell culture supernatant was collected, the cells remaining in the plate were fixed and the nuclei were stained with Hoechst 33248 for cell counting by LSC. TSLP protein release was measured by analysing culture supernatants with ELISA. Data were normalized as TSLP release per cell. Somewhat surprisingly, CSE did not induce TSLP release in either COPD (Figure 17 A) or non-COPD HLFs (Figure 17 B) over 72 h.

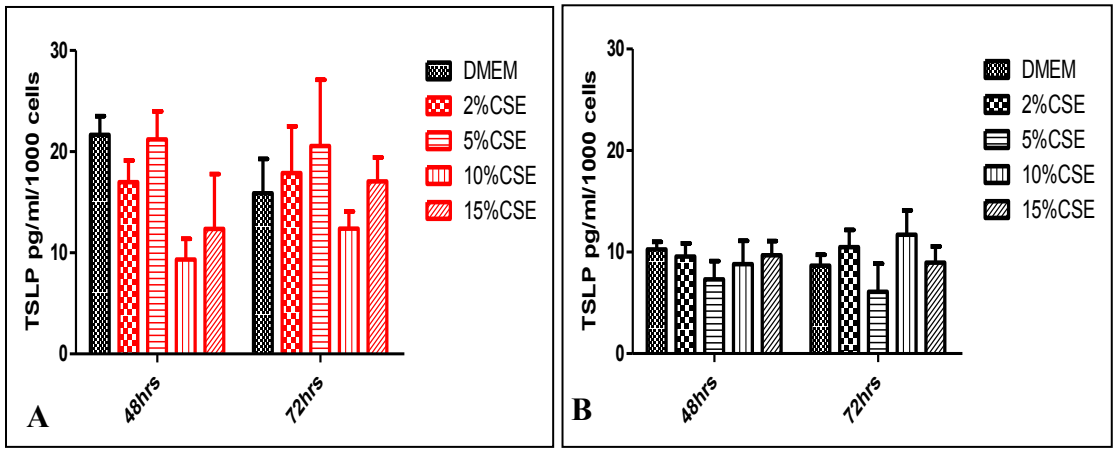

Figure 17: CSE induced TSLP protein secretion. CSE (cigarette smoke extract) was added to the culture medium at a concentration of 2% to 15% after 48 h of serum deprivation to induce TSLP release. Cell culture supernatant was collected at 48 h and 72 h. TSLP protein release was determined by ELISA through assessing cell culture supernatant in COPD HLFs (A) and non-COPD HLFs (B). TSLP reading was normalized to the cell number. Data represent the ± SEM (standard error of means) of triplicate values of three independent experiments done on three COPD and three non-COPD cell lines. Effect of CSE was compared with un-induced DMEM controls. Statistical analysis: Two-way ANOVA, Bonferroni post hoc analysis using Graph Pad Prism version 5. $P<0.05$ was considered significant.

4.1.11 CSE induced IL-8 release as determined by ELISA

ELISA showed that CSE did not induce TSLP protein release so we wanted to confirm whether the properties of CSE were not altered with the protocols we established. To confirm that, we used the culture supernatant from one CSE exposed COPD HLF from the above experiment and assessed IL-8 release as it has been reported [182, 183] that CSE induces IL-8 release in other cell types. Our ELISA result indicated that CSE induces IL-8 release and thereby suggested the bioactive capacity of the CSE though we could not perform a statistical analysis due to only one sample (Figure 18).

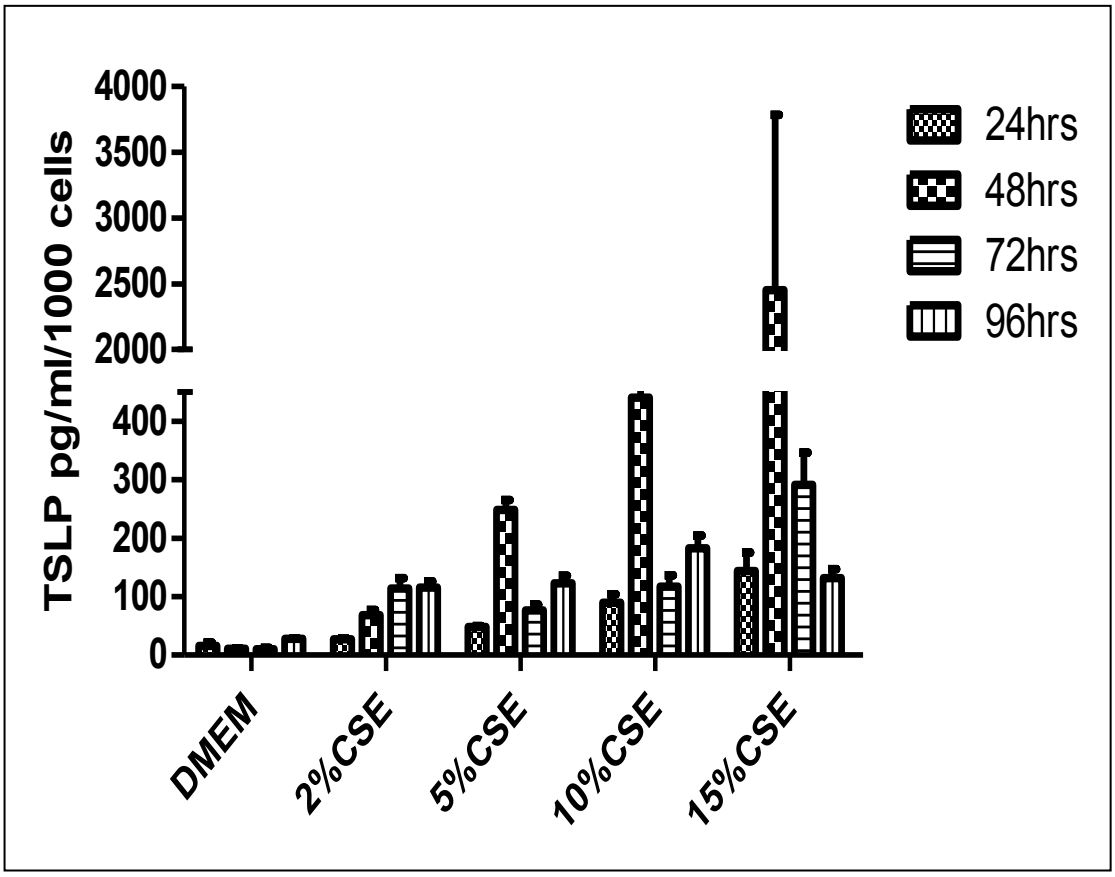

Figure 18: CSE induced IL-8 protein secretion. IL-8 protein release was determined by ELISA through assessing cell culture supernatant. CSE was added to the culture medium at a concentration of 2% to 15% to induce IL-8 release. Cell culture supernatants were collected at 24, 48, 72 and 96 h to analyse IL-8 release by ELISA. Data represent values the ± SEM (standard error of means) of triplicate values from one COPD HLF. IL-8 secretion was compared to un-induced DMEM controls.

4.1.12 COPD derived human lung fibroblasts exhibit increased basal and cytokine stimulated TSLP release

From our experiments we concluded that pro-inflammatory cytokines such as TNFα and IL-1β can stimulate TSLP release in both COPD and non-COPD HLFs. We further compared TSLP release in COPD and non-COPD HLFs at basal levels (Figure 19 A), as well as upon cytokine stimulation (Figure 19 B). We found that at baseline COPD HLFs release approximately 2-fold more TSLP per cell compared to non-COPD HLFs ($P<0.05$). TNFα and/or IL-1β (10ng/ml) stimulated TSLP release at 48 and 72h. COPD HLFs released 1.8-fold to 2.3-fold more TSLP at 48h compared to non-COPD HLFs (IL-1β; $P<0.01$, TNFα and TNFα + IL-1β; $P<0.001$). We broadened our investigation by screening 6 different cell lines each for COPD and non-COPD HLFs. To have a more precise result, we restricted ourselves to select one stimulation (TNFα) and the best time point (48h) based on our above experiment. After stimulation with TNFα (10ng/ml) for 48h we affirmed that TSLP release was significantly higher in COPD HLFs, both un-stimulated ($P<0.05$), and TNFα-stimulated ($P<0.001$) (Figure 20).

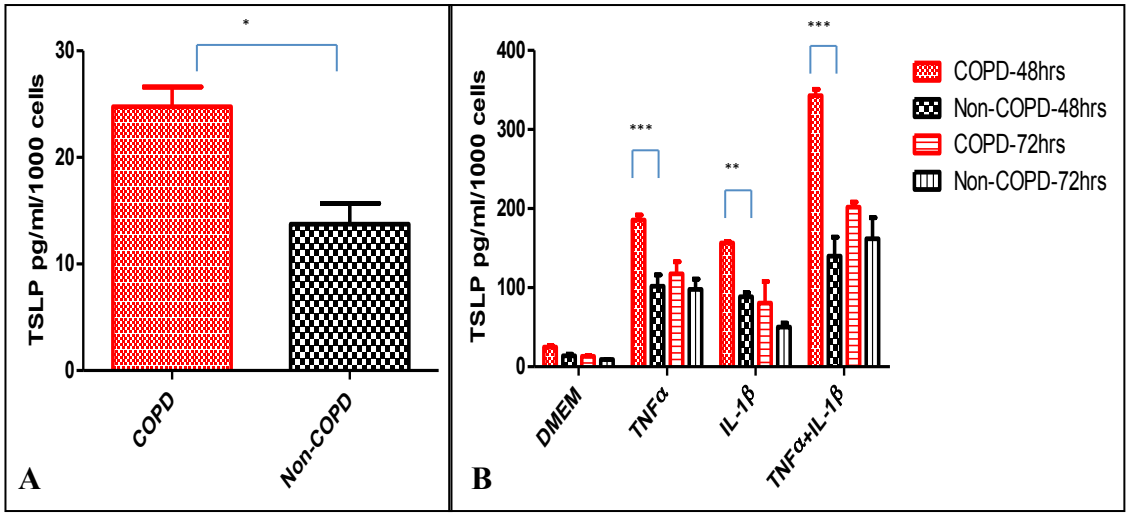

Figure 19: TSLP protein secretion in COPD and non-COPD HLFs. TSLP protein secretion was measured in COPD HLFs and non-COPD HLFs by ELISA through assessing cell culture supernatant. TNFα (10ng/ml), IL-1β (10ng/ml), TNFα & IL-1β (10ng/ml each) were added to the cell cultures after 48 h of serum deprivation. TSLP secretion was measured at baseline (A) and at 48, 72 h after adding treatments (B). Data represent the ± SEM (standard error of means) of three independent experiments done on three COPD and non-COPD HLFs. TSLP release was compared to DMEM controls. Statistical analysis: Two-way ANOVA, Bonferroni post hoc analysis using Graph Pad Prism version 5. $p<0.05$ was considered significant. Note:* $p<0.05$;** $p<0.01$;*** $p<0.001$.

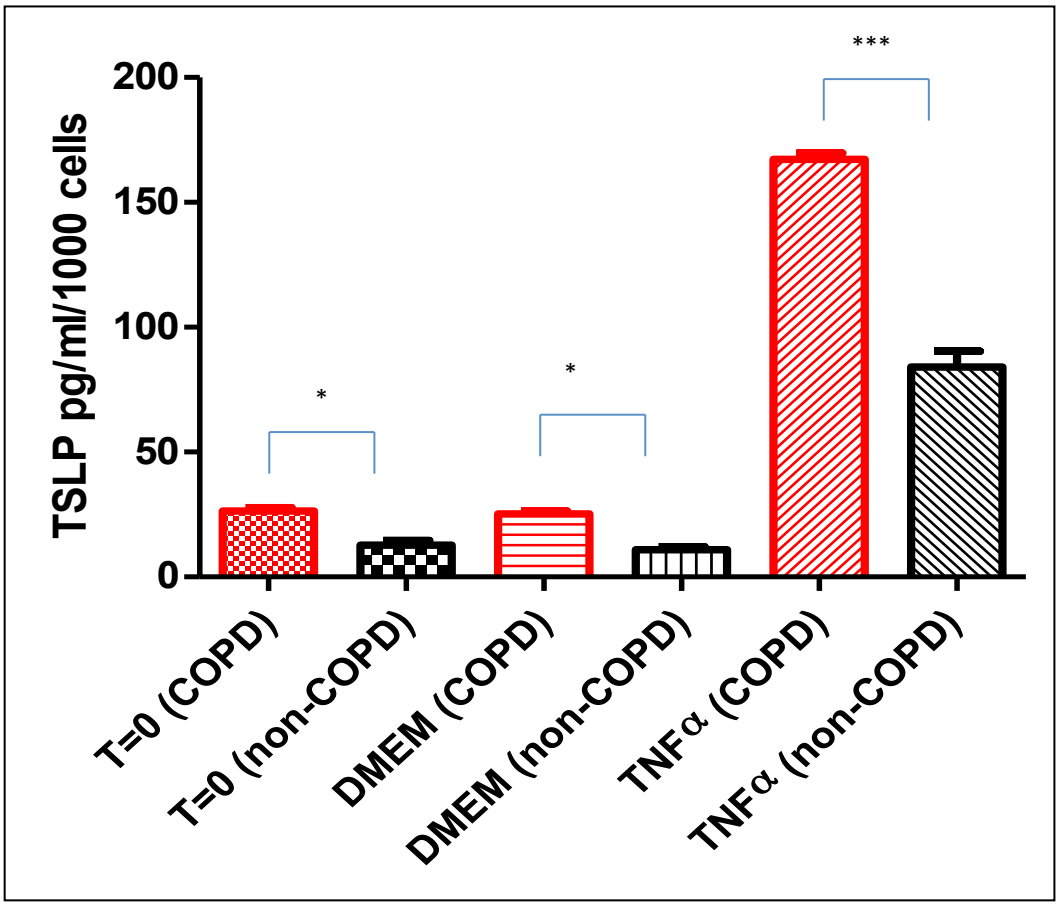

Figure 20: Screening of COPD and non-COPD HLFs for TSLP release. COPD and non-COPD HLFs were analyzed for TSLP release at basal level (T=0) and after stimulation with TNFα (10ng/ml). Cell culture supernatant was collected at T=0 h and 48 h after stimulation and analyzed by ELISA for TSLP release. Data represent the ± SEM (standard error of means) of triplicate values from six COPD and non-COPD HLFs. Statistical analysis: one-way ANOVA, Bonferroni multiple comparisons test using Graph Pad Prism version 5. $P<0.05$ was considered significant. *Note:* Note: * $p<0.05$;*** $p<0.001$.

4.1.13 COPD derived lung fibroblasts exhibit increased cytokine stimulated TSLP mRNA accumulation

We wanted to investigate HLFTSLP expression, thus measured relative TSLP mRNA levels after stimulating COPD and non-COPD HLFs with TNFα. HLF cultures were treated with TNFα (10ng/ml) and RNA was isolated 6-to-24h thereafter. Quantitative PCR (qRT-PCR) was used to analyse TSLP mRNA abundance. TSLP mRNA abundance increased markedly upon TNFα stimulation, reaching maximum levels 6hrs after stimulation, and declining thereafter, in both COPD and non-COPD HLFs. Notably, relative TSLP mRNA was 15-fold higher in COPD HLFs compared to non-COPD HLFs (Figure 21), (P <0.01).

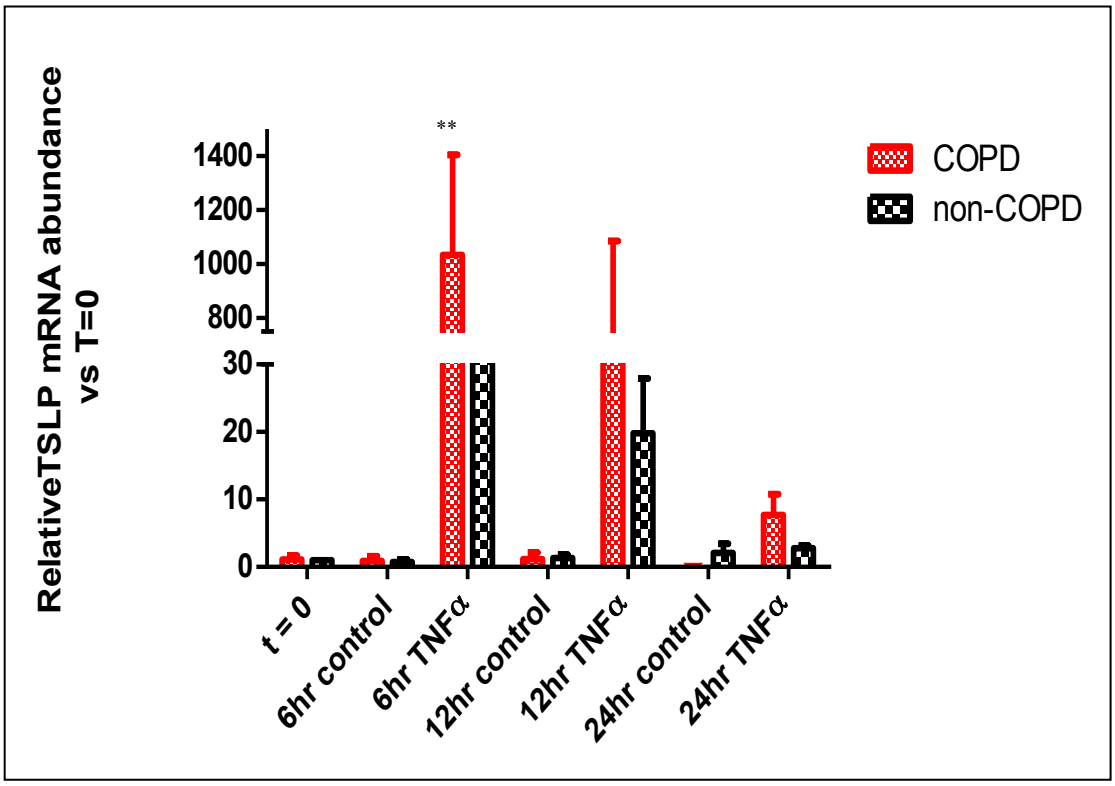

Figure 21: TSLP mRNA expression in COPD and non-COPD HLFs. COPD and non-COPD HLFs were induced with TNFα (10ng/ml) at 6, 12 or 24 h after 48 h of serum deprivation. mRNA was isolated and converted to CDNA for qRT-PCR. Data represent the ± SEM (standard error of means) of values of three independent experiments done on three COPD and non-COPD cell lines (n=3). Statistical analysis: Two-way ANOVA, Bonferroni post hoc analysis using Graph Pad Prism version 5. $p<0.05$ was considered significant. *Note:*$^{**}p<0.01$

4.1.14 COPD derived lung fibroblasts exhibit increased TSLP transcriptional activity

As our findings indicate that both TSLP mRNA and protein levels are elevated in COPD HLFs, we wanted to explore whether this may be due to increased TSLP transcriptional activity. We used a human TSLP promoter luciferase reporter assay to compare transcriptional activity for TSLP in COPD and non-COPD HLFs. We found that both basal and TNFα-induced (10ng/ml) TSLP promoter activity was higher in COPD HLFs compared to non-COPD HLFs (Figure 22). Baseline transcription in COPD HLFs was 17-fold higher than COPD-free HLFs. In TNFα-stimulated cultures, TSLP luciferase activity was increased by more than three-fold from basal levels and was 10-fold higher in COPD HLFs compared to HLFs from COPD free donors ($P<0.001$).

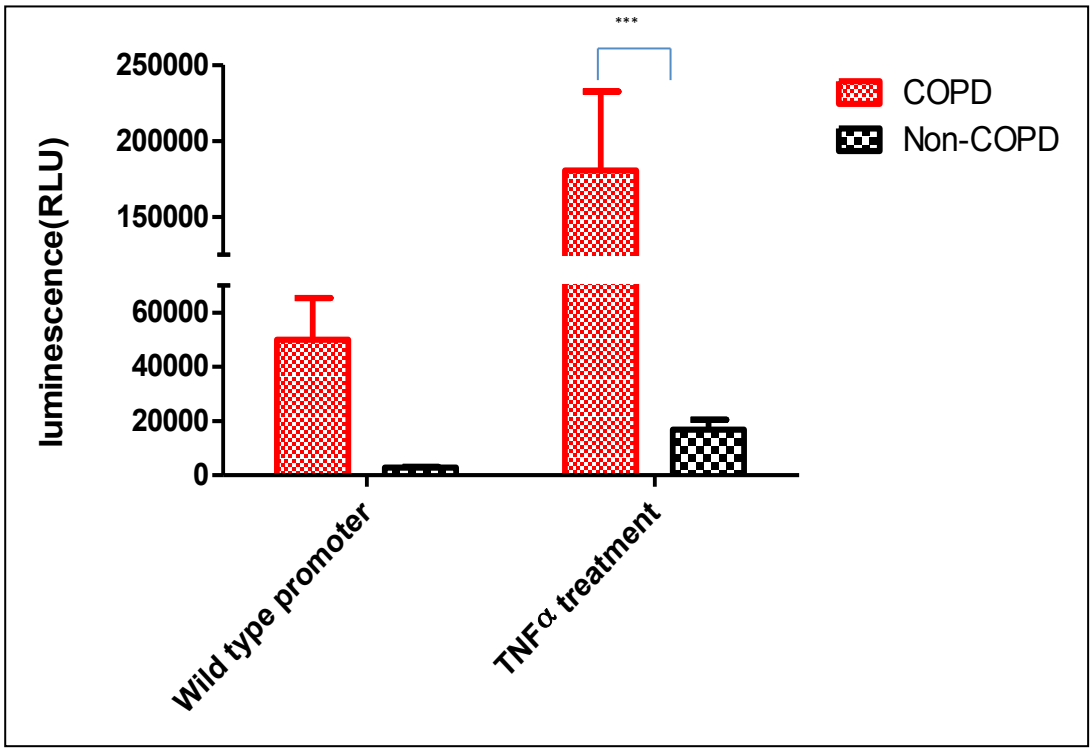

Figure 22: TSLP transcriptional activity in COPD and non-COPD HLFs. Human TSLP promoter luciferase reporter assay was used to compare transcriptional activity in COPD and non-COPD HLFs. RLU- Relative luciferase unitsData represent the ± SEM (standard error of means) of duplicate values from three independent experiments done with cells from three COPD and non-COPD HLFs. Statistical analysis: Two-way ANOVA, Bonferroni post hoc analysis using Graph Pad Prism version 5. $P<0.05$ was considered significant. *Note:****$P<0.001$

4.1.15 Effect of simvastatin on TSLP release

As an initial objective of this project was to determine the impact of simvastatin of secretory function of HLFs, we measured the effect of simvastatin on TNFα-induced TSLP release. Simvastatin was added at a concentration of 10μM, 5μM and 1μM along with TNFα (10ng/ml) after 48h of serum deprivation. Cultures with no added simvastatin and only TNFα were used as controls. TSLP secretion was determined by ELISA through assessing cell culture supernatant. Simvastatin had no inhibitory effect on TSLP release both in COPD and non-COPD HLFs, and surprisingly at 72h, for COPD HLFs we detected a statistically significant enhancement of TNFα-induced TSLP in the presence of 1 and 5μM simvastatin ($P<0.01$ at both times) as well as in non-COPD HLFs treated with 1μM simvastatin for 72 h ($P<0.05$) (Figure 23).

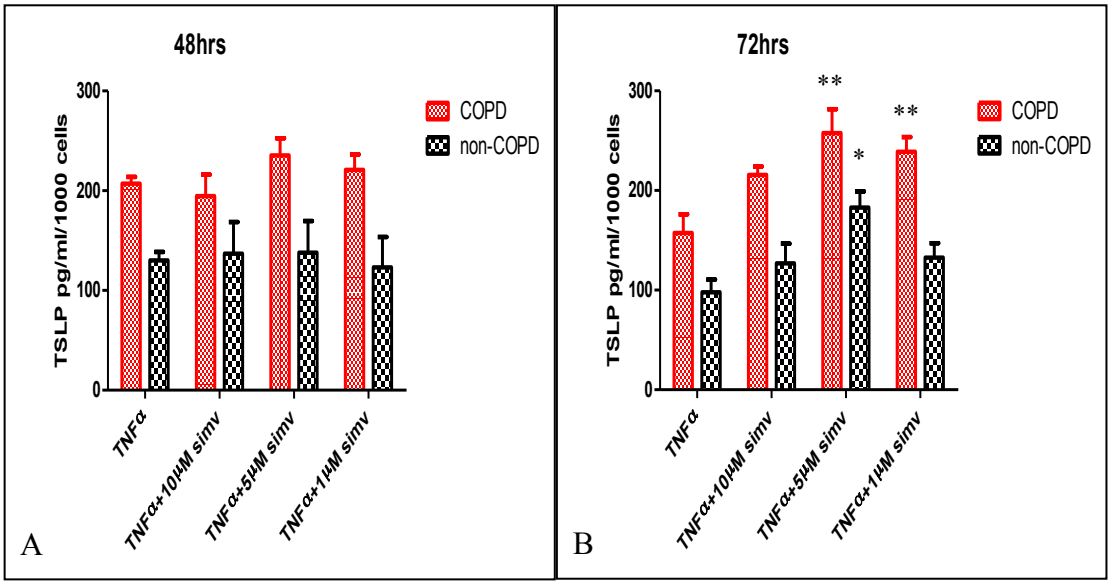

Figure 23: Effect of simvastatin on TSLP protein release in COPD and non-COPD HLFs. COPD and non-COPD HLFs were stimulated with TNFα (10ng/ml) and at the same time treated with increasing concentrations of simvastatin (1μM, 5μM and 10μM). Cultures with no added simvastatin were used as controls. TSLP secretion was determined by ELISA through assessing cell culture supernatant at 48 h (A) and 72 h (B). Data represent the ± SEM (standard error of means) of triplicate values from three independent experiments done with cells from three COPD and non-COPD HLFs. Statistical analysis: Two-way ANOVA, Bonferroni post hoc analysis using Graph Pad Prism version 5. $P<0.05$ was considered significant. *Note:***$p<0.01$; :*$p<0.05$.

4.1.16 Presence of TSLP in lung structural cells - immunohistochemistry

We wanted to determine which cells in the lung express TSLP and whether this differs between COPD and non-COPD subjects. To achieve this we performed immunohistochemistry on deparaffinised sections of COPD and non-COPD lung tissue – tissue blocks had been prepared from peripheral lung samples that had been adjacent to those used to isolate fibroblasts for primary culture. Thus all histological analyses were performed with samples from the same tissue donors used to establish the primary cultures that were used in all other studies in this project. Sections were incubated with primary TSLP antibody overnight followed by incubation with biotinylated secondary antibody. The sections were than analyzed for the presence of TSLP. Staining suggested more robust TSLP expression in COPD lungs as compared to non-COPD lungs (Figure 24). We performed additional immunostaining to determine the cell types, in particular lung fibroblasts, that expressed TSLP in human lung. For this purpose, deparaffinized sections were incubated with TSLP antibody overnight followed by incubation with rhodamine-conjugated anti-rabbit IgG which as secondary antibody. In the tissue sections we analyzed locations in the lung tissue where fibroblasts are commonly found and tried to identify cells with spindle-shaped morphology (Figure 25). It should be noted that immunostaining with specific markers is required to ascertain that they are indeed fibroblasts.

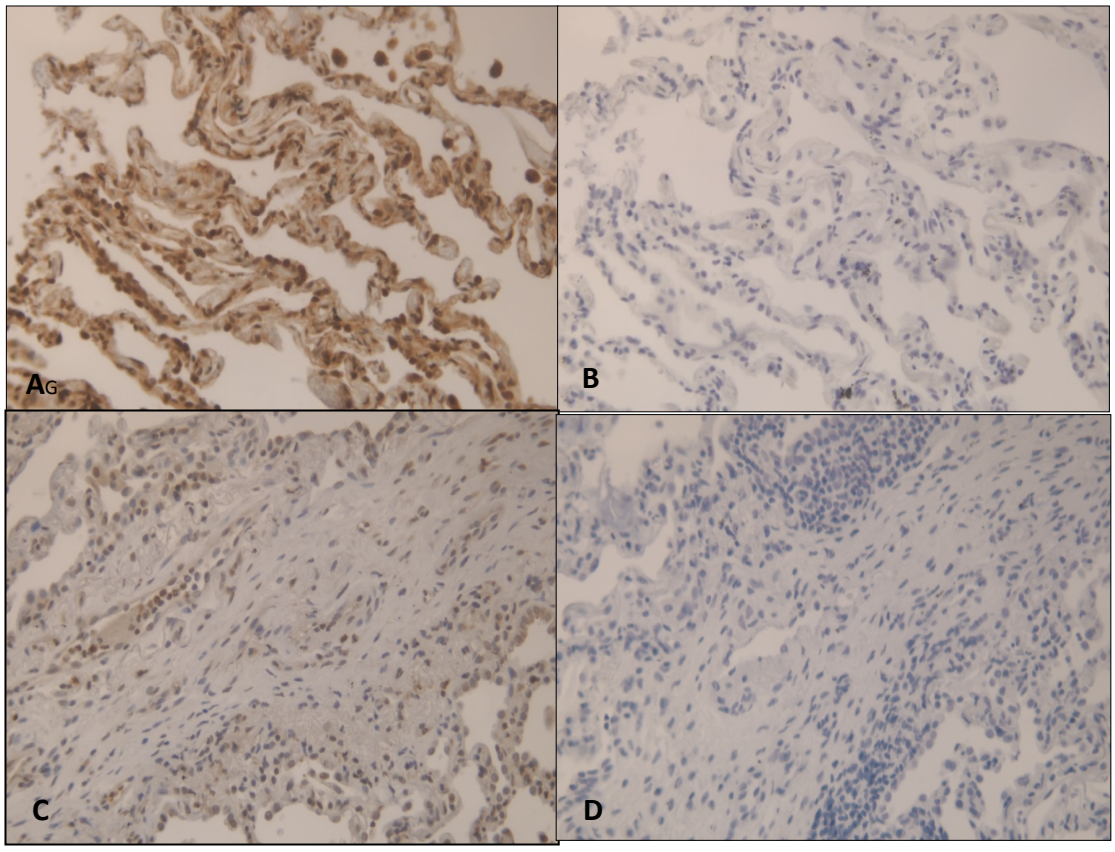

Figure 24: Immunohistochemistry to determine TSLP protein in lung.
Presence of TSLP protein was identified by brown staining with TSLP antibody in COPD lung (A) as compared to non-COPD lung (C). Negative controls for COPD (B) and non-COPD lung (D).

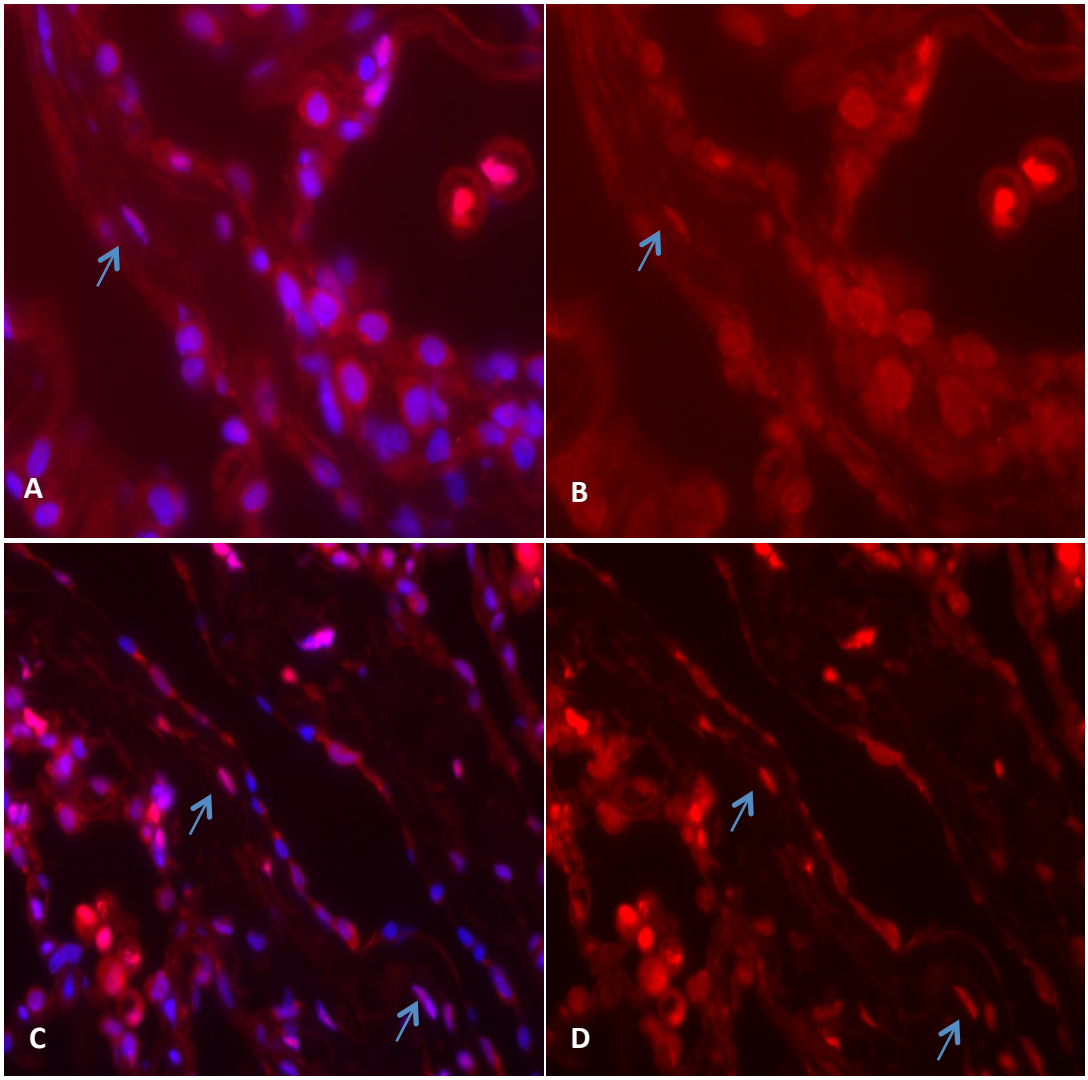

Figure 25: Immunofluorescence immunostaining for TSLP in structural cells in COPD lung. Fibroblast-shaped cells expressed TSLP are evident from the red staining from two separate specimens (top, A & B, and bottom , C & D, rows). Blue staining in left hand column panels is for DNA, using H33342 to demonstrate nuclei. Images on the right are the same as on the left, but the nuclear labelling has been omitted for clarity.

4.2 Signaling pathways induced by TNFα in human lung fibroblasts.

4.2.1 TNFα and IL-1β receptor abundance is similar in COPD and non-COPD HLFs

We examined whether the enhanced cytokine-induced release of TSLP from COPD HLFs might be due to differences in expression of TNFα and IL-1β receptors. To ascertain this we measured TNFα and IL-1β receptor subunit expression using qRT-PCR. Specifically, we assayed mRNA levels of the TNF receptor superfamily member 1 (TNFRSF1A) and the interleukin receptor type 1 (IL1R1) in 6 different cell lines of COPD HLFs and of non-COPD HLFs. As all specimens were from subjects who were cigarette smokers, we further assayed 2 HLFs from donors who had no history of smoking and who were not diagnosed with COPD. We found that there were no differences in mRNA abundance of TNFRSF1A or IL1R1 between groups, suggesting that elevated TSLP release by COPD HLFs is not attributable to increased receptor abundance (Figure 26). IL-1β receptor abundance was extremely higher in one COPD cell line as compared to the remaining cell lines so Grubbs test was performed to determine the outlier. Grubbs test confirmed the value to be a significant outlier ($P<0.05$). When we repeated the analysis there was still no significant higher IL-1β receptor abundance in the overall COPD. Since we had only 2 HLFs from non-smoking donors, therefore no statistical analysis was carried out for that group.

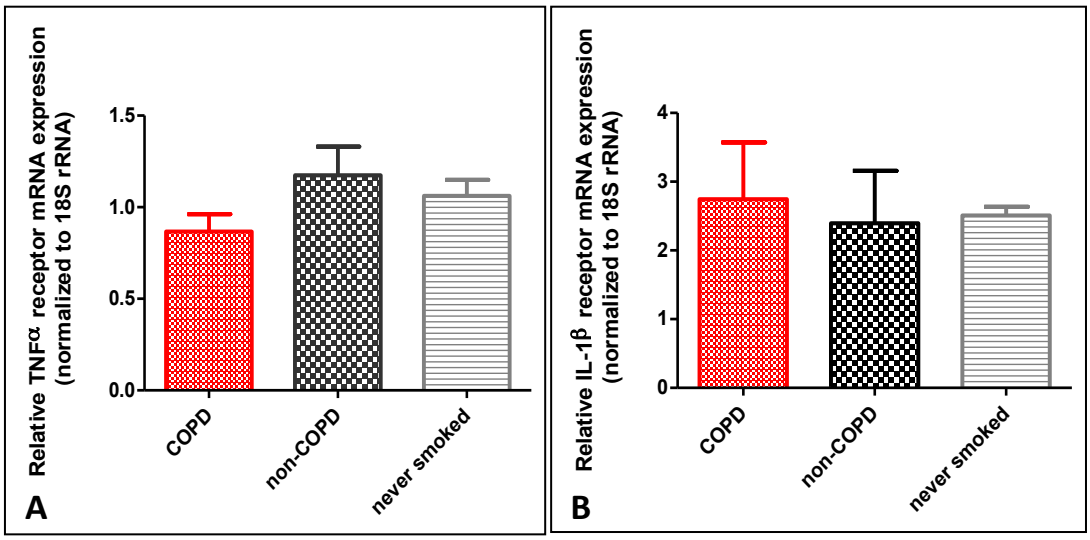

Figure 26: TNFα and IL-1β receptor abundance. Receptor abundances of TNFα and IL-1β were compared by analysing mRNA abundance of human TNF receptor (A)superfamily member 1 (TNFRSF1A) and interleukin receptor type 1(B) (IL1R1) in 6 cell lines each of COPD HLFs, non-COPD HLFs and 2 never smoked HLF controls by RT-PCR. There was no difference in mRNA abundance of either receptor in both the COPD and non-COPD groups. Data represent the ± SEM (standard error of means) of triplicate values from three independent experiments done with cells from three COPD and non-COPD HLFs. Statistical analysis: One-way ANOVA, Bonferroni multiple comparison analysis using Graph Pad Prism version 5. $P<0.05$ was considered significant. No statistical analysis was carried on for the never smoked group due to less number of cell lines (n=2).

4.2.2 TNFα induces increased NF-kB transcriptional activity in COPD derived lung fibroblasts

As we found that the receptor abundance of TNFα does not vary in COPD and non-COPD HLFs, we next wanted to know if there were any differences in the signaling pathways mediated via the TNF receptor in COPD and non-COPD HLFs. To ascertain this, we analysed the induction of intracellular signaling pathways by TNFα. One of the signaling cascades known to be typically activated by TNFα is the NF-κB pathway. The final step of this pathway involves translocation of NF-κB p65 domain to the nucleus where it binds to NF-κB binding sites in the DNA and activates transcription of an array of genes associated with tissue repair and inflammation. It has been reported that TNFα induces TSLP transcriptional activation via the IKK pathway of NF-κB [184]. Thus, we performed transient transfection of HLFs with 0.1μg of wild type NF-κB promoter construct. NF-κB-driven transcriptional activity was compared between COPD and non-COPD HLFs. ExGen 500 in vitro transfection reagent (MBI Fermentas, Ontario, Canada) was used for transfection of cells. After 24 h, cells were washed with PBS and then stimulated with TNFα (10ng/ml) or media alone. Then after 12 h of cytokine stimulation, cells were washed with PBS and cell lysates were obtained which were used to measure luciferase activity. All the values obtained were normalized to renilla luciferase activity. In TNFα-stimulated cultures, NF-κB-driven transcriptional activity was 2.6 fold higher in COPD HLFs as compared to non-COPD HLFs (Figure 27).

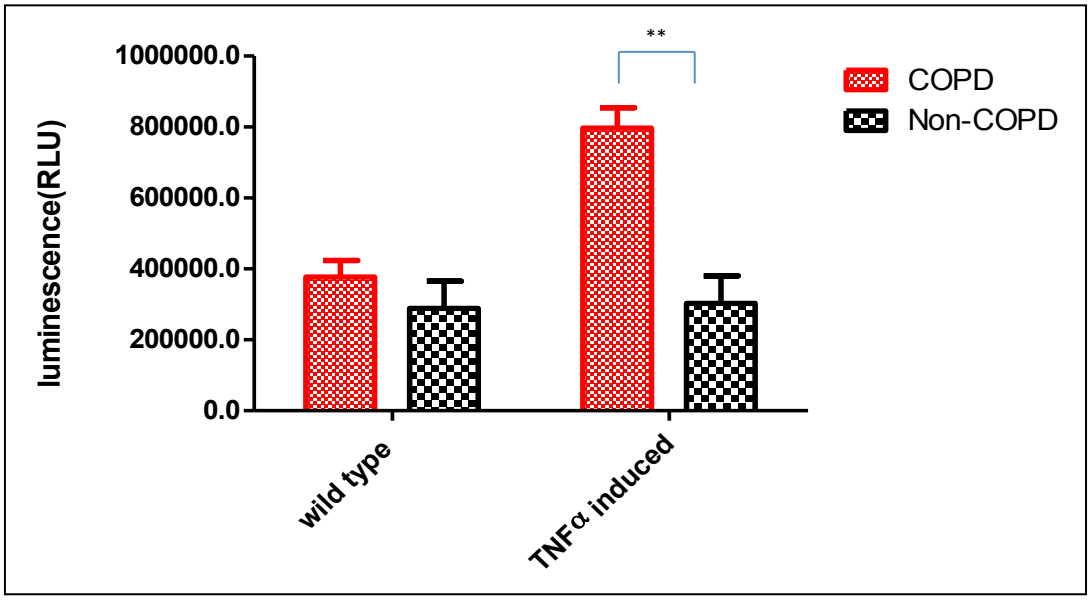

Figure 27: Human NF-κB promoter luciferase reporter assay in COPD and non-COPD HLFs. Luciferase reporter assay was used to compare transcriptional activity in COPD and non-COPD HLFs. NF-κB transcriptional activity was measured in wild type and compared to TNFα induced HLFs. NF-κB driven transcriptional activity was compared between COPD and non-COPD HLFs**.** RLU-relative luciferase units. Data represent the ± SEM (standard error of means) of duplicate values from three independent experiments done with cells from three COPD and non-COPD HLFs. Statistical analysis: Two-way ANOVA, Bonferroni post hoc analysis using Graph Pad Prism version 5. $P<0.05$ was considered significant. *Note:***$p<0.01$.

4.2.3 TNFα induces MAPK signaling pathways in COPD and non-COPD derived lung fibroblasts

In different cell types TNFα induces multiple intracellular signaling cascade, including MAPK pathways, most prominently p38 MAPK and ERK [156]. We compared p38 MAPK and ERK phosphorylation in COPD and non-COPD HLFs after stimulating cultures with 10ng/ml TNFα and collecting total cell lysates 30 min later. Western blotting and densitometry (Figure 28) was used to analyze phosphorylated and total MAPK proteins in three COPD HLFs and three non-COPD HLFs. This analysis revealed that ERK (Figure 29) and p38 MAPK (Figure 30) induction was significantly higher in COPD HLFs compared to non-COPD HLFs. In association with our findings using the NF-κB reporter (Figure 27), these data confirm that intracellular signaling pathways are more readily induced by TNFα in COPD HLFs, which may explain the increased TSLP release we observed in the same cells.

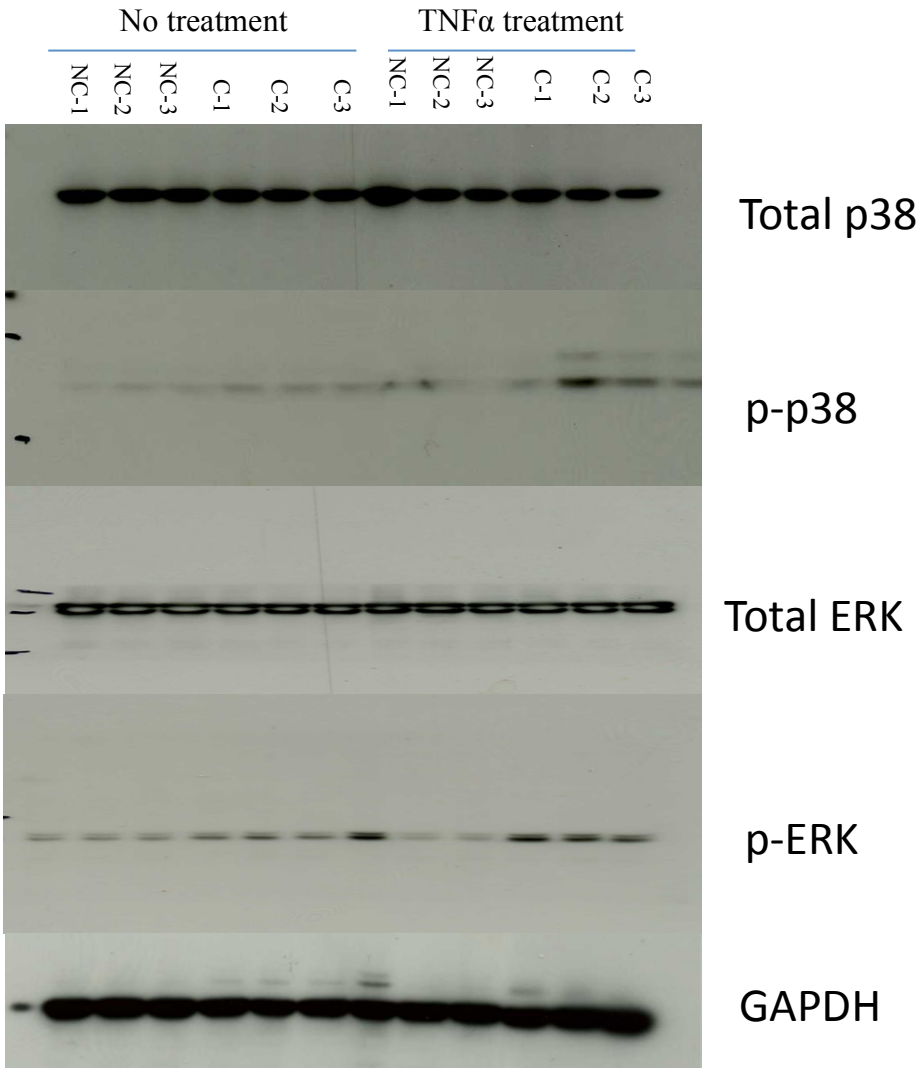

Figure 28: ERK and p38 MAPK phosphorylation in HLFs.
Non-COPD cell line 1(NC1), Non-COPD cell line 2 (NC2), Non-COPD cell line 3(NC3), COPD cell line 1 (C1), COPD cell line 2(C2), COPD cell line 3(C3). HLFs from three COPD and non-COPD donors were treated with TNFα (10ng/ml) and cell lysates were collected at 30 min to analyse ERK and p38 MAPK phosphorylation.

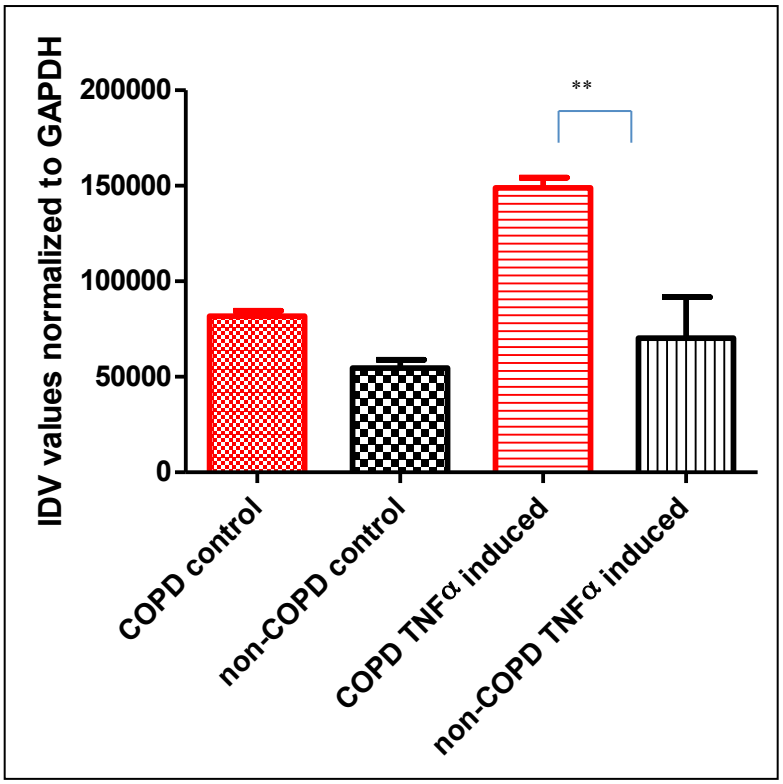

Figure 29: ERK phosphorylation in COPD and non-COPD HLFs. HLFs from three COPD and non- COPD donors were treated with TNFα (10ng/ml) and cell lysates were collected at 30 min to analyse ERK phosphorylation. Western blotting was utilized to estimate ERK and p-ERK. Data represent the ± SEM (standard error of means) of values from experiment done with cells from three COPD and three non-COPD HLFs. Statistical analysis: one-way ANOVA, Bonferroni multiple comparison analysis using Graph Pad Prism version 5. $P<0.05$ was considered significant. *Note:***$P<0.01$

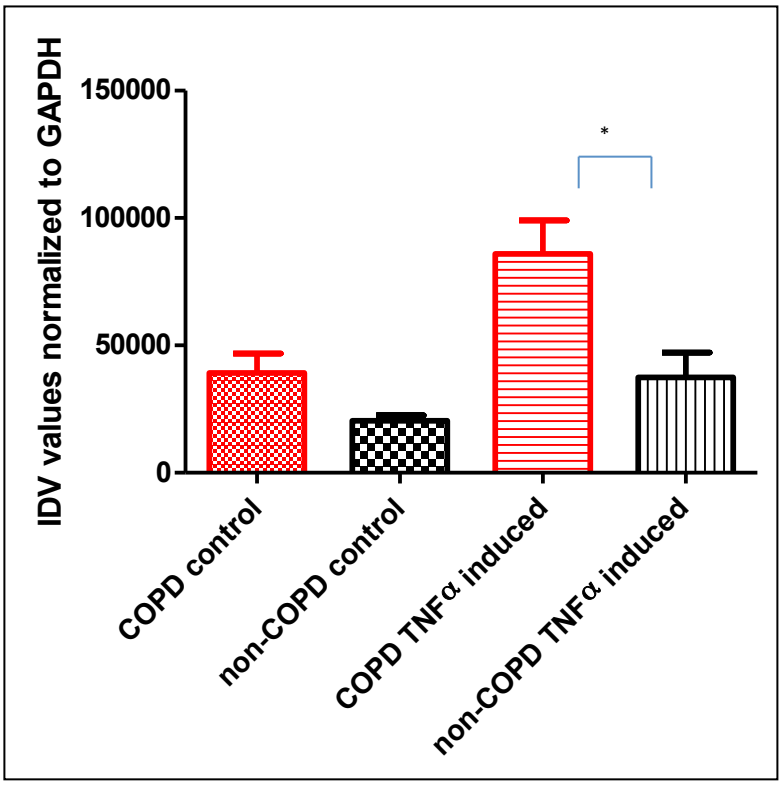

Figure 30: p38 MAPK phosphorylation in COPD and non-COPD HLFs . HLFs from three COPD and non- COPD donors were treated with TNFα (10ng/ml) and cell lysates were collected at 30 min to analyse ERK phosphorylation. Western blotting was utilized to estimate ERK and p-ERK. Data represent the ± SEM (standard error of means) of values from experiment done with cells from three COPD and three non-COPD HLFs. Statistical analysis: one-way ANOVA, Bonferroni multiple comparison analysis using Graph Pad Prism version 5. $P<0.05$ was considered significant. *Note:* $*P<0.05$

5. Discussion

Discussion

COPD is characterized by partially reversible airway flow limitation and chronic inflammation of the airways and parenchyma. COPD pathogenesis has been associated with an enhanced numbers of neutrophils and macrophages along with CD8 and CD4 T- and B-cell lymphocytes. The inflammatory process induced by tobacco smoking results in the degradation of extracellular matrix and also structural alterations in lung parenchyma. Alteration of tissue repair activity has been considered a major driving factor for emphysema. Human lung fibroblasts are major structural cells of the lung responsible for repair and remodeling in COPD. There is increasing evidence suggesting that the impaired tissue repair capacity of lung fibroblasts to maintain extracellular matrix is decreased in COPD. Notably, lung fibroblasts isolated from COPD patients showed a decline in their proliferation and chemotaxis ability along with a decline in the production of the extracellular matrix macromolecules [55, 185, 186]. Reduced proliferation of lung fibroblasts was seen in response to lipopolysaccharide (LPS) treatment; LPS being an important catalyst for COPD development [181]

In our study, we investigated differences in proliferation of lung fibroblasts from COPD and COPD free donors in response to a complex mitogen (5% fetal bovine serum (FBS)).We showed that FBS directly induces proliferation of lung fibroblasts from both COPD and COPD-free donors, and confirmed that proliferation of COPD HLFs is significantly lower compared to HLFs from COPD-free donors. We assessed the impact of simvastatin on proliferation – as expected HLF grow was suppressed, but we observed no difference in response of COPD or COPD-free HLFs to this compound. Simvastatin

and other statins which are widely used as cholesterol lowering drugs, can exert anti-proliferative and anti-inflammatory effects, and their use has been linked to improved lung health in aging smokers, non-smokers and COPD patients. Our data suggest this positive impact is not a result of direct effects on HLF proliferation.

Traditionally, inflammation in COPD is believed to trigger and perpetuate a vicious circle of tissue 'injury and repair' resulting in tissue remodelling. Lung-infiltrating immune cells and a variety of locally released pro-inflammatory mediators such as TNFα and IL-1β are widely studied to gain insight of the mechanism for inflammation. Although the role of T cells and T helper cytokines (Th1, Th2, Th17, and Treg) have been widely studied, the structural components of the lung are also believed to be a major factor in promoting local inflammation (in the lung) [187]. Though some key inflammatory mediators linked to COPD include TNFα, IL-1β and the chemokine IL-8/CXCL8, we focussed our study on thymic stromal lymphopoietin (TSLP)), an IL-7 like cytokine, originally isolated from a murine thymic stromal cell line, characterized as a lymphocyte growth factor. TSLP is expressed by immune and structural cells associated with COPD and asthma, and is a determinant of inflammation, including release of COPD-associated cytokines, and tissue repair. To this end, we treated HLFs from COPD and COPD-free donors with different concentration of pro-inflammatory cytokines (TNFα and IL-1β) and investigated TSLP expression and release. Our results confirmed that TNFα and IL-1β can induce TSLP release in HLFs from COPD and COPD-free donors. However, our findings are unique in that they revealed COPD HLFs mount a significantly greater synthesis and secretion of TSLP compared HLFs from COPD–free donors. In line with

these observations, our real time PCR and luciferase promoter activity reporter assays suggested that the increased TSLP release in COPD HLFs is linked with increased transcription of TSLP, leading to mRNA accumulation. We also observed that basal (i.e. not stimulated by exogenous cytokine) TSLP release and TSLP promoter activity was elevated in COPD HLFs. This suggests that lung fibroblasts from COPD subjects carry stable, intrinsic differences in function and the molecular pathways that regulate them. In the course of our studies we also assessed the effects of simvastatin on TSLP release and found that the compound was without effect on TSLP release. One study [188] reports that simvastatin can selectively inhibit TSLP production in COPD epithelium, but our data did not confirm this to be the case in COPD HLFs.

Increased TSLP expression has been observed in the mouse lung in response to repeated intra-nasal exposure to cigarette smoke extract (CSE) [163]. Enhanced TSLP expression in response to CSE has been attributed to oxidative stress and TNFR activation. Interestingly, in airway smooth muscle (ASM) cells, chronic CSE exposure augments TSLP and TSLP receptor expression [164]. We were interested to know whether CSE can differentially induce TSLP release in COPD lung fibroblasts, CSE being an important player in activating inflammation in COPD. Thus, we treated COPD and COPD-free HLFs with different concentrations of CSE and measured TSLP release; surprisingly, CSE did not induce TSLP release in HLF regardless of the clinical phenotype of the donor. In my work I did not measure whether CSE exposure had any impact on TSLP mRNA abundance or TSLP promoter activity – despite lack of impact on TSLP protein

release, it is still necessary to determine whether CSE might have impact on upstream pathways and processes that could ultimately impact of pathogenic features of COPD.

As we demonstrate that TSLP expression is elevated in COPD HLFs, we further investigated the factors that may be responsible. As we used TNFα and IL-1β to induce TSLP expression, we profiled expression of receptors for these cytokines in lung fibroblasts. Our data show that there is no difference in TNFα- and IL-1β-receptor abundance between COPD and COPD-free lung fibroblasts. This confirms that elevated TSLP levels in COPD HLFs is not related to differences in the abundance of receptors for pro-inflammatory cytokines that mediate TSLP release. We next surveyed canonical signaling pathways for the response to TNFα and IL-1β and found that enhanced activation of some TNFα-mediated pathways may underpins increased TSLP release in COPD fibroblasts: we observed increased phosphorylation of MAPKs ERK1/2 and p38, as well activation of the inflammation-associated transcription factor NFκB. Notably these pathways have previously been shown to be required for TSLP expression in several cell types, including human airway smooth muscle. It has been reported [157] that pro inflammatory cytokine-induced TSLP expression requires activation of Erk1 and 2 and other MAPKs, thus we compared MAPK signaling between COPD- and COPD-free HLFs. Our data demonstrate that phosphorylation of MAPK pathway signaling molecules are elevated in COPD lung fibroblasts. We also assessed NFkB activation, which has been attributed to the release of many cytokines. Using luciferase reporter assay we show that NF-κB-driven transcription is increased in COPD lung fibroblasts upon TNFα stimulation. Thus, increased TSLP release in these cells is likely underpinned, at least in

part, by increased activation of signaling pathways, such as NF-κB and MAPKs, in response to TNFα exposure.

Concluding comments and future direction

Altogether we found that proliferation is reduced in COPD lung fibroblasts and TSLP release, both basal and cytokine-induced, is increased. A recent study done on pulmonary fibroblasts from COPD patients by Boczkowski et al [52] indicates that prostaglandin E2 synthesized by COPD lung fibroblast induces a senescent, secretory phenotype that contributes directly to disease pathogenesis. It would be interesting to know whether decreased lung proliferation could be a result of increased fibroblast senescence. Moreover, inhibition of the an autocrine PGE2 pathway could be a potential target in improving COPD proliferation, and perhaps even reverse the hypersecretory state of these cells. Our data demonstrate that TSLP is among the cytokines that HLFs from COPD donors secrete in excess. The mechanism by which lung structural cells produce excess TSLP at basal levels and upon pro-inflammatory cytokine exposure is not yet to be fully understood but our work provides a platform to move forward. To our best knowledge, our findings are the first to reveal increased TSLP transcription and release in primary human COPD lung fibroblasts, suggesting that these cells carry stable intrinsic differences, perhaps epigenetic in nature and consistent with recent reports that COPD fibroblasts acquire a senescent-hyper secretory phenotype. Though numerous reports demonstrate that simvastatin possesses anti-inflammatory properties, we did not observe any direct impact on TNFα-induced TSLP release in COPD and COPD-free HLFs pre-

treated with simvastatin. This suggests that the positive effects of statin use may be linked to their impact on systemic immunity, rather than from direct effects on the cells of the lung. The precise role of TSLP in COPD and its response to therapy need further investigation.

As a collective our observations suggest that there is a defect in the mesenchymal cell populations in COPD that can lead to altered local inflammation and maintenance of lung tissue, including extracellular matrix and other lung structural cells. Due to its emerging importance as a central determinant of immunity and inflammation, our findings indicate that TSLP released by lung structural cells (fibroblasts), should be considered in future work for its possible role in sustaining and orchestrating inflammation in the lung in COPD patients, This should include individuals who have ceased smoking, for it is established that local lung inflammation persists in subjects with COPD, even long after cigarette smoking is halted. That increased TSLP release may be tied to evidence that inflammation persists for prolonged periods after cigarette smoking is stopped should be an area for direct investigation in the future as this may reveal fundamental features for the chronic, progressively debilitating character of COPD.

6. References

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Appendix I

List of chemicals and reagents.		
Material	**Company**	**Catalog number**
DMEM	GIBCO	12800-017
FBS	Hyclone	SH30396.03
Antibiotics	GIBCO	15140
Sterile DMSO	Sigma - Aldrich	D2650
Cell culture plates	Corning	430167
ITS	GIBCO	51300-044
MTT	Sigma - Aldrich	M2128
Hoechst reagent	Cell signaling	4082S
ELISA plates	Costar	3369
TSLP ELISA kit	R&D	DY1398
TNFα	Peprotech	300-01A
IL-1beta	Peprotech	200-01B
Simvastatin	Sigma - Aldrich	S6196
Harris Haematoxylin	Sigma - Aldrich	HHS16
Eosin Y solution alcoholic	Sigma - Aldrich	HT110116

Appendix II

List of antibodies		
Name	**Company**	**Catalog number**
Vimentin	Sigma - Aldrich	V6630
E-cadherin	Cell Signaling	4065
Von Willebrand Factor	Abcam	ab9378
GAPDH	Santa Cruz	sc-47724
TSLP	Pro Sci	4023
p44/42 MAPK	Cell Signaling	9102S
Phospho p44/42 MAPK	Cell Signaling	91065
p38 MAPK	Cell Signaling	9212S
Phospho p38 MAPK	Cell Signaling	4511S

Appendix III

Krebs – Henseleit Buffer	
Material	**Molarity**
NaCl	112.6mM
$NaHCO_3$	25mM
Anhydrous NaH_2PO_4	1.38mM
KCl	4.7mM
$MgSO_4.7H_20$	2.46mM
$CaCl_2$	1.91mM
Dextrose	5.56mM
Solution was oxygenated for 30 min and pH adjusted to 7.2 after oxygenation and was sterile filtered with a 0.2µm filter and stored cold at 4°C.	

Hank's Buffered Salt Solution (HBSS)	
Material	**Molarity**
$CaCl_2$	1.26mM
$MgCl_2.6H_2O$	0.493mM
$MgSO_4.7H_2O$	0.407mM
KCl	5.33mM
KH_2PO_4	0.441mM
$NaHCO_3$	4.17mM
NaCl	137.93mM
Anhydrous Na_2HPO_4	0.338mM
Dextrose	5.56mM
Solution was sterile filtered with a 0.2µm filter after pH adjustment to 7.2 and stored cold at 4°C.	

Digestion buffer	
Material	**Molarity**
HBSS	50ml
Collagenase	600 units/ml
Protease	2 units/ml
Papain	2 units/ml
Calcium chloride	0.0207g/50ml of HBSS
Solution was sterile filtered with a 0.2µm filter into a sterile 50 mL tube just prior to use and keep the solution warm at 37^0C.	

10X SDS- PAGE running buffer	
Material	**Molarity**
Tris-base	250 mM
Glycine	1.92 M
SDS	1%

SDS- PAGE transfer buffer	
Material	**Molarity**
Tris-base	25mM
Glycine	192mM
methanol	20%

Cytoskeletal buffer (CB) Buffer	
Material	**Molarity**
MES	10mM
NaCl	150mM
EGTA	5mM
$MgCl_2$	5mM
Glucose	5mM

Maleic Acid Buffer (1X)	
Material	**Molarity**
Maleic Acid	100mM
NaCl	150mM

Radio immunoprecipitation assay (RIPA) buffer	
Material	**Molarity**
Tris-HCl	10mM
EDTA	1mM EDTA
EGTA	0.5mM
Triton X 100	1%
Sodium deoxycholate	0.1%
SDS	0.1%
NaCl	140mM

Blocking Buffer	
Material	**Volume**
Maleic Acid Buffer (1X)	1.5ml
Fetal bovine serum (FBS)	0.5ml
10% Roche stock blocking solution	0.5ml
PBS (1X)	2.5ml
Tween-20	50µl

Citrate Buffer	
Material	**Molarity**
Citric Acid	0.1M
Sodium citrate	0.1M