Acute Effect of Cigarette Smoke on TNF-α and Proteases Released by Macrophages

By

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I. ABSTRACT

Pulmonary emphysema is a major cause of mortality and morbidity in chronic obstructive pulmonary disease (COPD). Cigarette smoking is a major risk factor in the development of pulmonary emphysema. In this study we investigated the acute effect of cigarette smoke in vitro on the production of tumour necrosis factor-α (TNF-α) using differentiated U937 cells, a human monocyte cell line used as a macrophage model system and human alveolar macrophages (AM). We found that stimulation of the macrophages with cigarette smoke media (CSM) leads to a rapid activation of extracellular-regulated kinases 1 and 2 (ERK1/2), p90RSK, and a transient decrease in phosphorylation of PKB/akt in differentiated U937 cells and alveolar macrophages. The CSM also caused the subsequent induction of TNF-α release in U937 differentiated macrophages, whereas alveolar macrophages showed no consistent change in TNF-α release. Our studies revealed that U0126, an inhibitor of the ERK1/2 pathway, markedly suppressed CSM-induced TNF-α release in U937 differentiated macrophages. Consistent with this finding, U0126 blocked CSM-stimulated ERK1/2 phosphorylation, as well as phosphorylation of the downstream kinase, p90RSK. On the other hand, the PI3-K inhibitor, LY294002 and epidermal growth factor receptor (EGFR)-specific inhibitor, AG1478 did not suppress the release of TNF-α by U937 differentiated macrophages. Thus, CSM induction of TNF-α production by U937 differentiated macrophages is regulated primarily via the ERK1/2 pathway. In contrast, CSM suppressed or had no effect on TNF-α release by alveolar macrophages, even though CSM induced the phosphorylation of ERK1/2. This suggests that alveolar macrophages may utilize a

different signaling pathway for TNF-α production. In addition, this study also investigated the acute effect of cigarette smoke solution in vitro on the release of Cathepsin L, Matrix Metalloprotease-1, 7, 9 and 12 by AM, from which only Cathepsin L showed a slight but statistically significant increase after smoke exposure.

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Abbreviation	Definition
AM	Alveolar macrophages
BAL	Bronchoalveolar lavage
BM	Broncho-alveolar macrophages
COPD	Chronic obstructive pulmonary disease
CSC	Cigarette smoke condensate
CSM	Cigarette smoke media
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
elF4E	Eukaryotic initiation factor 4E
ELISA	Enzyme Linked Immunosorbent Assay
ERK1/2	Extracellular-regulated kinases 1 and 2
FEV_1	Forced expiratory volume in one second
FVC	Forced vital capacity
GM-CSF	Granulocyte macrophage colony stimulating factor
GOLD	Global Initiative for Chronic Obstructive Lung Disease
HBMC	human peripheral blood mononuclear cells
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule-1
IL	Interleukin

INF-γ	Interferon gamma
IP-10/CXCL10	Interferon-γ inducible protein
I-TAC	Interferon-inducible T cell α chemoattractant
JNK/SAPK	c-Jun NH_2-terminal kinase /stress activated protein kinase
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MEKK	Mitogen activated protein kinase kinase
Mig	Monokine-induced by interferon
MIP-2	Macrophage inflammatory protein 2
MMP	Matrix metalloprotease
NE	Neutrophil elastase
NF-κB	Nuclear factor-kappa β
p70RSK	p70 ribosomal S6 kinase
p90RSK	p90 ribosomal S6 kinase
PBS	Phosphate buffered saline
PDK1	Phosphoinositide-dependent protein kinase 1
PH	Pleckstrin homology
PI 4,5-P_2	Phosphatidylinositol 4,5-diphosphate
PI-3K	Phosphatidylinositol 3-kinase
PIP_3	Phosphatidylinositol 3,4,5-triphosphate
PKB	Protein kinase B
PMA	Phorbol myristate acetate

PTEN	phospholipids phosphatase
RIP	Receptor interacting protein kinase
S6	S6 Ribosomal protein
TBST	Tris-buffered saline containing 0.05% Tween 20
TGF-α	Transforming growth factor-α
TIMP	tissue inhibitors of matrix metalloprotease
TMB	3, 5, 3', 5'-tetramethylbenzidine
TNFR	Tumour necrosis factor-α receptor
TNF-α	Tumour necrosis factor-α
TRADD	TNF-α associated death domain protein
TRAF2	TNF-α receptor associated factor 2
VCAM-1	Vascular cell adhesion molecule-1

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VII. INTRODUCTION

A. COPD

A major health problem which causes a vast amount of mortality and morbidity in the world today is chronic obstructive pulmonary disease (COPD). Currently, COPD is the fourth leading cause of death in the United States and worldwide incidences are rising due to a widespread increase in tobacco consumption, particularly among women and adolescents [1] . COPD research has been relatively neglected for the past few decades, being one of the most under-funded research topics in relation to disease prevalence. There are currently no therapies which reduce the progression of the disease, however there is now new interest in understanding the cellular and molecular mechanisms involved in pathogenesis, and in searching for new therapies [2] .

The recent Global Initiative for Chronic Obstructive Lung Disease (GOLD) report classified COPD as "a disease state characterized by airflow limitation that is not fully reversible. The airflow limitation is usually both progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases"[1]. The airflow limitation is due to small airway narrowing, emphysema and excessive mucus secretion within the lumen. All three processes can influence airflow limitation; however the contribution of each factor varies according to differences between individuals and different stages of the disease. The airflow limitation is accompanied by respiratory symptoms, such as chronic cough, shortness of breath, sputum production and wheeze [3]. Patients with these symptoms are tested for COPD by measurement of their forced

expiratory volume in one second (FEV_1) and forced vital capacity (FVC) using a spirometer. COPD is diagnosed when the ratio of FEV1/FVC is less than 70 %, indicating airflow obstruction. The severity of COPD is determined by the degree of lung function impairment. The GOLD criteria categorize COPD into 4 stages (Table 1): Stage 0 (at risk), stage I (mild COPD), stage II (moderate COPD) and stage III (severe COPD). As the severity increases there is a decline in the predicted FEV_1 value.

Pathogenesis of COPD is characterized by chronic inflammation throughout the airway and parenchyma, and an imbalance between proteases and anti-proteases in the lung. Inflammatory cells such as neutrophils, macrophages and T lymphocytes, (predominately CD8+ (cytotoxic) cells)[4], infiltrate into the various parts of the lung resulting in an overall increase in the number of inflammatory cells. Activated inflammatory cells release a variety of cytokines or mediators that are able to damage the lung tissue and recruit more inflammatory cells to the lung.

COPD commonly refers to chronic bronchitis and emphysema, and cigarette smoking is a major contributor in the development of pulmonary emphysema.

Table 1- Classification of COPD by severity

Stage	Characteristics
0: At Risk	Normal spirometry Chronic symptoms (cough, sputum production)
I: Mild COPD	$FEV_1/FVC < 70\%$ $FEV_1 \geq 80\%$ predicted With or without chronic symptoms (cough, sputum production)
II: Moderate COPD	$FEV_1/FVC < 70\%$ $30\% \leq FEV_1 < 80\%$ predicted (IIA: $50\% \leq FEV_1 < 80\%$ predicted) (IIB: $30\% \leq FEV_1 < 50\%$ predicted) With or without chronic symptoms (cough, sputum production, dyspnea)
III: Severe COPD	$FEV_1/FVC < 70\%$ $FEV_1 < 30\%$ predicted, or the presence of respiratory failure,* or clinical signs of right heart failure

* Respiratory failure: $Pa_{O_2} < 8.0$ kPa (60 mm Hg) with or without $Pa_{CO_2} > 6.7$ kPa (50 mm Hg) while breathing air at sea level.

(Source: Romain A. Pauwels, 2001) Ref #:1

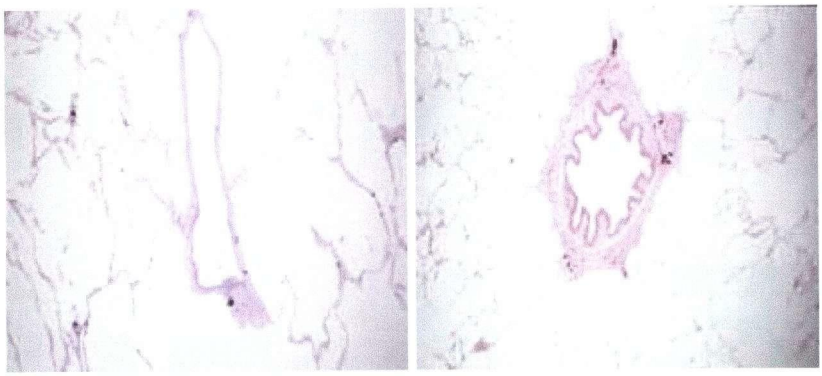
B. PULMONARY EMPHYSEMA

Pulmonary emphysema is defined as "permanent airspace enlargement beyond the terminal bronchioles, with destruction of alveolar walls and without obvious fibrosis" [5] (Fig 1). This lung damage results in increased resistance in the small airways and decrease in the lung elastic recoil force available to drive air out of the lung, thereby reducing the maximum expiratory flow in emphysematous patients [6]. Cigarette smoking is by far the major risk factor for development of pulmonary emphysema; however other factors, such as air pollution, α1-antitrypin deficiency and occupational factors also play a role.

Emphysema occurs in different forms based on anatomical destruction of the lung. There are four pathological types of emphysema, centriacinar (or centrilobular) emphysema, panacinar (or panlobular) emphysema, periacinar (or paraseptal) and irregular (or scar) emphysema (Table 2). The pulmonary acinus consists of the respiratory bronchioles, alveolar ducts, alveolar sacs and alveoli (Fig 2). The two major forms of emphysema are centriacinar and panacinar emphysema. Centriacinar emphysema is the most common type of emphysema and is mostly associated with cigarette smoking. This type of emphysema involves dilation and destruction of the respiratory bronchioles, commonly affecting the upper lobes of the lung and producing enlarged airspaces within the centre of the acinus. Panacinar emphysema is mostly associated with α1 anti-trypsin deficiency [7], and affects younger patients. This type of emphysema is common in the lower lobes of the lung and produces many smaller diffuse airspace enlargements throughout the whole of the acinus.

A commonly accepted theory for the pathogenesis of cigarette smoke–induced emphysema is the protease-anti-protease hypothesis. Cigarette smoke induces an inflammatory response within the lung; these recruited inflammatory cells are composed of a heterogeneous population of neutrophils, macrophages and CD 8+ T cells. However, macrophages and neutrophils are the two major types of inflammatory cells that release various proteolytic enzymes in excess of their natural inhibitor, leading to tissue destruction and airway enlargement [8-10] . Thus, excessive and persistent inflammation within the lung is the initiating factor in the pathogenesis of emphysema.

Figure 1- Anatomical changes in emphysema



Emphysema ***Normal***

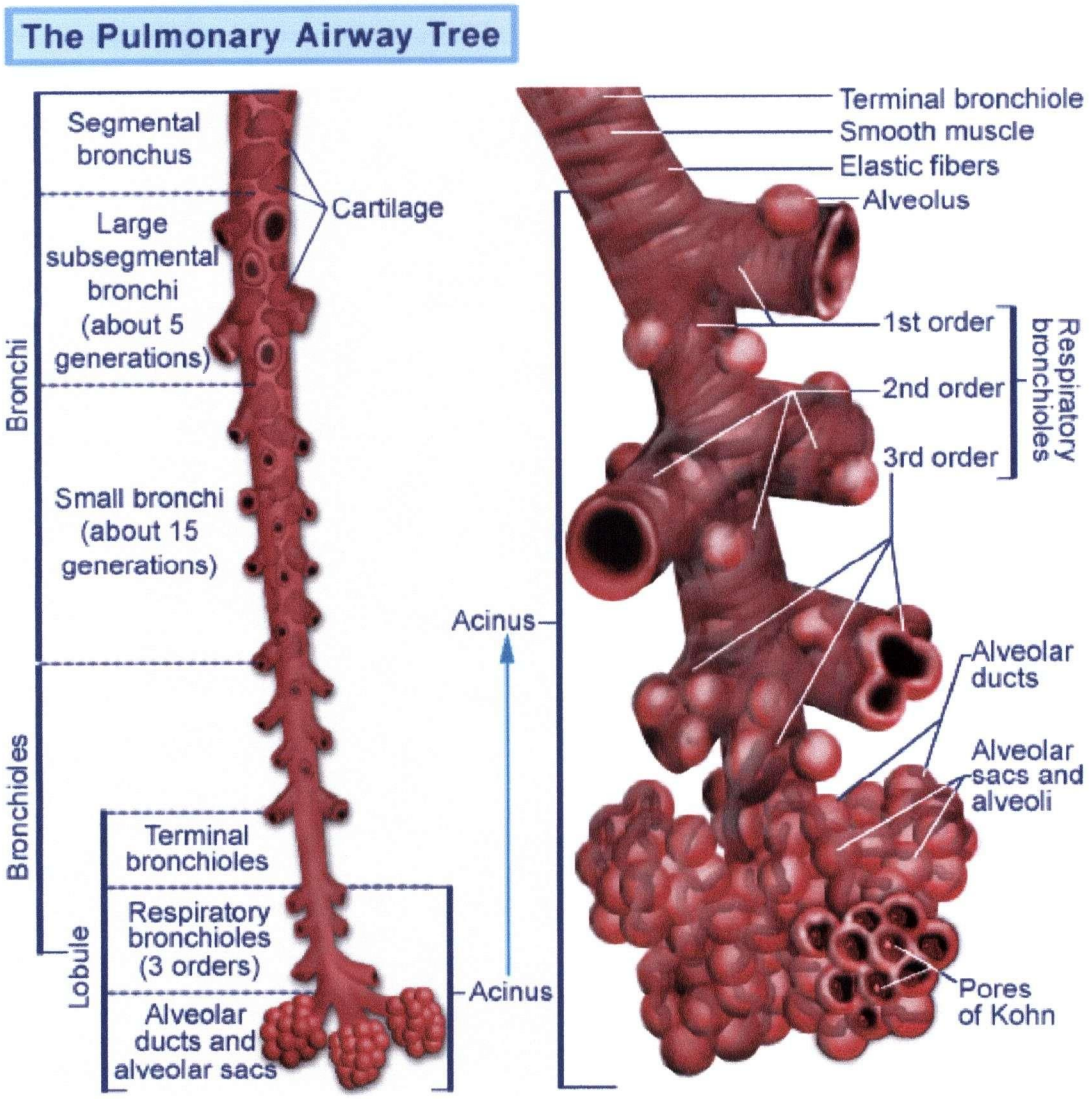
Loss of alveolar walls, enlargement of alveolar spaces and decreased alveolar wall attachments in emphysema

(Source:http://www.thoracic.org/)

Table 2- Anatomical types of emphysema

	Panacinar	Centriacinar	Periacinar	Irregular
Involvement	Whole acinus	Central region of acinus	Edge of acinus	Irregular
Distribution in lungs	Mainly lower zones	Mainly upper zones	Upper zones	Variable
Chest radiographic changes	Apparent	Not apparent	Not apparent	Variable
Sex	Men& women	Men & Women	Men more	Men & Women

Figure 2- The pulmonary airway tree



"The pulmonary airway tree begins with the trachea which consists of a series of cartilage horseshoes connected together by soft tissue. The trachea bifurcates at the carina, behind the sternum, to produce the two mainstem bronchi. These in turn divide into the lobar bronchi (2 on the left, 3 on the right). The lobar bronchi again bifurcate, and so on for about 23 generations. The first 16 or so of these generations act merely as conduits for passage of gas, and together constitute the conducting zone. Beyond the 16th generation, alveoli start to appear in the airway walls, becoming more numerous until the process terminates in an acinus consisting of a large collection of alveoli."

(Source: http://www.mmi.mcgill.ca/mmimediasampler2004/)

C. MACROPHAGES

Macrophages orchestrate the primary response to external stimuli when defending the body; they are the major defence cells in the lower airspace of the lung in healthy non-smokers and appear to have an essential role in the pathogenesis of COPD by accounting for most known features of the disease[11]. Smokers and patients with COPD have a 5-10 fold increase in the number of macrophages in their bronchoalveolar lavage (BAL) fluid, sputum and airways of the lung, compared to non-smokers. BAL fluid from smokers compared to non-smokers show a fivefold increase in the number of inflammatory cells in the lung, of which 85-90% are alveolar macrophages (AM). A morphometric analysis of the lung parenchyma of patients with emphysema showed a 25 fold increase in the number of macrophages in the lung tissue and alveolar space compared to non-smokers [12]. Macrophages are the predominant cells in the respiratory bronchioles of smokers. Studies have shown a correlation between alveolar macrophage number and the presence of emphysema[13], and also with the extent of lung destruction in patients with emphysema, where macrophages were localised at the sites of alveolar wall destruction [13, 14]. In addition, the severity of COPD in smokers is correlated with the number of macrophages in the airways of the lung [15]. The chronic increase in macrophages at sites of tissue damage in the lung corresponds with the slow progression and chronicity of COPD, suggesting macrophage involvement in pathogenicity.

The increase in alveolar macrophages within the lung is likely due to increased recruitment of monocytes from circulating blood. Blood monocytes migrate through the blood vessel walls into the lung in response to monocyte-selective chemokines produced

in the lung. An example is MCP-1 (monocyte chemoattractant protein-1), a monocyte selective chemokine which attracts monocytes to the lung where they differentiate into macrophages. In patients with COPD, MCP-1 is increased in the sputum and BAL fluid, suggesting its involvement in the migration of macrophages to the lung [16, 17]. In addition, adhesion molecules, such as ICAM-1 (intercellular adhesion molecule-1) on endothelial cells play an important role in cellular interaction and migration of monocytes and neutrophils to the lung. ICAM-1 is constitutively expressed in low levels in most organs; however the lung expresses the largest accumulation of ICAM-1, approximately 30 fold higher than other organs [18], suggesting its importance in the recruitment of monocytes to the lung. In a study using cigarette smoke condensate (CSC), the particulate fraction derived from cigarette smoke, exposure resulted in a 70-90% increase in the adherence of human peripheral blood monocytes to cultured human umbilical vein endothelial cells (HUVEC)[19]. The expression of CD11 ligand on the surface of the monocytes and expression of ICAM-1 on the HUVEC were also increased following exposure to CSC [19]. In addition, exposure to CSC increased the rate of trans-endothelial migration of monocytes across HUVEC by 200% [20].

The high numbers of macrophages in the lungs of smokers and COPD patients may also be due to prolonged survival of the macrophages, ineffective mucociliary clearance, and increase in macrophage proliferation. Cigarette smoking results in ingestion of substances in the inhaled smoke by macrophages. This leads to the development of characteristic smoker inclusion bodies within the cytoplasm of the cell; this is an important defence mechanism in the neutralization and clearance of toxic

cigarette smoke particles by macrophages. Macrophages have a life span of several months under normal circumstances, however in a study by Costabel and coworkers, smoker's inclusion bodies were detected in AM over two years after smoking cessation [21]. This can be due to increased survival of the cells and/or reduced effectiveness of mucociliary clearance in smokers. Macrophages within the lung normally have a low proliferation rate. However, it has been demonstrated that there is an increase in macrophage proliferation in smokers compared to non-smokers [22]. Nevertheless, the major contributing factor in the increase in the number of macrophages in the lungs of smokers remains the increase in recruitment of circulating monocytes to the lung.

Alveolar macrophages have the ability to release various inflammatory proteins, such as cytokines, chemokines, growth factors and proteolytic enzymes. Alveolar macrophages can also be activated by a variety of stimuli including bacterial endotoxins, pro-inflammatory cytokines and cigarette smoke. AM activated by cigarette smoke release various chemokines, including the neutrophil chemotatic cytokine interleukin-8 (IL-8), the expression of this cytokine is increased in macrophages of smokers compared to non-smokers following LPS stimulation. Macrophages can also secrete chemo-attractant cytokines for CD8 +T cells, such as Interferon-γ inducible protein (IP-10/CXCL10), which interacts with the CXCR3 receptor. The expressions of CXCR3/CXCL10 were found to be increased in smokers with COPD compared to nonsmoking subjects [23]. Other cytokines secreted by macrophages are TNF-α, MCP-1, interferon-inducible T cell α chemoattractant (I-TAC) and monokine-induced by interferon (Mig). In addition, AM produce growth factors, such as transforming growth

factor-α (TGF-α), a major endogenous activator of the EGF (epidermal growth factor) receptor which is involved in mucus secretion after inhalation of cigarette smoke [24]. AM also release various proteolytic enzymes including matrix metalloproteases (MMP) and cathepsins that can degrade the extracellular matrix (ECM) of the lung

D. TNF-α

Inflammation is a protective response of the body against foreign agents. However, excessive or persistent inflammation may cause tissue damage and contribute to the pathogenesis of a disease. The mechanism of inflammation involves the accumulation and subsequent activation of inflammatory cells in response to signaling proteins. Cytokines are important signaling proteins which regulate inflammatory responses against various foreign agents.

Cytokines are produced by a variety of cells, and function as extracellular signaling proteins between cells. They can act as long distance signals (endocrine signaling), short distance signals (paracrine signaling), or act on the cell which originally secreted the cytokine (autocrine signaling) [25]. Cytokines affect cells via binding to high-affinity cell surface receptors, initiating changes in cellular activity.

TNF-α is a key mediator of inflammation; it plays an important role in defence of the body against foreign pathogens such as viruses, bacteria and fungi. In humans, TNF-α is synthesized as a 31-kDa precursor molecule, however prior to secretion the N-terminal sequence is removed, leading to the release of a 17-kDa polypeptide [26].

TNF-α initiates signaling by binding to one of two cell surface receptors, p55 (TNFR1) or p75 (TNFR2). The main mediator for the TNF-α response is the p55 receptor which induces a variety of biological effects whereas the p75 receptor is only responsible for cytotoxic effects (Fig 3). The cytoplasmic domains of TNF-α receptors lack intrinsic protein kinase activity, however interaction with TNF-α causes a rearrangement of the clustered cytosolic tails of the receptors and recruitment of intracellular signaling proteins which interact with the cytoplasmic domain of the receptors and initiate signaling pathways. The intracellular signaling proteins that interact with the cytoplasmic domain of TNF-α receptors include two adaptor proteins, TNF-α receptor associated factor 2 (TRAF2) and TNF-α associated death domain protein (TRADD), and a receptor interacting protein kinase (RIP) [27]. The exact role of RIP is unclear; however it is known to be required for TNF-α signalling.

The intracellular signals triggered by TNF-α in the noncytotoxic signaling process serve primarily to modulate gene expression of a variety of proteins within the cell. This occurs through activation of the mitogen activated protein kinase kinase (MEKK) pathway, leading to activation of transcription factors such as AP-1, cJun and nuclear factor-kappa β (NF-κB). (Fig. 3) NF-κB, a prominent transcription factor, is activated after dissociating from an inhibitor protein IκB, and translocating into the cell nucleus where it can mediate alteration of gene expression [28].

The major function of TNF-α is to modulate gene expression for products that influence cell proliferation, cell differentiation, growth stimulation and inflammation.

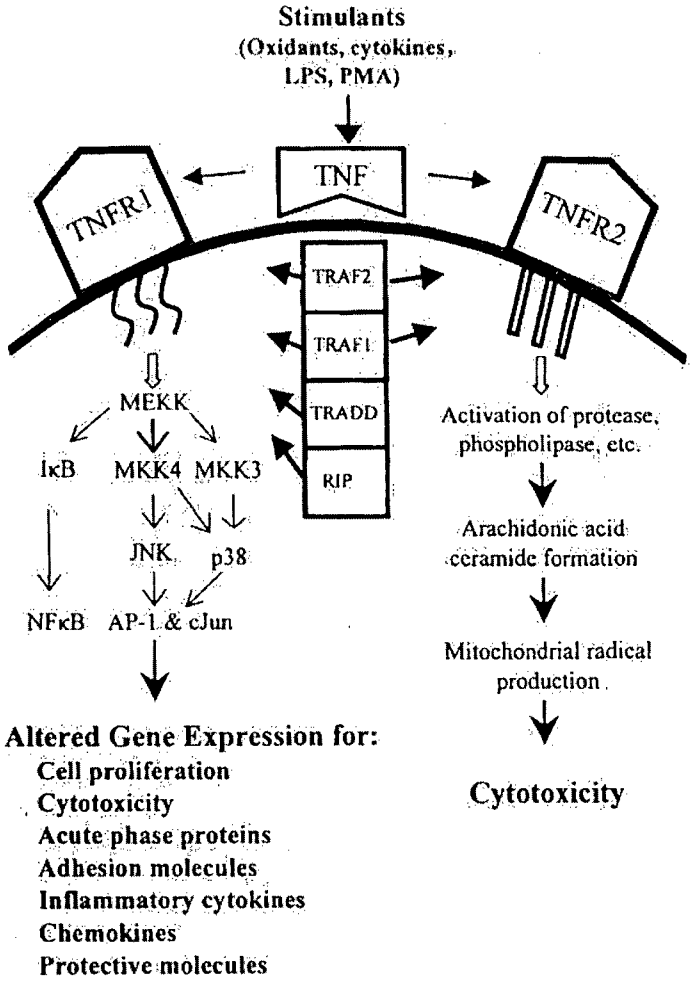
Inflammation is a major contributor in development of COPD thus increased understanding of how the inflammatory process is regulated will be beneficial to development of therapies. TNF-α has the ability to regulate the expression of inflammatory mediators such as IL-1, IL-6, IL-8, platelet derived growth factor (PDGF), granulocyte macrophage colony stimulating factor (GM-CSF), MCP-1, macrophage inflammatory protein 2 (MIP-2), and adhesion molecules including ICAM-1, E-selectin and vascular cell adhesion molecule-1 (VCAM-1) [29-32] .

A variety of cells produce TNF-α, including neutrophils, T cells, macrophages, monocytes, epithelial cells, fibroblasts and smooth muscle cells [33, 34] ; however, the principle source of pulmonary TNF-α remains the alveolar macrophages. A major stimulant of TNF-α production is bacterial endotoxins, however other agents such as cytokines and noxious particle can also be effective. Cytokines, including IL-1, GM-CSF and INF-γ (interferon gamma), induce TNF-α secretion by monocytes and macrophages. Many extracellular toxins such as bleomycin, silica and asbestos are also associated with increased TNF-α production in animals and humans. Studies have demonstrated that pre-treatment of mice with neutralizing antibodies against TNF-α results in resistance to these pulmonary toxins [28].

Studies have shown that patients with COPD have higher expression levels of TNF-α, due to either a genetic factor and/or cigarette consumption. For example, a polymorphism at position 308 in the promoter region of the TNF-α gene results in increased TNF-α production and has been associated with development of smoke-

induced COPD [35] ; however this association is not yet confirmed because other groups have shown no association between this TNF-α polymorphism and COPD development [36]. The effect of cigarette smoke exposure on TNF-α production is similarly inconsistent, and will be discussed in detail in the Discussion section of this thesis.

Figure 3- Model for TNF-α action



(Source: Micheal I. Luster, 1999) Ref #:28

E. PROTEASES

For the past four decades the protease/anti-protease hypothesis has been the dominant theory in explaining the pathogenic mechanism of COPD. It proposes that the equilibrium between proteases and anti-proteases in the lung is damaged as a result of increase in pulmonary proteases and/or reduction in pulmonary anti-proteases.

Early studies focused on the role of neutrophil elastase (NE) in the pathogenesis of emphysema, since patients with inherited deficiency in α1-anti-trypsin, an endogenous inhibitor of NE, were likely to develop early onset emphysema. Furthermore, it was demonstrated that cigarette smoke oxidatively inactivates α1-anti-trypsin, which raised the question of whether NE is an important factor in development of emphysema in smokers with normal levels of α1-anti-trypsin. Experimental observations in animal models showed that intratracheal instillation of NE resulted in the development of emphysema [37]. These early studies led to the idea that neutrophils and NE are two important agents in emphysema development [2, 10]. However the association of neutrophils and NE with human emphysema has been inconsistent and new proteases have also been shown to be important players in emphysema.

Macrophages have the ability to secrete a variety of matrix metalloproteases and cathepsins which are known to degrade various components of the ECM. In recent years there is growing evidence that macrophages and macrophage derived proteases may be more important in emphysema development than neutrophils and NE. Finkelstein et al. have shown a correlation between alveolar macrophage number and the extent of lung

destruction in patients with emphysema, where macrophages were localised at the sites of alveolar wall destruction. No such correlation has been found with neutrophil numbers [14]. Exposure of wild type mice to cigarette smoke for periods up to six months results in the development of smoke induced emphysema. In two different studies using MMP-12 $^{-/-}$ and NE $^{-/-}$ knockout mice, it was found that exposure to cigarette smoke led to development of emphysema in 40% of NE $^{-/-}$ knockout mice [9] whereas the MMP-12$^{-/-}$ knockout mice were completely protected against long term smoke-induced emphysema [38]. In our studies we will further investigate macrophage protease release in response to cigarette smoke exposure.

1. MATRIX METALLOPROTEASES

Currently, there are 24 MMPs that have been identified and are believed to play essential roles in healthy tissue development and in remodelling and repair of damaged tissues. However, over expression of these enzymes is associated with tissue destruction in diseases such as arthritis, atherosclerosis, tumour cell progression and pulmonary emphysema.

MMPs are a structurally related family of zinc-dependent enzymes capable of degrading ECM components, including basement membrane collagen, fibronectin, interstitial collagen, elastin and various proteoglycans. They can be divided into five classes: collagenases, gelatinases, stromelysins, membrane type MMPs and others (Table 3) [39]. MMPs are secreted by alveolar macrophages, neutrophils , airway epithelial cells and eosinophils in the lung. Macrophage-derived MMPs are considered to be critical in the development of COPD, as a result of increased turnover of macrophages within the

lungs of smokers. MMPs are secreted as inactive proenzymes and are activated by proteolytic cleavage of the N-terminal domain with proteases such as other MMPs, plasmin, neutrophil elatase and cathepsin G. Proteolytic activity of MMPs is regulated by specific tissue inhibitors of MMPs (TIMPs). Currently, four types of TIMPs have been characterized, TIMP1, 2, 3 and 4.

MMPs have the ability to induce morphological changes within the lung and there is evidence to suggest MMP involvement in the pathogenesis of COPD. Studies in humans have shown increases in the expressions of pulmonary MMP-1 [40], MMP-2 [41], MMP-8 [42], MMP-9 [43] and MMP-12 [44] in COPD patients.

Table 3- The MMP family of enzymes

NAME	MMP number	Molecular wt. latent/active (kDa)	Chromosome location	Substrates
Interstitial collagenases				
Collagenase-1	MMP-1	52 / 41	11q22-q23	I, II, III, VII, VIII, X, aggrecan, gelatin, pro-MMP-2, 9
Collagenase-2	MMP-8	85 / 64	11q21-q22	I, II, III, VII, VIII, X, aggrecan, gelatin
Collagenase-3	MMP-13	65 / 55	11q22.3	I, II, III, aggrecan, gelatin
Collagenase-4	MMP-18	53 / 42	Not applicable	I
Gelatinases				
Gelatinase A	MMP-2	72 / 66	16q13	I, II, III, IV, V, VII, X, XI, XIV, gelatin, elastin, fibronectin, aggrecan
Gelatinase B	MMP-9	92 / 82, 65	20q11.2-q13.1	IV, V, VII, X, XIV, gelatin, pro-MMP-9, -13, elastin, aggrecan
Stromelysins				
Stromelysin-1	MMP-3	57 / 45, 28	11q23	II, III, IV, IX, X, XI, elastin, pro-MMP-1, -7, -8, -9, -13, fibronectin, aggrecan, laminin, gelatin
Stromelysin-2	MMP-10	56 / 47, 24	11q22.3-q23	III, IV, V, gelatin, fibronectin
Stromelysin-3	MMP-11	58 / 28	22q11.2	fibronectin, laminin, gelatin, aggrecan
Membrane-type MMPs				
MT1-MMP	MMP-14	66 / 60	14q11-q12	pro-MMP-2, -13, I, II, III, gelatin, aggrecan, laminin, fibronectin
MT2-MMP	MMP-15	68 / 62	16q13-q21	pro-MMP-2, gelatin, laminin, fibronectin
MT3-MMP	MMP-16	64 / 55	unknown	pro-MMP-2
MT4-MMP	MMP-17	57 / 53	12q24.3	unknown
MT5-MMP	MMP-24	63 / 45	20q11.2-q12	pro-MMP-2
MT6-MMP	MMP-25	unknown	Not applicable	gelatin
Others				
Matrilysin-2 (PUMP-1)	MMP-7	28 /19	11q21-q22	II, III, IV, IX, X, XI, elastin, pro-MMP-1, -7, -8, -9, -13, , fibronectin, aggrecan, laminin, gelatin
Matrilysin	MMP-26	28 / unknown	Not applicable	IV, gelatin, fibronectin
Metalloelastase	MMP-12	54 / 45, 22	11q22.2-22.3	elastin
No name	MMP-19	57 / 45	12q14	tenascin, gelatin, aggrecan
Enamelysin	MMP-20	54 / 22	11q22.3	enamel, gelatin
No name	MMP-21	70 / 53	Not applicable	unknown
No name	MMP-23	unknown	Not applicable	unknown
No name	MMP-27	unknown	Not applicable	unknown
Epilysin	MMP-28	unknown / 58, 55	Not applicable	unknown

(Source: Ohbayashi H., 2002) Ref #: 39

2. CATHEPSINS

Cathepsins are enzymes that contain cysteine in their active site and are as also known as cysteine proteases. Cathepsins are synthesized as inactive 30-50 kDa pre-pro-enzymes which are glycosylated post-translationally and directed into the lysosomes where they catalyze protein hydrolysis [45]. Cathepsins can degrade components of the ECM intracellularly and extracellularly. Lysosomes containing cathepsins will fuse with phagosomes to intracellularly degrade fragments of ECM proteins which have been phagocytosed by macrophages [46]. Cathepsins can also be released extracellularly into the ECM by a variety of cells, including macrophages, mast cells, smooth muscle cells, tumour cells and fibroblasts. Cathepsins B, H, L and S are elastolytic enzymes that have optimal activities at an acidic pH and are expressed in alveolar macrophages [47, 48].

Our lab has previously studied the effect of cigarette smoke gas phase on the release of Cathepsin L, MMP-1 and MMP-9. Therefore we were interested to investigating the cellular mechanisms leading to the release of proteases by cigarette smoke media.

F. SIGNAL TRANSDUCTION

1. PKB

PKB (protein kinase B) is a serine/threonine kinase and a key mediator of signal transduction processes. PKB plays an important role in regulating signaling pathways in response to growth factors, such as insulin and other extracellular stimuli to control many biological processes, including proliferation, apoptosis, survival and growth [49]. PKB is

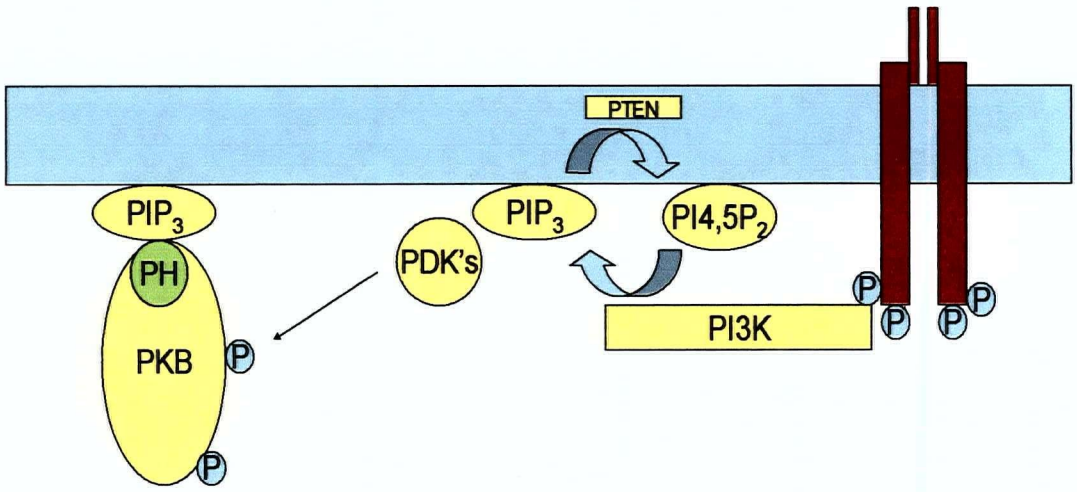
composed of an N-terminal PH domain, a central kinase domain and a C-terminal regulatory domain.

PKB acts downstream of PI3K (phosphatidylinositol 3-kinase), which is activated by tyrosine kinase and G-protein coupled receptors [50]. PI3K is activated once it is recruited to these receptors on their adaptor proteins at the plasma membrane where it phosphorylates the D3 position of membrane associated PI 4,5-P_2 (phosphatidylinositol-4,5-diphosphate) to form PIP_3 (PI 3,4,5-triphosphate) which is an important intracellular second messenger and is necessary for the activation of PKB. The level of PIP_3 is tightly regulated by the action of the 3 phosphatase, PTEN, which dephosphorylates PIP_3 to generate PI-4,5P_2 [51] (Fig 4). In addition SHIP1/2 or other 5'-phosphatases can dephosphorylate the D5 position of PIP3 to generate PI-3,4P2 forming another second messenger for PKB.

PKB gets translocated to the plasma membrane through direct contact of its pleckstrin homology (PH) domain with the 3'-phosphorylated PIP3 and PI-3,4-P_2 [52]. Upon membrane localization the confirmation of PKB is altered and this allows subsequent phosphorylation of the protein. PKB first gets phosphorylated at Thr308 by the 3-phosphoinositide-dependent protein kinase 1 (PDK1) which partially activates PKB, followed by phosphoryaltion of Ser 473, which is required for maximal PKB activity. The mechanism of Ser473 phosphorylation is not fully understood [52]. Activated PKB has a wide range of substrates and mediates the activation and inhibition of several targets that control cellular growth, proliferation and survival. Once fully activated, PKB

will phosphorylate numerous proteins in the nucleus and cytoplasm. Activated PKB is a well known survival factor, that phosphorylates and inactivates the pro-apoptotic factors BAD [53] and caspase-9 [54]. In addition it prevents the release of cytochrome c from mitochondria and inactivates the forkhead transcription factor which is known to induce pro-apoptotic factors such as Fas ligand [55]. In addition, activated PKB can induce phosphorylation of p70RSK which is known to phosphorylate the S 6 ribosomal protein. This signalling pathway plays an important role in translational regulation and protein synthesis.

Figure 4- Activation of PKB



2. MITOGEN ACTIVATED PROTEIN KINASE, ERK1/2

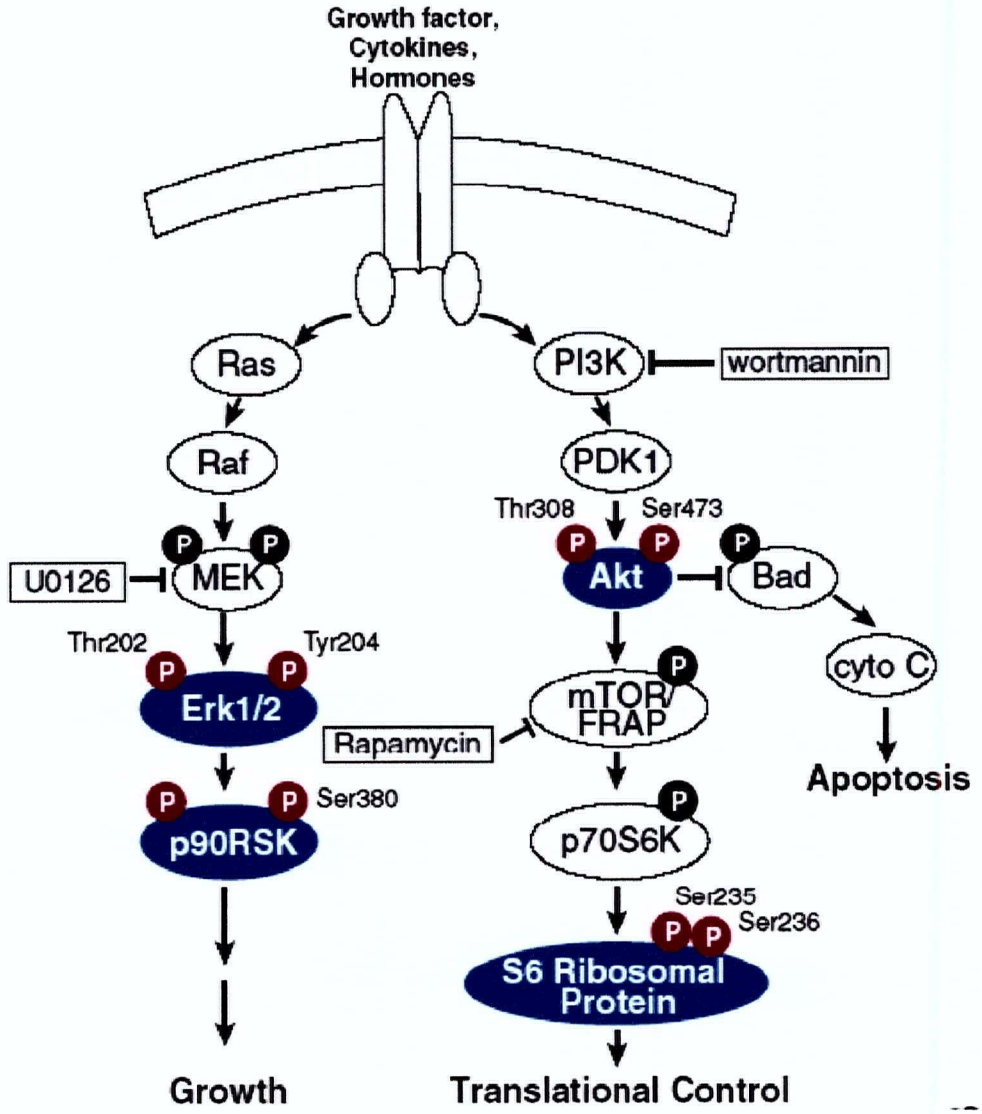
Mitogen activated protein kinases (MAPK's) are considered major players in numerous signal transduction pathways. They are activated by a variety of stimuli including, growth factors, cytokines and environmental stress resulting in a number of

physiological changes within a cell, such as, proliferation, cell survival, cell growth, differentiation and regulation of cell death in eukaryotic cells [56]. The three major and well characterized MAPK's are the extracellular-regulated kinase (ERK1/2), c-Jun NH_2-terminal kinase /stress activated protein kinase (JNK/SAPK) and p38 kinase, all of which are activated by LPS.

ERKs, or extracellular signal regulated kinases are activated by growth factors, G-protein linked receptors and phorbol esters, whereas the JNK/SAPK and p38 are stress activated MAP kinases, activated by inflammatory cytokines, or exposure to UV light [57, 58]. MAPK's are fully activated when phosphorylated at both threonine and tyrosine residue, and thereby activating a variety of protein kinases and several transcription factors. ERK has many substrates both in the cytosol and the nucleus, and can affect gene expression directly by phosphorylating transcription factors, such as Ets, Elk and Myc, as well as indirectly by targeting other substrates, such as p90-RSK (Fig. 5).

Few studies have investigated the effect of cigarette smoke on signaling pathways; therefore we were interested to evaluate the in vitro acute effect of CSM on the activation of signaling proteins specially, PKB, ERK1/2, p90RSK and S6 ribosomal protein.

Figure 5- Activation of ERK1/2 and PKB pathways



(Source: Cell Signaling Technology)

VII. WORKING HYPOTHESIS AND SPECIFC AIMS

Hypothesis:

Acute exposure to cigarette smoke contributes to lung inflammation and tissue destruction in pulmonary diseases such as emphysema, by inducing the release of TNF-α and matrix degrading enzymes by macrophages.

Specific Aims:

1. To determine the acute effect of cigarette smoke on TNF-α release by U937 differentiated macrophages, human alveolar macrophages derived from distal lavage of resected lungs and broncho-alveolar macrophages derived from volunteers.

2. To investigate the acute effect of cigarette smoke on the activation of ERK1/2, PKB, p90RSK and ribosomal S6 protein in U937 differentiated macrophages and human alveolar macrophages.

3. To investigate the mechanism by which cigarette smoke induces TNF-α release by macrophages.

4. To determine the acute effect of cigarette smoke on the release of cathepsin L, and matrix metalloproteases – 1, 7, 9 and 12 by alveolar macrophages.

IX. MATERIALS AND METHODS

A. Materials

The human monocytic cell line, U937, was obtained from the American Type Culture Collection (Manassas, USA). RPMI-1640, MSFM, L-glutamine, streptomycin-penicillin and Fetal Bovine Serum were purchased from Stem Cell Technologies (Vancouver, BC, Canada). The PathScan Multiplex western cocktail I consisted of: phopho-p90RSK (Ser-380), Phospho-PKB (Ser-473), Phospho-ERK1/2 (Thr-202/Tyr-204), Phospho-S6 Ribosomal protein (Ser 235/236), elF4E antibody; the cocktail and MEK 1/2 inhibitor U0126 were purchased from Cell Signaling Technology (Beverly, MA). Anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody, EGFR-specific inhibitor AG1478 and PI3-K inhibitor LY294002 were purchased from Calbiochem (La Jolla, CA). The high molecular weight protein standard for western blotting was purchased from Bio-Rad (Hercules, CA). ECL (enhanced Chemiluminescence) reagent was purchased from Amersham (Buckinghamshire, UK). Human TNF-α Elisa kit and the Fluorokine MAP multiplex assay kit were purchased from R&D Systems (Minneapolis, MN). Cathepsin L ELISA kit was purchased from Bender MedSystems (Vienna, Austria).

B. U937 Cell Culture

U937 cells were cultured in 75 cm^2 tissue culture flasks with RPMI medium supplemented with 10% heat-inactivated fetal bovine serum, 2mM L-glutamine, 10mM HEPES and streptomycin-penicillin in a humidified atmosphere of 95% air and 5% $C0_2$

at 37°C. Cells in the culture flasks were maintained at a density of $1x10^5$ cells/ml to 2 $x10^6$ cells/ml. Cells were harvested, then plated in a 6-well tissue culture plates ($2x10^6$ cells/2ml/well) and stimulated with 20ng/ml of phorbol myristate acetate (PMA, Sigma) for 48 hours resulting in adherent differentiated macrophages. Adherent cells were washed twice with RPMI-1640, followed by the addition of fresh RPMI-1640 medium in the absence of PMA for an additional 24 hours. On the day of smoke exposure, cells were washed twice with RPMI followed by the addition of fresh RPMI and incubated for 2 hours prior to smoke exposure. For the experiments with MEK1/2 inhibitor U0126, EGFR-specific inhibitor AG1478 and PI3-K inhibitor LY294002, cells were pre-incubated with 10 μM of U0126, 10 nM of AG1478 and 6 μM of LY294002 for 1 hour prior to smoke exposure and compared to control cultures that were not treated with inhibitors.

C. Isolation and culture of human alveolar macrophages from resected lungs

AM were recovered from resected human lungs from smokers or exsmokers with localized lung cancer. BAL was preformed by injecting large volume up to 1 liter of cold 0.9% sterile saline into a segmental or lobar bronchus of the resected lobe or lung that did not contain any tumor. The BAL fluid was then filtered through sterile gauze to remove mucus and centrifuged at 1000 rpm, 4°C for 10 min, cell pellets were then resuspended with macrophage serum free medium (MSFM) (GIBCO, Ontario). Mononuclear cells were purified from red blood cells and granulocytes using Hypaque-Ficoll centrifugation. Briefly, the cell suspension was layered over Histopaque 1077 and centrifuged at 1600rpm, 25°C for 30 minutes. Mononuclear cells were held at the surface of the

Histopaque and collected. Cells were then washed twice with MSFM, counted and loaded onto cell culture plates at a concentration of $1x10^6$/ml. After 4h of incubation in a humidified incubator at 37°C, non-adherent cells were removed by washing twice with MSFM. The adherent macrophages were then incubated overnight and washed again with MSFM prior to experimental exposure.

D. Isolation and culture of human broncho-alveolar macrophages from volunteers

Broncho-alveolar macrophages were kindly provided by Dr. S. Lam from his study screening lung cancer from volunteer smokers and exsmokers. Because Dr. Lam was interested in studying Bronchial epithelial cells, lavage was done using fiberoptic bronchoscopie under local xylocaine anesthesia from a segmental bronchus using three 20 ml aliquots of prewarmed (37°C) sterile isotonic saline solution and collecting only the first 30 ml of recovered lavage fluid. The lavage fluid was filtered, centrifuged and suspended in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES and streptomycin-penicillin. Macrophages were counted and plated onto cell culture plates at a concentration of $1x10^6$/ml. The cells were allowed to adhere for 2 hours at 37°C, non-adherent cells were removed by washing twice with RPMI. We estimated from the presence of epithelial cells that the alveolar macrophages had 5-15% contamination by bronchial macrophages, and we termed them as broncho-alveolar macrophages (BM). The adherent broncho-alveolar macrophages were then incubated for an additional 2 hours and washed again, followed by the addition of fresh RPMI and incubated overnight prior to experimental exposure.

E. Exposure of Cultures to Cigarette Smoke Medium

Cigarette smoke generated from two medium tar commercial filtered cigarettes was bubbled through 25 ml of RPMI-1640. Eight puffs of 30ml of smoke from each cigarette withdrawn over 2 seconds using a 60 ml syringe where bubbled through 25 ml of RPMI over 30 seconds. The pH of the medium was adjusted to 7.2-7.4 and diluted to achieve an optical density reading of 1.0 at 320 nm. This was defined as 100% CSM. The wavelength 320 was used to standardize the CSM, because cigarette smoke treated RPMI showed a maximal absorbance at 320nm. To remove particles, the CSM was filtered through a 0.2 μm pore filter. The CSM was freshly prepared within an hour prior to macrophage exposure and diluted with medium to achieve the desired % concentration of CSM. Cells were treated with different percentages of CSM, and cell viabilities following treatment were always greater than 95% as measured by trypan blue exclusion. Media were collected at 18, 24 and 48 hours, centrifuged at 1000rpm for 10 minutes, and then stored at -70°C.

F. TNF-α Enzyme Linked Immunosorbent Assay (ELISA)

TNF-α levels in the cell culture supernatant of control and CSM-exposed differentiated U937 cells, AMs and BMs were quantified by ELISA following the manufacturer's instructions (R&D Systems). Briefly, 96 well plates (NUNC,MaxiSorp^{TM}Surface) were coated with monoclonal anti-human TNF-α antibody (4μg/ml) at 4 °C overnight. The wells were then washed 3 times with PBS-0.05% Tween 20 and then blocked with 1% BSA in PBS for 1 hour. The plate was washed again, followed by the addition of samples and standards in duplicate, and incubated for 2 hours

at room temperature. The washing step was repeated and biotinylated goat anti-human TNF-α (75 ng/ml) was added and incubated for 2 hours at room temperature. The wells were washed again, and streptavidin-HRP was added and incubated for 30 minutes at room temperature. After a final wash, TMB (3, 5, 3', 5'-tetramethylbenzidine) was added as a substrate and incubated for 1 hour at room temperature, the reaction was stopped by the addition of H_2SO_4 (2N) and read at 450nm.

G. Western blot analysis

Immunoblot analyses for phospho-proteins were performed on control and CSM-exposed differentiated U937 cells and AMs. The cells were serum-deprived for two hours prior to stimulation with CSM. Control and treated cells ($2x10^6$ cells) were washed 3 times with ice cold phosphate buffered saline (PBS) followed by the addition of 125µl of ice-cold lysis buffer (50 mM Tris buffer pH 7.4, 1% Triton X-100, 10% glycerol, 100 mM NaCl, 2.5 mM EDTA, 10 mM NaF, 0.2 mM Na orthovanadate, 1 mM Na molybdate, 40 µg/ml PMSF, 1 µM pepstatin, 0.5 µg/ml leupeptin, 10 µg/ml soybean trypsin inhibitor). Lysates were collected and centrifuged at 13,000 rpm for 10 min at 4°C, the supernatant containing protein was collected and the precipitate discarded. The protein concentrations of extracts were quantified by the Pierce BCA (bicinchoninic acid) protein assay. 5X sample buffer was added to each sample and heated at 90°C for four minutes. High molecular weight standard (BioRad) and samples of equal protein quantities were loaded and separated on a 12% SDS polyacrylamide gel.

The proteins were then transferred onto nitrocellulose paper by semi-dry blotting. After transfer, blots were stained with Ponceau Red for one minute to confirm equal protein transfer to each blot. The membrane was then blocked with 5% skim milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) for 1.5 hours followed by incubation with the primary antibody overnight in 5% BSA/TBST at 4°C. Membranes were then washed twice in TBST for 15 minutes at room temperature, then incubated with horseradish peroxidase-conjugated secondary antibody (1:2000 dilution) for 1 hour at room temperature. The immunoblots were visualized with ECL reagent.

H. Fluorokine MAP multiplex assay

For simultaneous quantitative determination of the concentrations of multiple human matrix metalloproteases in cell culture supernatant, the human fluorokine MAP multiplex assay kit was used to detect MMP-1, MMP-7, MMP-9 and MMP-12 released by AM. First, the filter-bottomed microplate was pre-wetted with wash buffer. The liquid was then removed through the filter at the bottom of the plate using a vacuum manifold designed to accommodate a microplate. Analyte-specific antibodies (MMP-1, MMP-7, MMP-9 and MMP-12) pre-coated onto color coded microparticles were loaded in each well of the filtered-bottomed microplate, followed by the addition of standards and samples (diluted 1:5) into the wells in duplicate. The plate was securely covered with a foil plate sealer and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker set at 500 rpm. Using a vacuum manifold device the plate was washed three times to remove any unbound substances. Biotinylated antibodies specific to the analytes of interest were added to each well, covered with a foil plate sealer and

incubated for 1 hour at room temperature on a microplate shaker set at 500 rpm. The washing step was then repeated, followed by the addition of streptavidin-phycoerythrin conjugate to all wells. After a final wash, the microparticles were resuspended in wash buffer and incubated for 2 minutes on the plate shaker and read using the Luminex 100 analyzer.

I. Cathepsin L ELISA

Cathepsin L levels in the cell culture media of AMs were quantified by ELISA following manufacturer's instructions (Bender MedSystems, Vienna, Austria). After treatment with different percentages of CSM, cell culture media were collected at 24 and 48 hours, centrifuged and stored at -70°C. Briefly, 96 well plates (NUNC ,MaxiSorp^{TM}Surface) were coated with monoclonal anti-human Cathepsin L antibody (1µg/ml), sealed with a plate cover and incubated at 4 °C overnight. Wells were washed 3 times with PBS-0.05% Tween 20 to remove any unbound substances. The plate was then blocked with PBS/1% BSA for 4 hours at room temperature. The plate was washed again, followed by the addition of samples and standards in duplicates, and incubated for 2 hours at room temperature. The washing step was repeated, then biotin-conjugated antibody was added, sealed and incubated overnight at 4 °C. Wells were washed again; streptavidin-HRP was added and incubated for 1 hour at room temperature. After a final wash, TMB was added as a substrate and incubated for 1 hour at room temperature, the reaction was stopped by H_2SO_4 (2N) and read at 450nm on a microplate reader.

J. Statistics

The data for TNF- α release by U937 differentiated macrophages are expressed as mean ± standard deviation. Results were analyzed by the student's t-test at a confidence level of $p \leq 0.05$. Primary human AMs and BMs data for TNF-α and MMPs were not normally distributed and tobacco smoke had no statistically significant effect. For the Cathepsin L release we used the Wilcoxon rank sum test as a non-parametric method to assay statistical significance of differences between smoke exposed and control results.

X. RESULTS

A. Cigarette Smoke Media Stimulates TNF-α Release by U937 Differentiated Macrophages.

To evaluate the time effect of CSM on TNF-α release, U937 differentiated macrophages were treated with 10 % CSM for 3, 6, 9, 18, 24 and 48 hours. Controls were macrophages treated with air-exposed media. TNF-α released in the media of the macrophages was assayed by ELISA. We observed that the effect of CSM on TNF-α release was time dependent. CSM induced TNF-α release as early as 3 hours and peaked at 24 and 48 hours (Fig. 6A). We then tested the effect of CSM on TNF-α release in a dose response experiment. Macrophages were treated with 0, 2.5, 5, 10, 15 and 20 % CSM for 18, 24 and 48 hours. Results indicate that CSM induces statistically significant release of TNF-α in a dose-dependent manner (Fig. 6B) following exposures to more than 2.5% CSM.

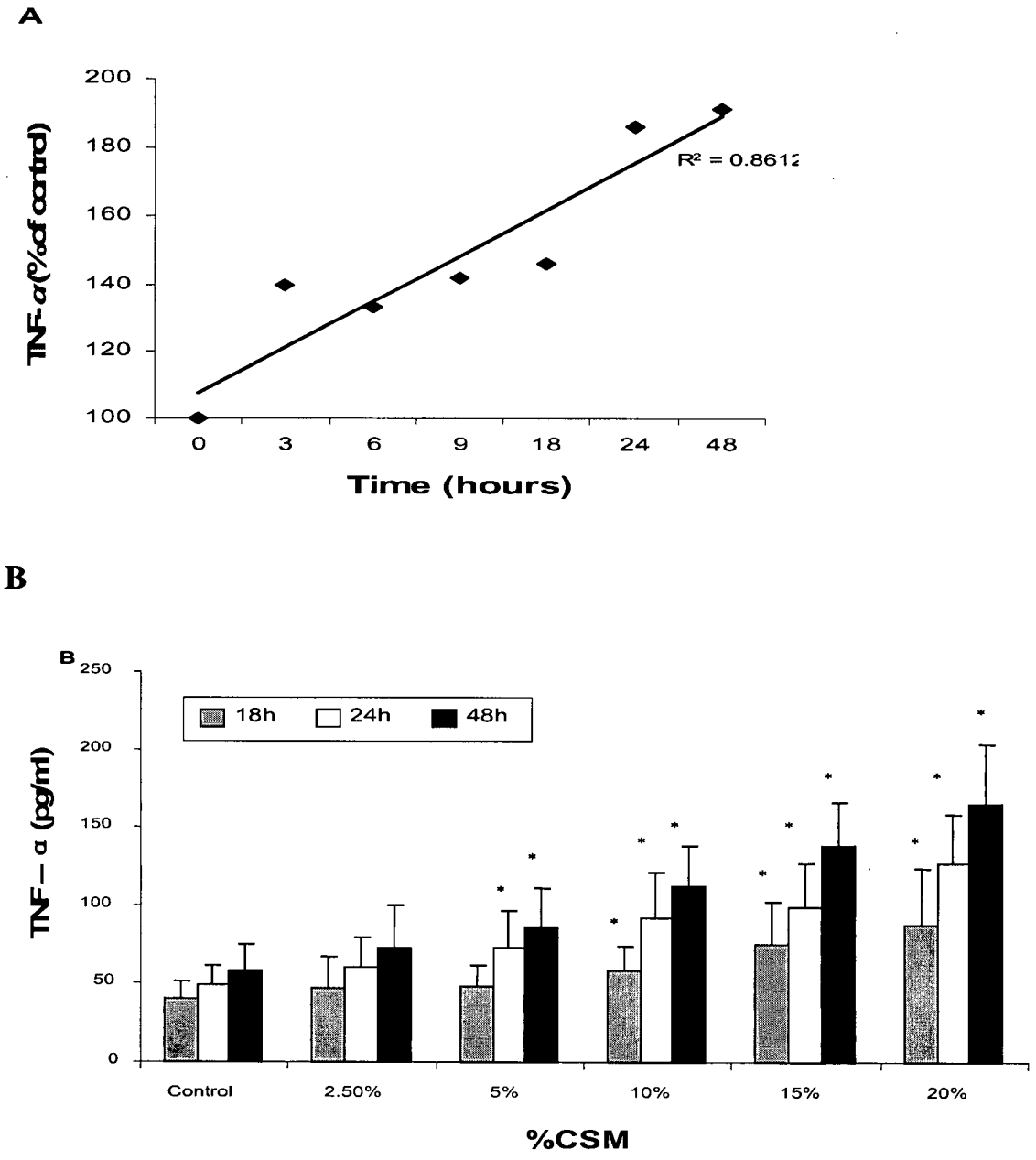
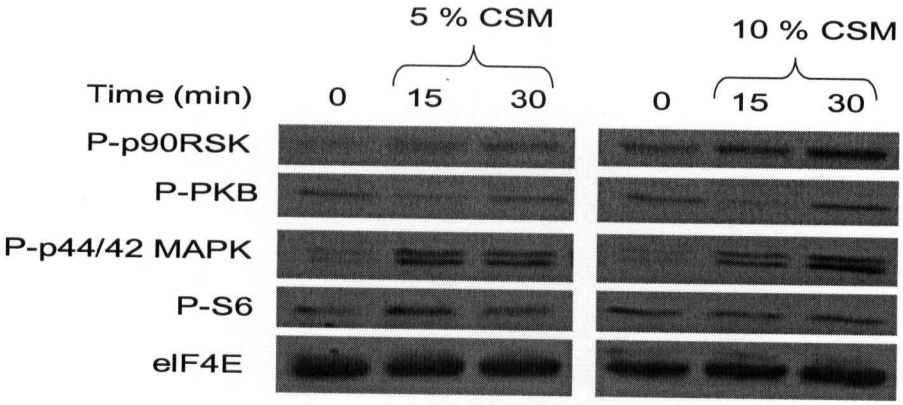


Figure 6 – Dose and time dependent effects of CSM on TNF-α release by U937 differentiated macrophages. [A] - TNF-α release by Macrophages treated with 10% CSM for 3, 6, 9, 18, 24 and 48 hours. CSM induced a time dependent release in TNF-α. [B] - Macrophages were treated with the indicated percentages of CSM for 18, 24 and 48 hours. CSM induced a dose-dependent release of TNF-α. Each bar represents the mean value ± S.D. from 7 independent experiments. CSM caused a significant increase in TNF-α ($p<0.05$) at concentrations of 5, 10, 15 and 20 %. TNF-α release in media was measured by ELISA and LPS was used as a positive control.

B. Activation of ERK1/2 MAP Kinases and Deactivation of PKB by CSM in U937 Differentiated Macrophages.

We investigated the effect of CSM on the activation of p90RSK, ERK1/2, PKB and p70 S6 kinase in U937 differentiated macrophages using a cocktail of specific antibodies. This was accomplished by immunoblotting with antibodies that detect only the phosphorylated and activated forms of the p90RSK, ERK1/2 and PKB kinases, as well as an antibody detecting phosphorylated S6 ribosomal protein, a target of p70 S6 kinase. Macrophages treated with 5 and 10 % of CSM showed a greater than two fold increase in the activity of ERK1/2 and a consistent decrease in the activity of PKB after 15 and 30 minutes of CSM exposure (Fig. 7). The activities of p90RSK and S6 ribosomal protein were also assayed with antibodies to the phosphorylated forms of these proteins. Activation of the p90RSK kinase is at least partially dependent upon ERK1/2 activation, while phosphorylation of the S6 ribosomal protein is one measure of the activation of p70S6K. Exposure to CSM was found to activate p90RSK, as well as induce slight activation of S6 Ribosomal protein (Fig. 7).

A



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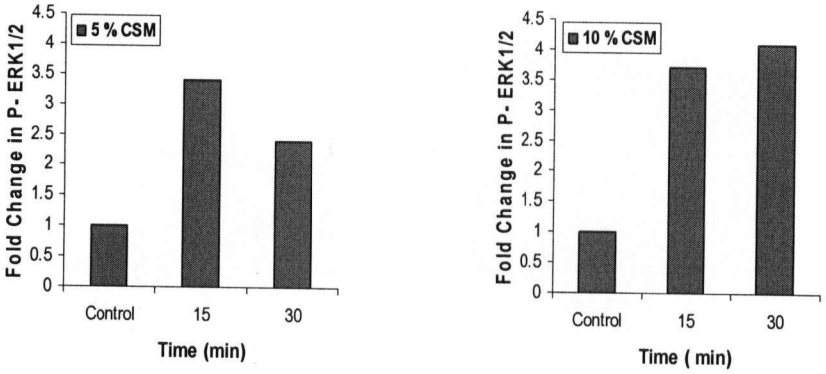
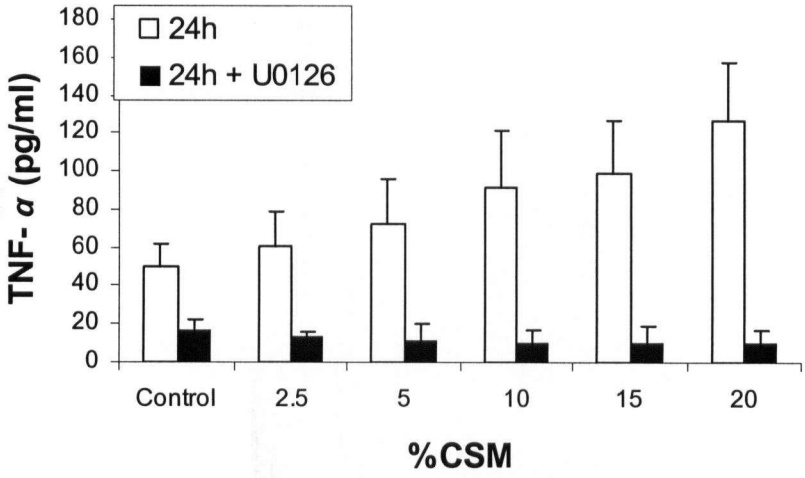


Figure 7 – CSM induces activation of ERK1/2 and p90RSK, and decreased activation of PKB in U937 differentiated macrophages. [A] - Phosphorylation of ERK1/2, PKB, p90RSK and S6 Ribosomal protein, was analyzed by Western blot. $2x10^6$ macrophages/well were treated with 5 and 10 % CSM for 15 and 30 minutes. Whole cell lysates were analyzed. Immunoblotting for elF4E was included as a control for protein loading. [B] Quantitative densitometry result of phospho-ERK1/2 was normalized against elF4E protein content.

C. Effect of MEK1/2 Inhibitor U0126 on TNF-α Release by CSM in U937 Differentiated Macrophages.

To elucidate the intracellular signaling mechanism by which CSM induces TNF-α release, we investigated whether the activation of the ERK1/2 pathway was necessary for CSM induced TNF-α release by U937 differentiated macrophages in vitro. Cells incubated with and without pre-treatment with MEK1/2 inhibitor U0126 (10μM) for 1 hour, were exposed to 2.5, 5, 10, 15 and 20 % of CSM for 24 hours. The inhibitor U0126 completely blocked TNF-α release, indicating that TNF-α release in response to CSM exposure is dependent on the activation of ERK1/2 (Fig. 8). In contrast, PI3-K inhibitor LY294002 and EGFR-specific inhibitor AG1478 had no effect on CSM- induced TNF-α release by macrophages (Fig. 9). U0126, AG1478 and LY294002 did not affect the viability of the macrophages as measured by the trypan blue exclusion test (data not shown).

A



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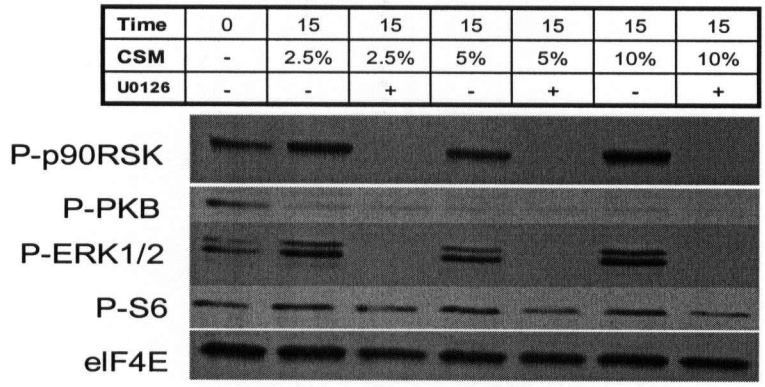


Figure 8 – ERK1/2 is a mediator in CSM-induced TNF-α release in U937 differentiated macrophages. [A] TNF-α release without (white bar) or with (black bar) pretreatment with MEK 1/2 inhibitor U0126 (10μM). The inhibitor blocked release of TNF-α following exposure to 2.5, 5, 10, 15 and 20 % CSM for 24 hours, indicating that release is dependent on ERK1/2 activation. Each bar represents the mean value ± S.D. from 3 independent experiments. TNF-α released in media was assayed by ELISA. (B) Western blot analysis from macrophages treated with U0126 for 1 hour prior to treatment with 2.5, 5 and 10 % CSM for 15 minutes. Treatment with U0126 did not affect the viability of U937 cells.

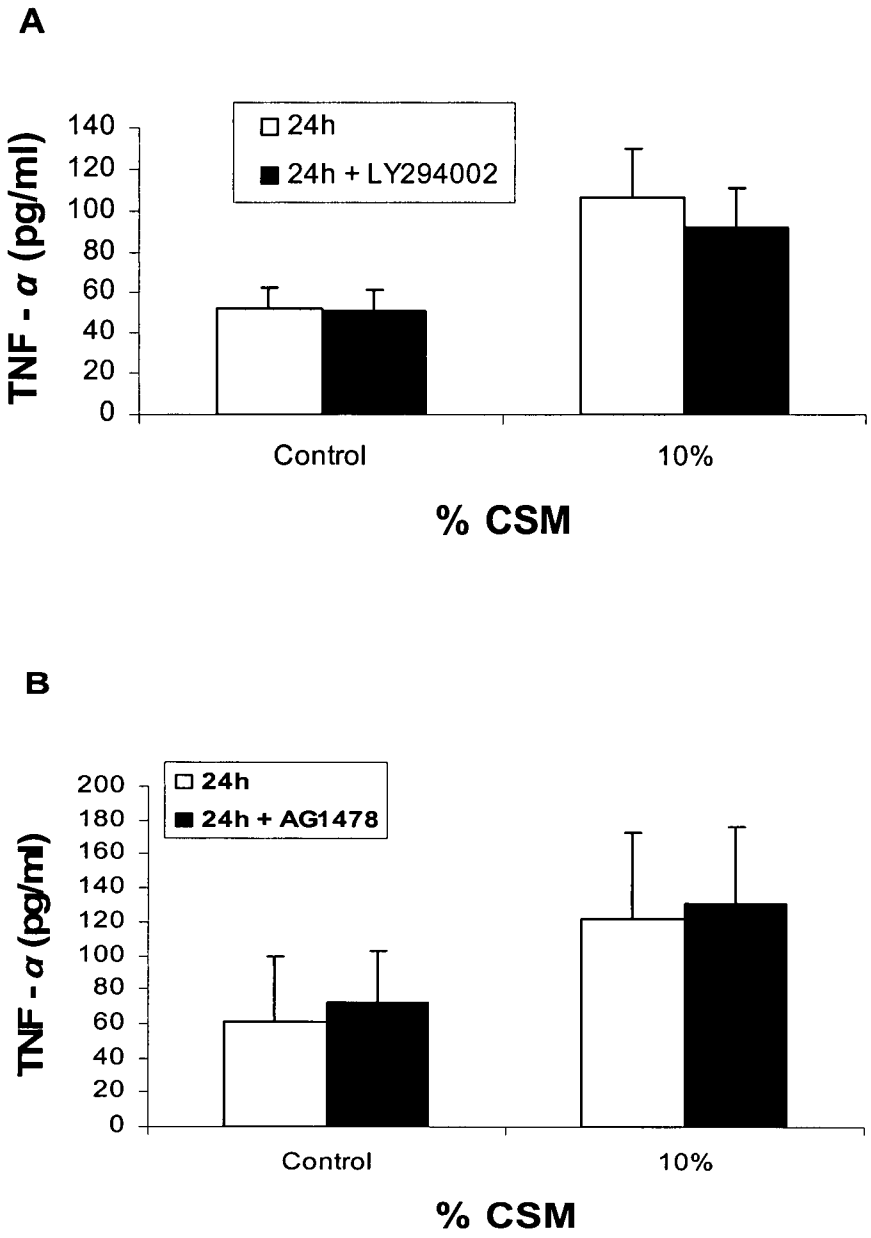


Figure 9- Effect of AG1478 and LY294002 on TNF- α release by U937 differentiated macrophages. TNF- α release without (white bar) or with (black bar) pretreatment with [A] PI3-K inhibitor U0126 (6μM), [B] and EGFR-specific inhibitor AG1478. Both inhibitors had no effect on CSM-induced TNF- α release. Each bar represents the mean value ± S.D. from 3 independent experiments.

D. Acute Effect of CSM on TNF-α Release by Human Alveolar and Broncho-alveolar Macrophages.

Levels of TNF-α were measured in macrophages collected from (1) BAL of resected human lungs from smokers and ex-smokers with small peripheral tumors, and from (2) bronchoalveolar lavage of volunteers who are smokers and ex-smokers. The effect of CSM on TNF-α release by AM and BM were quantified using ELISA assays.

(1)- All experiments on AM from resected human lungs were performed a few months prior to TNF-α analysis. Samples were aliquoted at the time of collection and stored at -70°C. AM were treated with 5 and 10 % of CSM for 24 and 48 hours and were compared to control unexposed AM. The level of TNF-α was undetectable for most of the samples at baseline and after treatment with 5 and 10 % CSM for 24 and 48 hours. Eight samples were tested from which only two samples released detectable amounts of TNF-α, furthermore there was great variability between the two results. One sample showed a trend of decrease in TNF-α, and the other sample showed no change after treatment with CSM (Fig. 10). We were unable to concentrate the cell culture supernatant from control and CSM treated AM from the undetectable samples because most of the samples were previously used to quantify the proteases released in the cell supernatant. In addition we had no more access to additional resected lungs; therefore we were unable to perform other experiment using AM derived from BAL of resected human lung of smokers.

(2) – BMs were treated with 10 and 20 % of CSM for 24 hours. The levels of TNF-α release were detectable at baseline and after treatment with CSM. However, there

was great variability between samples and the quantitative results were statistically insignificant. Nevertheless most samples treated with 10 % CSM showed either a trend of decrease or no change in TNF- α released by BM in the cell culture supernatant. Treatment with 20 % CSM suppressed TNF- α release in all the samples (Fig. 11). In addition, we examined the effect of exposure to CSM on TNF-α release by BM at an earlier time period of 4 hours after CSM exposure; however most samples had undetectable levels of TNF-α. The results are shown as individual data points and medians.

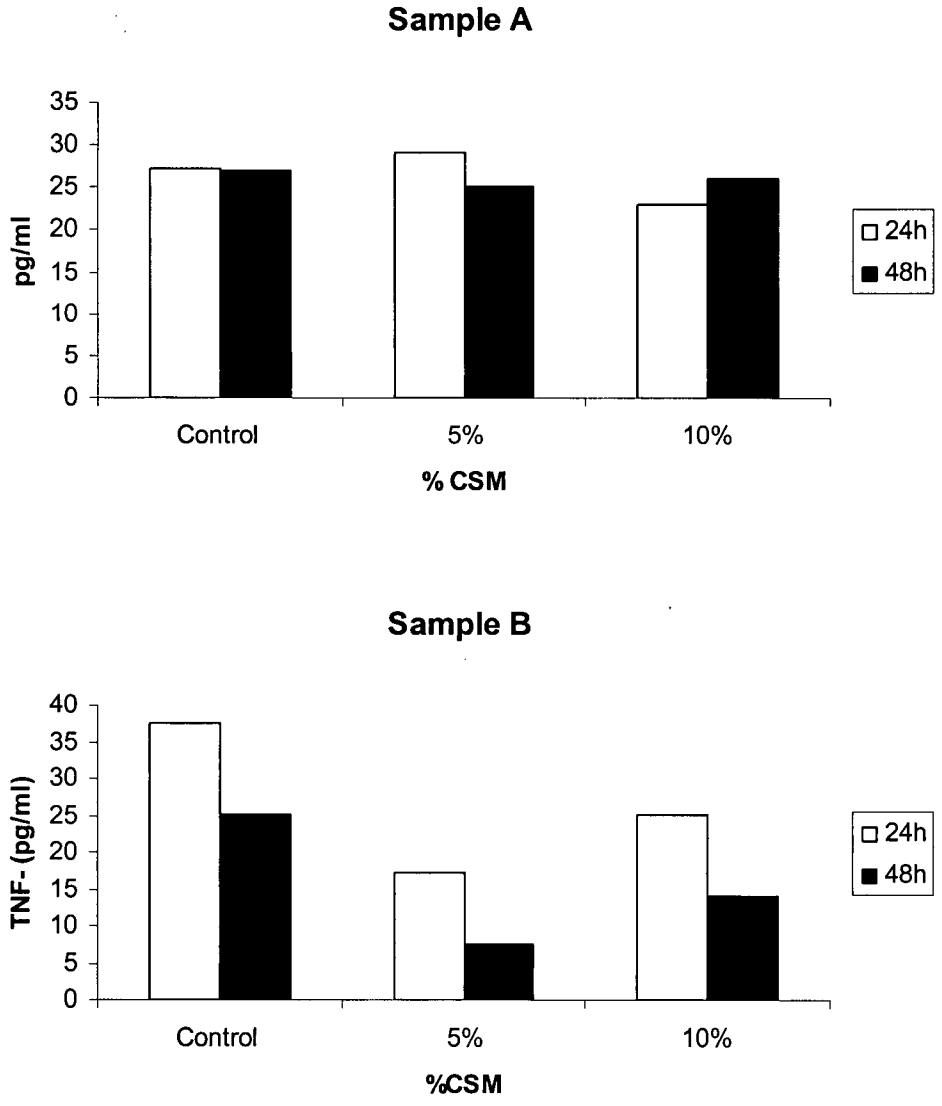


Figure 10- Acute effect of CSM on TNF-α release by alveolar macrophages. TNF- release by AM treated with 5 and 10% CSM for 24 and 48 hours. Eight samples were tested form which only two samples were in the detectable range of the TNF-α ELISA assay. Sample A showed no change in TNF- release after treatment with 5 and 10% CSM for 24 and 48 hours, whereas sample B showed a trend of decrease in TNF-α release at 24 and 48 hours to a point that it becomes undetectable.

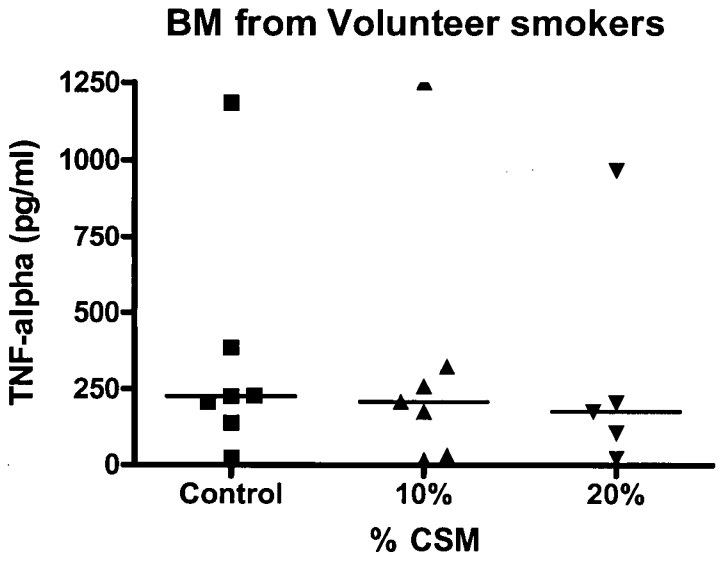
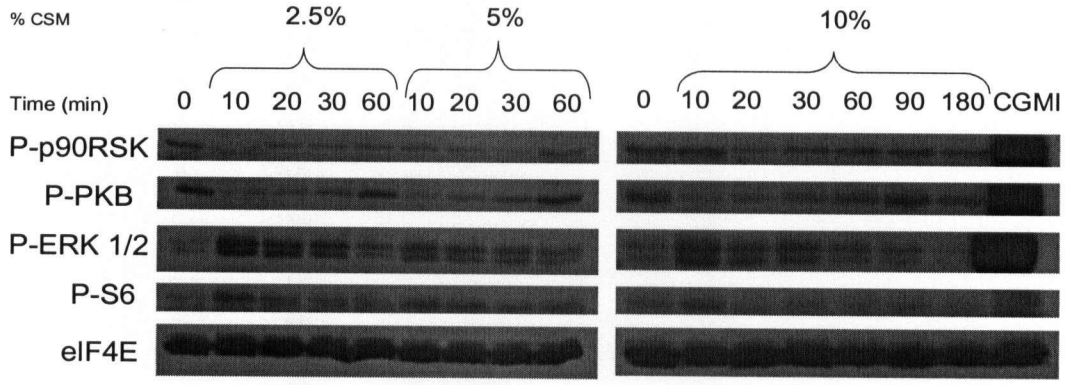


Figure 11- Acute effect of CSM on TNF-α release by broncho-alveolar macrophages. TNF- α release by BM treated with 10 and 20% CSM for 24 and 48 hours. CSM caused a decrease or no change in TNF-α release by BM however there was great variability between the results and there is no statistically significant change to control.

E. Effect of CSM on ERK1/2 and PKB Activity in Alveolar Macrophages.

CSM was found to induce the phosphorylation of ERK1/2 in AM after treatment with varying amounts of CSM. Stimulation of ERK1/2 phosphorylation by CSM was evident within 5 minutes and reached a maximum at 10 minutes. The activity of p90RSK, a downstream kinase of ERK1/2, was increased at 10 minutes after CSM treatment and showed decrease when assayed at subsequent time points. S6 ribosomal protein was also slightly phosphorylated at 10 minutes after CSM treatment. The phosphorylation of PKB was unaffected in some samples and decreased in others after CSM treatment. CSM inactivated PKB as early as 5 minutes after treatment, however after one hour the activity of PKB returned to baseline level (Fig. 12).

A


% CSM
2.5%
5%
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Time (min)
0 10 20 30 60 10 20 30 60 0 10 20 30 60 90 180 CGMI
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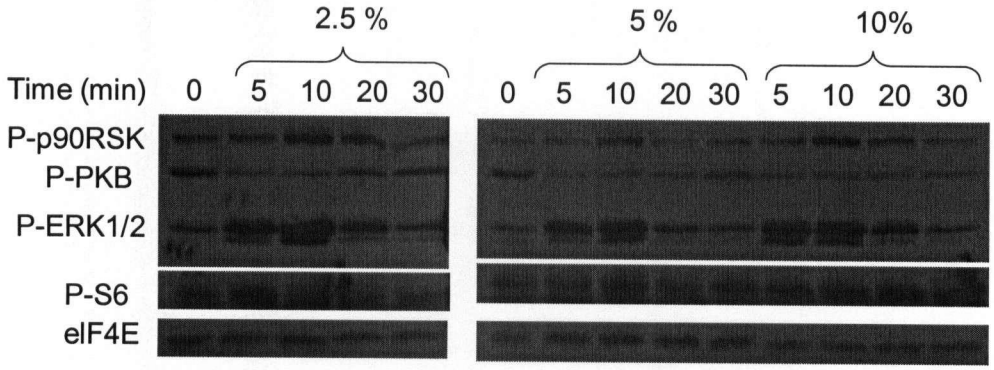
B


2.5 %
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P-p90RSK
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P-ERK1/2
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eIF4E

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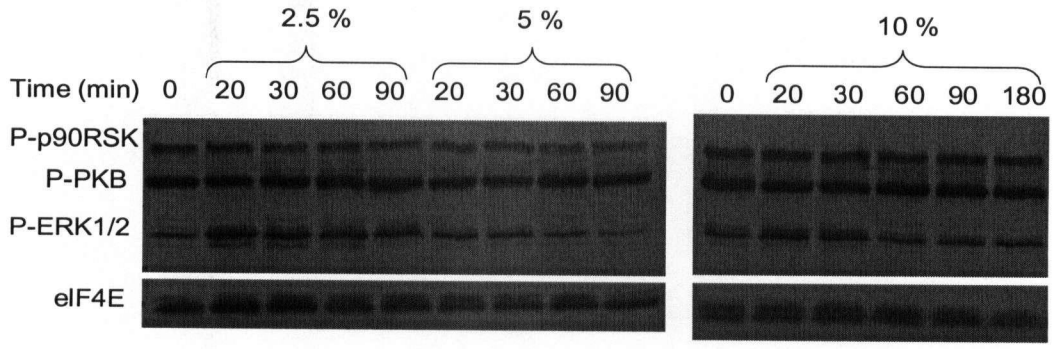
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P-ERK1/2
eIF4E

Figure 12- CSM induces activation of ERK1/2 and p90RSK, but decreased or had no effect on the activity of PKB in alveolar macrophages. Phosphorylation of ERK1/2, PKB, p90RSK and S6 Ribosomal protein analyzed by Western blot. Whole cell lysate were analyzed. immunoblotting for elf4E was included as a control for protein loading. Panels A, B and C represent three different samples exposed to Cigarette smoke.

F. Cathepsin L Release in Culture Media of CSM Exposed Alveolar Macrophages.

The levels of Cathepsin L in the media of cultured AM at 24 and 48 hours were compared in control and CSM treated cells. We observed a slight but statistically significant increase in Cathepsin L release at 24 and 48 hours after AM were incubated with 10% CSM (Table 4). Because the data points were not normally distributed due to variability in different subject response, the Wilcoxon signed-rank test was used to evaluate statistical significance.

Table 4- Cathepsin L in culture media of alveolar macrophages incubated with cigarette smoke media. (Means ± SD)

	ng/ml	% of Control
24 hrs	**n = 10**	
Control	24.3 ± 28.1	100
5 %	26.7 ± 29.9	91 ± 17.9
10 %	29 ± 36.2 **	109.7 ± 21.5
48 hrs	**n = 10**	
Control	26.3 ± 32.2	100
5 %	26.7 ± 32	106.7 ± 17.9
10 %	28.2 ± 34.3 *	111.2 ± 25.1

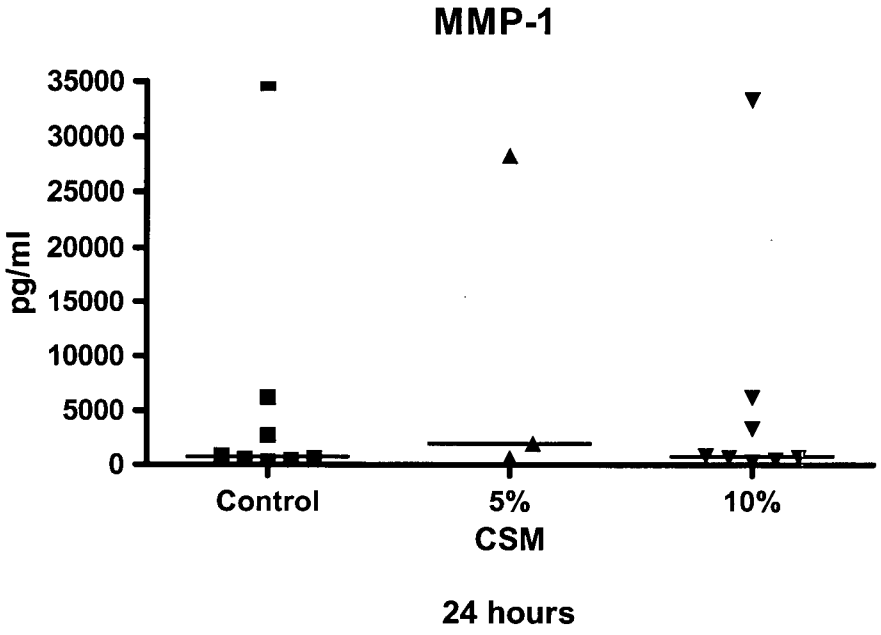
- There was a slight but statistically significant increase in Cathepsin L at 24 and 48 hours after AM incubation with 10% CSM.

- * $P<0.05$, ** $P<0.01$by the Wilcoxon rank sign test; this was used to evaluate statistical significance because data were not normally distributed

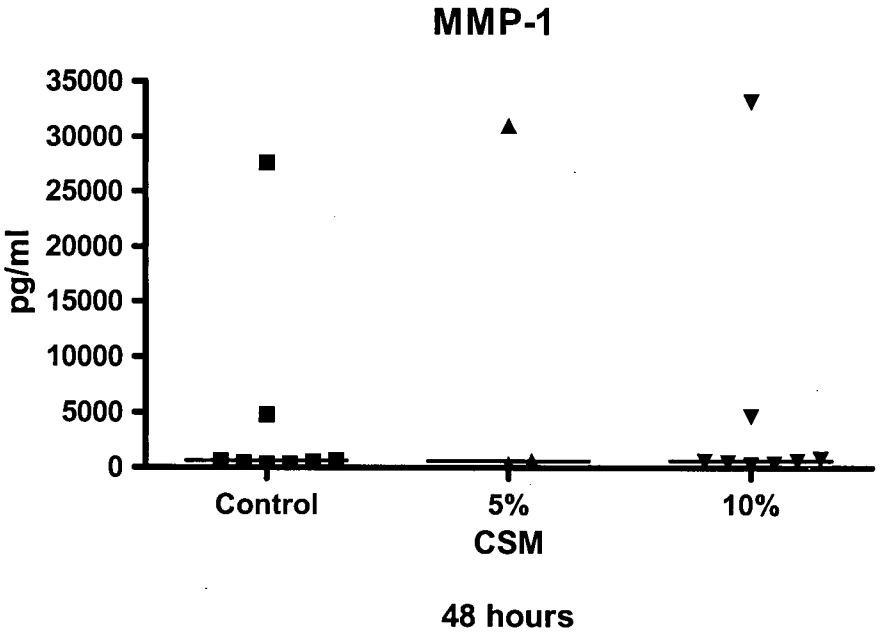
G. Acute Effect of CSM on Matrix Metalloproteases Release by Human Alveolar Macrophages.

Figure 13 summarizes the amounts of MMP-1, -7, -9 and -12 released in media of AM after 24 and 48 hours of exposure to 5 and 10 % of CSM. The effects of CSM on production of the four proteases were measured using the fluorokine MAP multiplex assay kit. Our results indicate that there was no statistically significant effect of CSM on protease levels in culture media of AM after 24 and 48 hours of exposure to CSM, compared to controls. The results are shown as individual data points and medians.

A

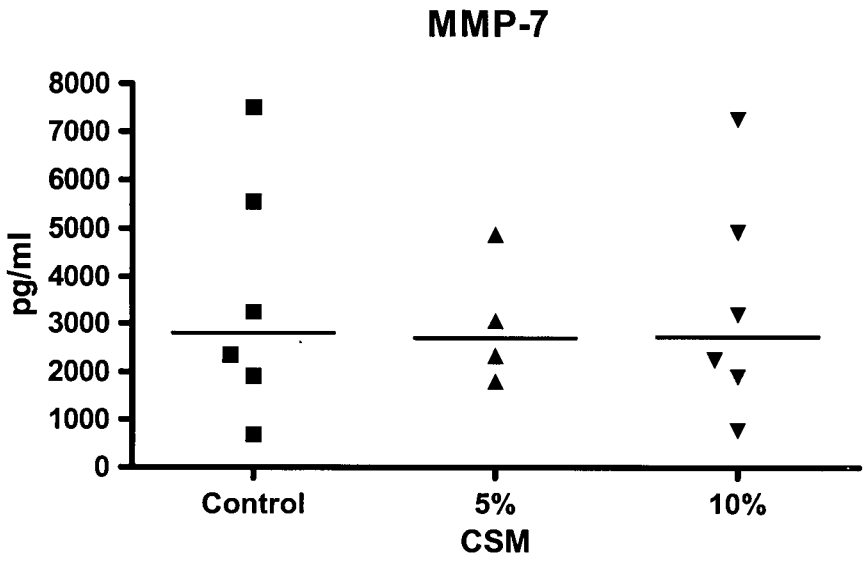


24 hours

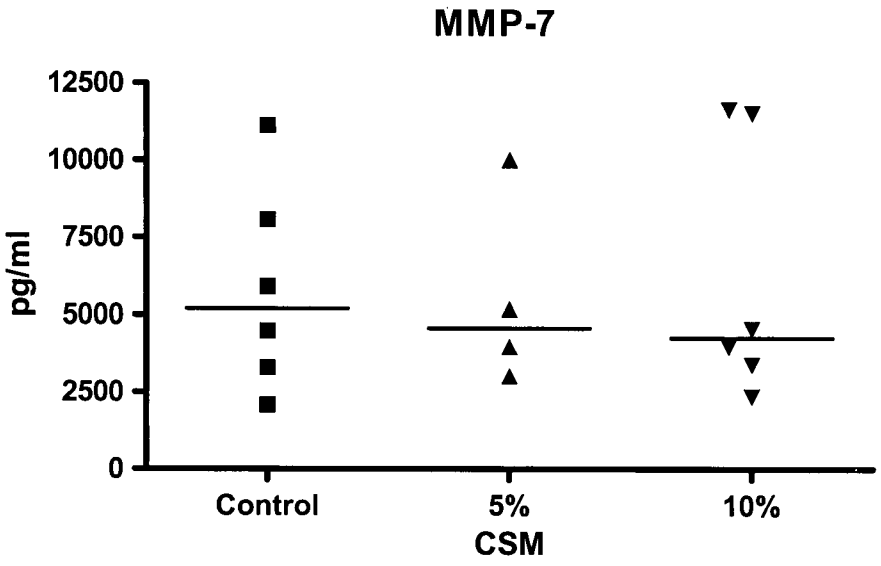


48 hours

B

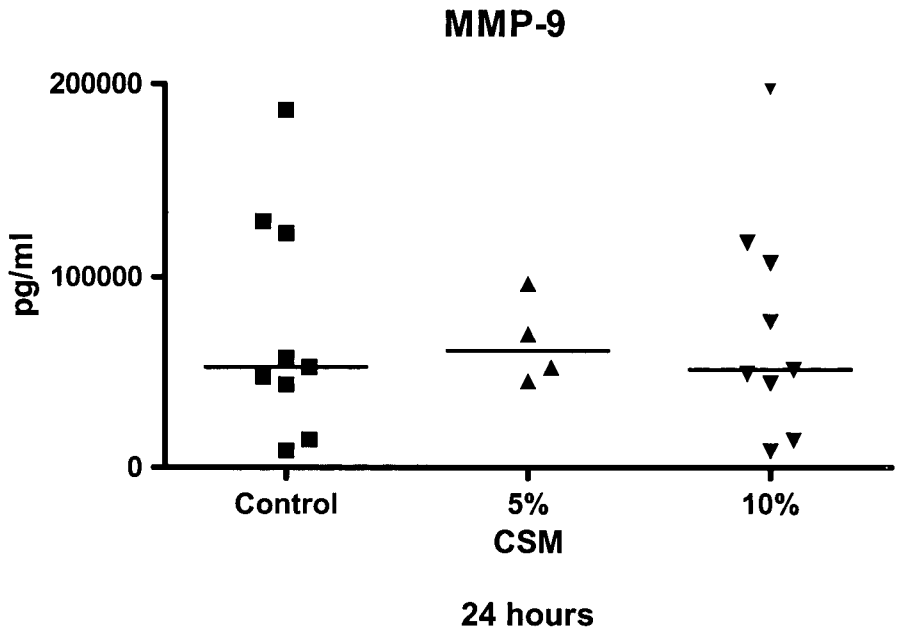


24 hours

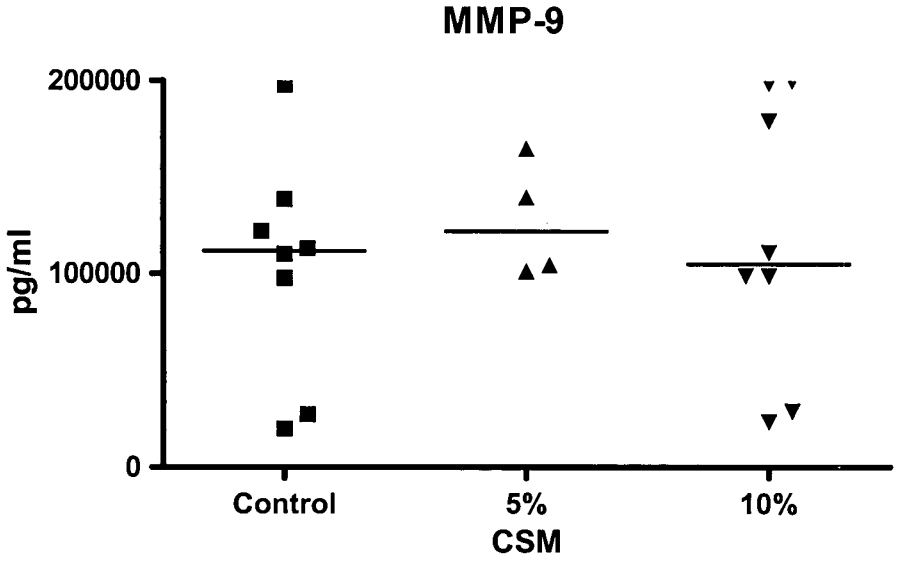


48 hours

C

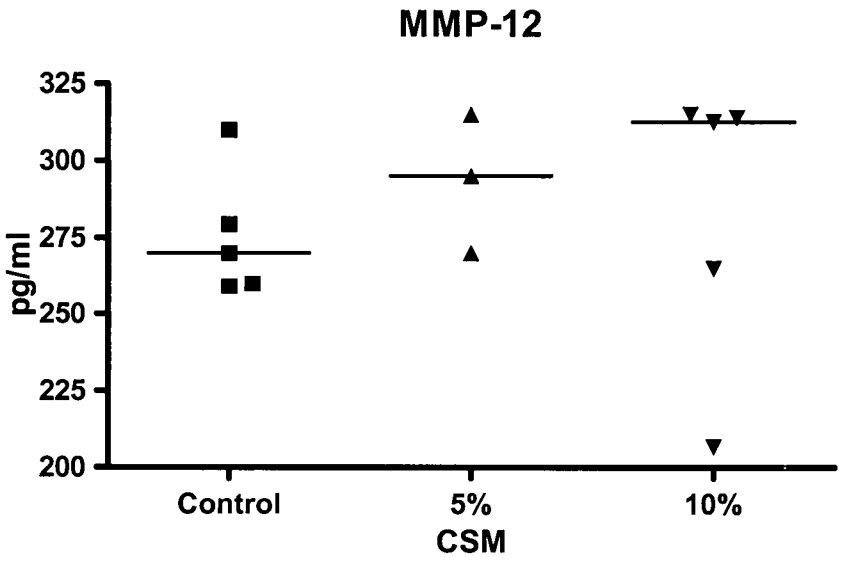


24 hours

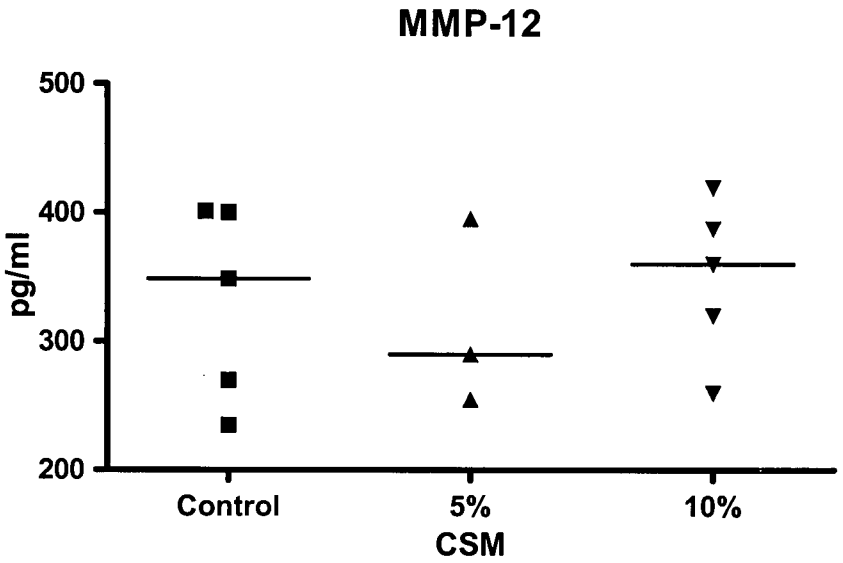


48 hours

D



24 hours



48 hours

Figure 13 - Levels of MMP-1, MMP-7, MMP-9 and MMP-12 in alveolar macrophages culture media at 24 and 48 hours after exposure to CSM.

[A] – MMP-1 release by AM treated with 5 and 10% CSM for 24 and 48 hours. [B] – MMP-7 release by AM treated with 5 and 10% CSM for 24 and 48 hours. [C] – MMP-9 release by AM treated with 5 and 10% CSM for 24 and 48 hours. [D] – MMP-12 release by AM treated with 5 and 10% CSM for 24 and 48 hours. There was no statistically significant increase in any of the MMPs tested at 24 and 48 hours after smoke exposure. The results are shown as individual data points and medians.

XI. DISCUSSION

A. Acute Effect of Cigarette Smoke on TNF-α Release by U937 Differentiated Macrophages Mediated Through the ERK1/2 Pathway.

TNF-α production has been studied in humans by comparing groups of smokers, non-smokers, smokers with COPD and healthy smokers without COPD, using samples from BAL fluid, induced sputum and serum. Kushner and coworkers, report that the concentration of TNF-α in the bronchoalveolar lavage was greater in healthy smokers compared to healthy non-smokers [59]. A study using induced sputum sample, a non-invasive method of studying airway secretion, showed that the concentration of TNF-α is greater in smokers with COPD compared to healthy smokers and non-smokers [60] . Takabatake and coworkers found increased serum concentration of TNF-α in patients with COPD compared to healthy non-smoking subjects [61]. The acute effect of smoke exposure on mice was studied by Churg and coworkers. A single acute smoke exposure from four cigarettes caused an increase in the gene expression of TNF-α from whole lung extract two hours after smoke treatment [62].

There have been several studies on the effect of cigarette smoke on TNF-α production. Wang and coworkers showed that nicotine, a component of cigarettes, induces the release of TNF-α by cultured Ana-1 macrophages; furthermore the TNF-α released induces the expression of adhesion molecules such as ICAM-1, VCAM-1 and E-selectin on endothelial cells which lead to an increase in adherence of monocytes to the endothelial cells [63]. In another study, alveolar macrophages from cigarette smokers

were shown to release greater amounts of TNF-α at baseline and after treatment with LPS and IL-1β, compared to alveolar macrophages from non-smokers [64]. However there are other studies using blood monocytes, cultured alveolar macrophages and BAL fluid which state that cigarette smoke exposure reduces or has no effect on TNF-α production [65-69]. Our results concur with the previous studies showing that cigarette smoke does induce TNF-α release by U937 differentiated macrophages, since we could easily detect increased TNF-α production in response to CSM exposure in both a time and dose-dependent fashion. We further explored the mechanism by which cigarette smoke induces TNF-α production in U937 differentiated macrophages by investigating a number of signaling enzymes.

The MAP kinases (ERK1/2, p38 and JNK) have been reported to mediate production of proteases and cytokines in response to a variety of stimuli. The effect of cigarette smoke or other components of cigarettes have been investigated for their roles in activation of MAPK–ERK1/2 in fibroblasts, smooth muscle cells, pulmonary epithelial cells and bronchoalveolar cells. In normal human bronchial epithelial cells and small airway epithelial cells, treatment with cigarette smoke extract increased phosphorylation of ERK1/2 [70, 71]. In addition a study by Zhang and coworkers showed that EGFR-specific inhibitor AG1478 blocked cigarette smoke induced ERK1/2 phosphorylation in nonmalignant human bronchial epithelial cell line [72] . In vascular smooth muscle cells and fibroblasts, treatment with nicotine also induced the activation of ERK1/2 [73]. However in a different study the ERK1/2 was phosphorylated by LPS in bronchoalveolar

cells (BAC) from smokers and nonsmokers and the kinetics of activation were similar in both groups [74].

In this study we showed that cigarette smoke activates human U937 differentiated macrophages and induces the release of TNF-α, and this release was dependent primarily on the activation of the ERK1/2 signaling pathway. Activation of ERK1/2 in turn induces the activation of the downstream kinase p90RSK and may have some effect on the activation of p70 S6 kinase. We could show that inhibition of MEK1/2, the upstream activators of ERK1/2, completely blocked CSM-induced ERK1/2 phosphorylation, as well as p90RSK phosphorylation. However, there was little or no effect on phosphorylation of S6 ribosomal protein, and thus no effect on p70S6 kinase. Macrophages exposed to CSM showed a consistent decrease in the level of PKB phosphorylation, particularly at early times of exposure. Inhibition of the PI 3-kinase enzymes with LY294002, which inhibits activation of PKB, had no effect on the release of TNF-α in response to CSM. Also EGFR-specific inhibitor AG1478 did not effect CSM-induced TNF-α release by macrophages.

In conclusion, U937 differentiated macrophages exposed to cigarette smoke products are activated and release TNF-α. Our results also show that the ERK1/2 pathway, which is activated by exposure to CSM, is necessary for the CSM-induced release of TNF-α in U937 differentiated macrophages.

B. Acute Effect of CSM on TNF-α Release by Human Alveolar and Broncho-alveolar Macrophages.

Alveolar macrophages are the major contributors in inflammation and destruction of the alveolar walls in the lung, thus they might be better models than broncho-alveolar macrophages with which to study the acute effect of cigarette smoke on TNF-α release in the lung. However, both cells may respond in a similar manner in terms of TNF-α release upon exposure to cigarette smoke. Several laboratories have shown that cigarette smoke decreases or has no effect on TNF- α release. McCrea and coworkers demonstrated that BAL fluid macrophages from smokers release less TNF-α during incubation with LPS compared to BAL fluid from nonsmokers [65]. Acute effects of cigarette smoke gas phase on AM include decreased TNF-α activity at 18-20 hours after smoke exposure, compared to air-exposed AM [66]. Another study using HBMC (human peripheral blood mononuclear cells) showed that treatment with cigarette smoke extracts suppressed the production of TNF-α in the cell culture supernatants [68]. In contrast, Morimoto and coworkers showed that TNF-α production by AM derived from rat lungs exposed to smoke were essentially the same as for the unexposed [69]. However, this issue is far from resolved, as other studies suggest that cigarette smoke can also increase TNF-α production by alveolar macrophages [63, 64].

We tested the acute effect of 5 and 10% CSM exposures for 24 and 48 hours on TNF-α release by primary human alveolar macrophages from resected smokers lungs with localized lung cancer; however, we were unable to reach a conclusion on the effect because most of the samples had undetectable amounts of TNF-α as assayed by ELISA.

Two samples out of eight had detectable levels of TNF-α, but there was great variability between the two samples. One sample showed a decrease while the other showed no change in TNF- α release after smoke exposure. The observed difference in the response of AM to CSM may be due to variability in individual patient responses, or perhaps due to effect of the cancer in the lungs. Unfortunately, we were unable to further investigate and confirm the acute effect of CSM on TNF- α release by AM because we had no further access to newly resected lungs. We were also unable to concentrate any of the samples with undetectable TNF-α because the original aliquots had been already used to quantify protease release in cell culture supernatant.

Interestingly, at that period of time, we had the opportunity to obtain human broncho-alveolar macrophages from volunteers who were smokers or exsmokers and decided to examine the acute effect of CSM on these cells. Due to the fact that most of the AM samples we previously tested had undetectable amounts of TNF-α, we decided to use higher concentrations of CSM with the BMs (10 and 20%), in the hope that it would effect an increase in release of TNF-α without affecting cellular viability. Surprisingly, the levels of TNF-α released by BMs were detectable and were much higher at baseline and after treatment with CSM, as compared to the levels detected in AM. However, even though exposure to CSM resulted in suppression or no effect on TNF-α release in most of the BM samples individually, the collective results were not found to be statistically significant.

In conclusion, our findings illustrate the caveat that primary cells and immortalized cell lines may respond very differently to the same stimuli. In our hands, exposure to CSM induced TNF-α release by differentiated U937 cell line whereas there seemed to be no effect or suppression of TNF- α release by primary human alveolar and broncho-alveolar macrophages. Even though we were unable to reach a conclusion on the acute effect of cigarette smoke on TNF-α release by AM, we proceeded to investigate the effect of CSM on the activation of signaling proteins in AM.

C. Acute Effects of CSM on the ERK1/2 Pathway in Human Alveolar Macrophages.

Using western blot analysis we examined the effect of CSM on the activities of ERK1/2, PKB, p90RSK and ribosomal S6 protein in AM. Our results showed an almost identical activation response of these signaling proteins to CSM in AM as with the differentiated U937 cells.

We show that cigarette smoke activates ERK1/2 in AM. In addition, the activity of p90RSK is increased after 10 minutes of treatment with CSM; this may be due to the activation of ERK1/2 which in turn induces the activation of the downstream kinase p90RSK. There is a slight increase in the phosphorylation of ribosomal S6 protein, the downstream target of p70 S6 kinase. We also show a consistent decrease in the activity of PKB at earlier times of exposure with CSM and cigarette smoke gas phase (appendix 1). Thus our results suggest that primary AM and the U937 differentiated macrophages respond acutely to CSM in a similar manner in regards to the activation of the signaling

proteins tested. Both cell types may also utilize different downstream signaling pathways to trigger TNF-α release; we showed that the U937 differentiated macrophages exhibit ERK1/2-dependent TNF-α release, but have not tested ERK1/2 dependency on TNF-α release in AM, due to the fact that CSM suppressed or had no effect on TNF-α release even thought it increased the phosphorylation of ERK1/2.

D. Acute Effects of CSM on Proteases Release by Human Alveolar Macrophages.

The ECM is composed of collagen, elastin, fibronectin and laminin, which can be degraded by proteolytic enzymes such as MMPs and cathepsins. Macrophage-derived proteases have the ability to degrade the ECM; excessive degradation eventually results in the development of emphysema. Earlier studies have focused on MMP-1, MMP-2, MMP-9 and MMP-12 secreted by macrophages, because of their critical roles in the degradation of the ECM.

The contribution of interstitial collagenase (MMP-1) in the pathogenesis of emphysema was first reported by D'Armiento et al using transgenic mice which expressed the human collagenase transgene in their lungs and subsequently developed morphological changes similar to human emphysema [75]. In addition, Imai et al detected the expression of MMP-1 mRNA, protein, and enzymatic activity in the lung parenchyma of patients with emphysema but not in the lungs of healthy control subjects [40]. Ohnishi et al [41] used an immunohistochemical analysis to show that the immunoreactivity of MMP-2 was more intense in emphysematous patients compared to those in the control group. This immunoreactivity was mainly observed in pneumocytes, fibroblasts and

alveolar macrophages. The level of MMP-2 protein was also elevated by more than 3 fold in the emphysematous samples compared to controls [41]. Segura-valdez et al report upregulation in the expressions of MMP-1, -2, -8 and -9 in the lung tissue of COPD patients compared to control subjects [76]. In a study by Molet et al, the expression of MMP-12 was greater in the BAL cells and bronchial biopsies of COPD patients compared to healthy control subjects. The level of secreted MMP-12 in BAL fluids was also greater in the COPD subjects [77].

Alveolar macrophages represent a significant source of matrix- degrading enzymes in the airways and lungs of patients with COPD. Finlay et al [78] reported elevation in mRNA expressions of MMP-1 and MMP-9 *in vitro* 24 hours following isolation of macrophages from BAL fluid of patients with and without emphysema. MMP-9 complexes were also found to be secreted in the conditioned medium of macrophages from emphysematous subjects but not in control macrophages [78]. Lim et al demonstrated that cultured airway macrophages from smokers release greater amounts of MMP-9 at baseline and in response to treatment with LPS and IL-1β, in comparison with non-smokers [64]. Russell et al measured the activity of macrophage-derived MMP-9 in smokers with COPD, healthy smokers and non smokers. They found that LPS, IL-1β and CSM-treated AM secreted greater amounts of MMP-9 with higher activity, in a dose-dependent manner, in smokers with COPD, as compared to healthy smokers and non-smokers. [79]. Another study showed a correlation between concentration of MMP-9 in lung parenchyma of smokers and their cigarette smoking history [80].

There have also been studies supporting the role of cathepsins in development of pulmonary emphysema. IL-13, a critical cytokine in asthma and a potent stimulator of the MMP and cathepsin proteolytic pathway in the lung, contributed to the development of emphysema in mice via a cathepsin–dependent mechanism [81]. This study which used transgenic mice to overexpress IL-13, demonstrated that cytokine-regulated activation of macrophages resulted in inflammation and release of MMPs and cathepsins B, K, L, and S, eventually leading to the development of emphysema; in addition, treatment with cysteine protease antagonists significantly reduced inflammation and the subsequent development of emphysema [81]. In another study, Wang et al. demonstrated that overexpression of IFN-γ in transgenic mice led to emphysema and increased expressions of cathepsins B, D, H, L and S as well as MMP-9 and MMP-12 [82]. Takeyabu et al. studied the role of cathepsin L in COPD by demonstrating increase in the levels of cathepsin L in the BAL fluid of patients with emphysema compared to healthy controls [83]. Takahashi et al. showed that cigarette smoking induces mRNA expression and activity of cathepsin L by alveolar macrophages from smokers [84]. In addition, alveolar macrophages of rats exposed to 10 puffs of cigarette smoke, twice a day, 7days a week, for 22 weeks showed a greater activity of cathepsin L compared to sham control rats [85].

We were also interested in investigating the effect of CSM on the production of Cathepsin L and MMP-1, -7, -9 and -12 by AMs, and to understand the mechanism by which these proteases are secreted. Therefore, AM were treated with 5 and 10 % of CSM for 24 and 48 hours and analyzed for MMPs and Cathepsin L release in the cell supernatants using ELISA.

CSM induced a slight but statistically significant release of Cathepsin L in the cell supernatant of AM, concurring with the results of previous studies. However, we observed no significant increase in MMP-1, 7, 9 and 12 releases by AM after treatment with CSM. This is not surprising because there are many conflicting reports regarding MMP-9 and MMP-12 expression. A study by Imai et al. reports that in the lung parenchyma of patients with smoke induced emphysema, one of 23 samples from was found to express MMP-12 [40]. Whereas Xu et al. report that cells from smoke-exposed rats demonstrated a significant increase in the expression of MMP-12 mRNA compared to the unexposed controls [86]. Imai et al. also analyzed the presence of MMP-9 in lung samples from patients with cigarette-induced emphysema, and found that both the smoker and control groups expressed MMP-9 in equal amounts [40]. In contrast, another study using rats exposed to cigarette smoke showed no expression of MMP-9 mRNA in either control or cigarette smoke-exposed groups [86]. Other studies report an increase in MMP-9 levels in lung tissue and BAL fluid from emphysematous patients [41, 43].

One possible explanation for us not observing an increase in the release of MMPs by AM after treatment with CSM, is that since all the AM were collected from resected lungs of smokers, there is the possibility that these cells might have already become resistant to the effect of cigarette smoke over time and would not have shown a strong response to CSM under our relatively short exposure times. In addition, the cells were collected from lungs which had small peripheral tumors, which could have affected the cells' normal responses to various stimuli. In future experiments, it might be interesting

to examine the effects of using higher concentrations of CSM or subjecting the cells to longer chronic exposures.

From our results, we conclude that in vitro exposure of macrophages to CSM induces a slight release in Cathepsin L but has no effect on the release of MMPs-1,-7,-9, and -12 by AM. While it might be important to investigate these results in greater detail in future studies, our current data does not support further investigations of the molecular mechanisms involved in the production of these proteases.

XII. REFERENCES

1. Pauwels, R.A., A.S. Buist, P.M. Calverley, C.R. Jenkins, and S.S. Hurd, *Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) Workshop summary.* Am J Respir Crit Care Med, 2001. **163**(5): p. 1256-76.

2. Barnes, P.J., S.D. Shapiro, and R.A. Pauwels, *Chronic obstructive pulmonary disease: molecular and cellular mechanisms.* Eur Respir J, 2003. **22**(4): p. 672-88.

3. Rennard, S.I., *COPD: overview of definitions, epidemiology, and factors influencing its development.* Chest, 1998. **113**(4 Suppl): p. 235S-241S.

4. Saetta, M., A. Di Stefano, G. Turato, F.M. Facchini, L. Corbino, C.E. Mapp, P. Maestrelli, A. Ciaccia, and L.M. Fabbri, *CD8+ T-lymphocytes in peripheral airways of smokers with chronic obstructive pulmonary disease.* Am J Respir Crit Care Med, 1998. **157**(3 Pt 1): p. 822-6.

5. Mannino, D.M., *Chronic obstructive pulmonary disease: definition and epidemiology.* Respir Care, 2003. **48**(12): p. 1185-91; discussion 1191-3.

6. Mead, J., J.M. Turner, P.T. Macklem, and J.B. Little, *Significance of the relationship between lung recoil and maximum expiratory flow.* J Appl Physiol, 1967. **22**(1): p. 95-108.

7. Kim, W.D., D.H. Eidelman, J.L. Izquierdo, H. Ghezzo, M.P. Saetta, and M.G. Cosio, *Centrilobular and panlobular emphysema in smokers. Two distinct*

morphologic and functional entities. Am Rev Respir Dis, 1991. **144**(6): p. 1385-90.

8. Senior, R.M. and N.R. Anthonisen, *Chronic obstructive pulmonary disease (COPD).* Am J Respir Crit Care Med, 1998. **157**(4 Pt 2): p. S139-47.

9. Shapiro, S.D., *Evolving concepts in the pathogenesis of chronic obstructive pulmonary disease.* Clin Chest Med, 2000. **21**(4): p. 621-32.

10. Shapiro, S.D., *Proteinases in chronic obstructive pulmonary disease.* Biochem Soc Trans, 2002. **30**(2): p. 98-102.

11. Shapiro, S.D., *The macrophage in chronic obstructive pulmonary disease.* Am J Respir Crit Care Med, 1999. **160**(5 Pt 2): p. S29-32.

12. Retamales, I., W.M. Elliott, B. Meshi, H.O. Coxson, P.D. Pare, F.C. Sciurba, R.M. Rogers, S. Hayashi, and J.C. Hogg, *Amplification of inflammation in emphysema and its association with latent adenoviral infection.* Am J Respir Crit Care Med, 2001. **164**(3): p. 469-73.

13. Abboud, R.T., A.F. Ofulue, R.H. Sansores, and N.L. Muller, *Relationship of alveolar macrophage plasminogen activator and elastase activities to lung function and CT evidence of emphysema.* Chest, 1998. **113**(5): p. 1257-63.

14. Finkelstein, R., R.S. Fraser, H. Ghezzo, and M.G. Cosio, *Alveolar inflammation and its relation to emphysema in smokers.* Am J Respir Crit Care Med, 1995. **152**(5 Pt 1): p. 1666-72.

15. Di Stefano, A., A. Capelli, M. Lusuardi, P. Balbo, C. Vecchio, P. Maestrelli, C.E. Mapp, L.M. Fabbri, C.F. Donner, and M. Saetta, *Severity of airflow limitation is*

associated with severity of airway inflammation in smokers. Am J Respir Crit Care Med, 1998. **158**(4): p. 1277-85.

16. Traves, S.L., S.V. Culpitt, R.E. Russell, P.J. Barnes, and L.E. Donnelly, *Increased levels of the chemokines GROalpha and MCP-1 in sputum samples from patients with COPD.* Thorax, 2002. **57**(7): p. 590-5.

17. Capelli, A., A. Di Stefano, I. Gnemmi, P. Balbo, C.G. Cerutti, B. Balbi, M. Lusuardi, and C.F. Donner, *Increased MCP-1 and MIP-1beta in bronchoalveolar lavage fluid of chronic bronchitics.* Eur Respir J, 1999. **14**(1): p. 160-5.

18. Panes, J., M.A. Perry, D.C. Anderson, A. Manning, B. Leone, G. Cepinskas, C.L. Rosenbloom, M. Miyasaka, P.R. Kvietys, and D.N. Granger, *Regional differences in constitutive and induced ICAM-1 expression in vivo.* Am J Physiol, 1995. **269**(6 Pt 2): p. H1955-64.

19. Kalra, V.K., Y. Ying, K. Deemer, R. Natarajan, J.L. Nadler, and T.D. Coates, *Mechanism of cigarette smoke condensate induced adhesion of human monocytes to cultured endothelial cells.* J Cell Physiol, 1994. **160**(1): p. 154-62.

20. Shen, Y., V. Rattan, C. Sultana, and V.K. Kalra, *Cigarette smoke condensate-induced adhesion molecule expression and transendothelial migration of monocytes.* Am J Physiol, 1996. **270**(5 Pt 2): p. H1624-33.

21. Marques, L.J., H. Teschler, J. Guzman, and U. Costabel, *Smoker's lung transplanted to a nonsmoker. Long-term detection of smoker's macrophages.* Am J Respir Crit Care Med, 1997. **156**(5): p. 1700-2.

22. Tomita, K., G. Caramori, S. Lim, K. Ito, T. Hanazawa, T. Oates, I. Chiselita, E. Jazrawi, K.F. Chung, P.J. Barnes, and I.M. Adcock, *Increased p21(CIP1/WAF1)*

and B cell lymphoma leukemia-x(L) expression and reduced apoptosis in alveolar macrophages from smokers. Am J Respir Crit Care Med, 2002. **166**(5): p. 724-31.

23. Saetta, M., M. Mariani, P. Panina-Bordignon, G. Turato, C. Buonsanti, S. Baraldo, C.M. Bellettato, A. Papi, L. Corbetta, R. Zuin, F. Sinigaglia, and L.M. Fabbri, *Increased expression of the chemokine receptor CXCR3 and its ligand CXCL10 in peripheral airways of smokers with chronic obstructive pulmonary disease.* Am J Respir Crit Care Med, 2002. **165**(10): p. 1404-9.

24. Takeyama, K., B. Jung, J.J. Shim, P.R. Burgel, T. Dao-Pick, I.F. Ueki, U. Protin, P. Kroschel, and J.A. Nadel, *Activation of epidermal growth factor receptors is responsible for mucin synthesis induced by cigarette smoke.* Am J Physiol Lung Cell Mol Physiol, 2001. **280**(1): p. L165-72.

25. Chung, K.F., *Cytokines in chronic obstructive pulmonary disease.* Eur Respir J Suppl, 2001. **34**: p. 50s-59s.

26. Kriegler, M., C. Perez, K. DeFay, I. Albert, and S.D. Lu, *A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: ramifications for the complex physiology of TNF.* Cell, 1988. **53**(1): p. 45-53.

27. Darnay, B.G. and B.B. Aggarwal, *Early events in TNF signaling: a story of associations and dissociations.* J Leukoc Biol, 1997. **61**(5): p. 559-66.

28. Luster, M.I., P.P. Simeonova, R. Gallucci, and J. Matheson, *Tumor necrosis factor alpha and toxicology.* Crit Rev Toxicol, 1999. **29**(5): p. 491-511.

29. Cromwell, O., Q. Hamid, C.J. Corrigan, J. Barkans, Q. Meng, P.D. Collins, and A.B. Kay, *Expression and generation of interleukin-8, IL-6 and granulocyte-macrophage colony-stimulating factor by bronchial epithelial cells and*

enhancement by IL-1 beta and tumour necrosis factor-alpha. Immunology, 1992. **77**(3): p. 330-7.

30. Standiford, T.J., S.L. Kunkel, S.H. Phan, B.J. Rollins, and R.M. Strieter, *Alveolar macrophage-derived cytokines induce monocyte chemoattractant protein-1 expression from human pulmonary type II-like epithelial cells.* J Biol Chem, 1991. **266**(15): p. 9912-8.

31. Pober, J.S., M.A. Gimbrone, Jr., L.A. Lapierre, D.L. Mendrick, W. Fiers, R. Rothlein, and T.A. Springer, *Overlapping patterns of activation of human endothelial cells by interleukin 1, tumor necrosis factor, and immune interferon.* J Immunol, 1986. **137**(6): p. 1893-6.

32. Osborn, L., C. Hession, R. Tizard, C. Vassallo, S. Luhowskyj, G. Chi-Rosso, and R. Lobb, *Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes.* Cell, 1989. **59**(6): p. 1203-11.

33. Dubravec, D.B., D.R. Spriggs, J.A. Mannick, and M.L. Rodrick, *Circulating human peripheral blood granulocytes synthesize and secrete tumor necrosis factor alpha.* Proc Natl Acad Sci U S A, 1990. **87**(17): p. 6758-61.

34. Warner, S.J. and P. Libby, *Human vascular smooth muscle cells. Target for and source of tumor necrosis factor.* J Immunol, 1989. **142**(1): p. 100-9.

35. Sakao, S., K. Tatsumi, H. Igari, Y. Shino, H. Shirasawa, and T. Kuriyama, *Association of tumor necrosis factor alpha gene promoter polymorphism with the presence of chronic obstructive pulmonary disease.* Am J Respir Crit Care Med, 2001. **163**(2): p. 420-2.

36. Higham, M.A., N.B. Pride, A. Alikhan, and N.W. Morrell, *Tumour necrosis factor-alpha gene promoter polymorphism in chronic obstructive pulmonary disease.* Eur Respir J, 2000. **15**(2): p. 281-4.

37. Senior, R.M., H. Tegner, C. Kuhn, K. Ohlsson, B.C. Starcher, and J.A. Pierce, *The induction of pulmonary emphysema with human leukocyte elastase.* Am Rev Respir Dis, 1977. **116**(3): p. 469-75.

38. Hautamaki, R.D., D.K. Kobayashi, R.M. Senior, and S.D. Shapiro, *Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice.* Science, 1997. **277**(5334): p. 2002-4.

39. Ohbayashi, H., *Matrix metalloproteinases in lung diseases.* Curr Protein Pept Sci, 2002. **3**(4): p. 409-21.

40. Imai, K., S.S. Dalal, E.S. Chen, R. Downey, L.L. Schulman, M. Ginsburg, and J. D'Armiento, *Human collagenase (matrix metalloproteinase-1) expression in the lungs of patients with emphysema.* Am J Respir Crit Care Med, 2001. **163**(3 Pt 1): p. 786-91.

41. Ohnishi, K., M. Takagi, Y. Kurokawa, S. Satomi, and Y.T. Konttinen, *Matrix metalloproteinase-mediated extracellular matrix protein degradation in human pulmonary emphysema.* Lab Invest, 1998. **78**(9): p. 1077-87.

42. Betsuyaku, T., M. Nishimura, K. Takeyabu, M. Tanino, P. Venge, S. Xu, and Y. Kawakami, *Neutrophil granule proteins in bronchoalveolar lavage fluid from subjects with subclinical emphysema.* Am J Respir Crit Care Med, 1999. **159**(6): p. 1985-91.

43. Finlay, G.A., K.J. Russell, K.J. McMahon, M. D'Arcy E, J.B. Masterson, M.X. FitzGerald, and C.M. O'Connor, *Elevated levels of matrix metalloproteinases in bronchoalveolar lavage fluid of emphysematous patients.* Thorax, 1997. **52**(6): p. 502-6.

44. Shapiro, S.D., *Elastolytic metalloproteinases produced by human mononuclear phagocytes. Potential roles in destructive lung disease.* Am J Respir Crit Care Med, 1994. **150**(6 Pt 2): p. S160-4.

45. Berdowska, I., *Cysteine proteases as disease markers.* Clin Chim Acta, 2004. **342**(1-2): p. 41-69.

46. Buhling, F., N. Waldburg, A. Reisenauer, A. Heimburg, H. Golpon, and T. Welte, *Lysosomal cysteine proteases in the lung: role in protein processing and immunoregulation.* Eur Respir J, 2004. **23**(4): p. 620-8.

47. Reddy, V.Y., Q.Y. Zhang, and S.J. Weiss, *Pericellular mobilization of the tissue-destructive cysteine proteinases, cathepsins B, L, and S, by human monocyte-derived macrophages.* Proc Natl Acad Sci U S A, 1995. **92**(9): p. 3849-53.

48. Punturieri, A., S. Filippov, E. Allen, I. Caras, R. Murray, V. Reddy, and S.J. Weiss, *Regulation of elastinolytic cysteine proteinase activity in normal and cathepsin K-deficient human macrophages.* J Exp Med, 2000. **192**(6): p. 789-99.

49. Brazil, D.P. and B.A. Hemmings, *Ten years of protein kinase B signalling: a hard Akt to follow.* Trends Biochem Sci, 2001. **26**(11): p. 657-64.

50. Wymann, M.P., M. Zvelebil, and M. Laffargue, *Phosphoinositide 3-kinase signalling--which way to target?* Trends Pharmacol Sci, 2003. **24**(7): p. 366-76.

51. Simpson, L. and R. Parsons, *PTEN: life as a tumor suppressor.* Exp Cell Res, 2001. **264**(1): p. 29-41.

52. Bellacosa, A., T.O. Chan, N.N. Ahmed, K. Datta, S. Malstrom, D. Stokoe, F. McCormick, J. Feng, and P. Tsichlis, *Akt activation by growth factors is a multiple-step process: the role of the PH domain.* Oncogene, 1998. **17**(3): p. 313-25.

53. Datta, S.R., H. Dudek, X. Tao, S. Masters, H. Fu, Y. Gotoh, and M.E. Greenberg, *Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery.* Cell, 1997. **91**(2): p. 231-41.

54. Cardone, M.H., N. Roy, H.R. Stennicke, G.S. Salvesen, T.F. Franke, E. Stanbridge, S. Frisch, and J.C. Reed, *Regulation of cell death protease caspase-9 by phosphorylation.* Science, 1998. **282**(5392): p. 1318-21.

55. Burgering, B.M. and R.H. Medema, *Decisions on life and death: FOXO Forkhead transcription factors are in command when PKB/Akt is off duty.* J Leukoc Biol, 2003. **73**(6): p. 689-701.

56. Camps, M., A. Nichols, and S. Arkinstall, *Dual specificity phosphatases: a gene family for control of MAP kinase function.* Faseb J, 2000. **14**(1): p. 6-16.

57. Cobb, M.H. and E.J. Goldsmith, *How MAP kinases are regulated.* J Biol Chem, 1995. **270**(25): p. 14843-6.

58. Lee, J.C. and P.R. Young, *Role of CSB/p38/RK stress response kinase in LPS and cytokine signaling mechanisms.* J Leukoc Biol, 1996. **59**(2): p. 152-7.

59. Kuschner, W.G., A. D'Alessandro, H. Wong, and P.D. Blanc, *Dose-dependent cigarette smoking-related inflammatory responses in healthy adults.* Eur Respir J, 1996. **9**(10): p. 1989-94.

60. Keatings, V.M., P.D. Collins, D.M. Scott, and P.J. Barnes, *Differences in interleukin-8 and tumor necrosis factor-alpha in induced sputum from patients with chronic obstructive pulmonary disease or asthma.* Am J Respir Crit Care Med, 1996. **153**(2): p. 530-4.

61. Takabatake, N., H. Nakamura, S. Inoue, K. Terashita, H. Yuki, S. Kato, S. Yasumura, and H. Tomoike, *Circulating levels of soluble Fas ligand and soluble Fas in patients with chronic obstructive pulmonary disease.* Respir Med, 2000. **94**(12): p. 1215-20.

62. Churg, A., J. Dai, H. Tai, C. Xie, and J.L. Wright, *Tumor necrosis factor-alpha is central to acute cigarette smoke-induced inflammation and connective tissue breakdown.* Am J Respir Crit Care Med, 2002. **166**(6): p. 849-54.

63. Wang, Y., L. Wang, X. Ai, J. Zhao, X. Hao, Y. Lu, and Z. Qiao, *Nicotine could augment adhesion molecule expression in human endothelial cells through macrophages secreting TNF-alpha, IL-1beta.* Int Immunopharmacol, 2004. **4**(13): p. 1675-86.

64. Lim, S., N. Roche, B.G. Oliver, W. Mattos, P.J. Barnes, and K.F. Chung, *Balance of matrix metalloprotease-9 and tissue inhibitor of metalloprotease-1 from alveolar macrophages in cigarette smokers. Regulation by interleukin-10.* Am J Respir Crit Care Med, 2000. **162**(4 Pt 1): p. 1355-60.

65. McCrea, K.A., J.E. Ensor, K. Nall, E.R. Bleecker, and J.D. Hasday, *Altered cytokine regulation in the lungs of cigarette smokers.* Am J Respir Crit Care Med, 1994. **150**(3): p. 696-703.

66. Dubar, V., P. Gosset, C. Aerts, C. Voisin, B. Wallaert, and A.B. Tonnel, *In vitro acute effects of tobacco smoke on tumor necrosis factor alpha and interleukin-6 production by alveolar macrophages.* Exp Lung Res, 1993. **19**(3): p. 345-59.

67. Kotani, N., H. Hashimoto, D.I. Sessler, Y. Yatsu, M. Muraoka, and A. Matsuki, *Exposure to cigarette smoke impairs alveolar macrophage functions during halothane and isoflurane anesthesia in rats.* Anesthesiology, 1999. **91**(6): p. 1823-33.

68. Ouyang, Y., N. Virasch, P. Hao, M.T. Aubrey, N. Mukerjee, B.E. Bierer, and B.M. Freed, *Suppression of human IL-1beta, IL-2, IFN-gamma, and TNF-alpha production by cigarette smoke extracts.* J Allergy Clin Immunol, 2000. **106**(2): p. 280-7.

69. Morimoto, Y., M. Kido, I. Tanaka, A. Fujino, T. Higashi, and Y. Yokosaki, *Synergistic effects of mineral fibres and cigarette smoke on the production of tumour necrosis factor by alveolar macrophages of rats.* Br J Ind Med, 1993. **50**(10): p. 955-60.

70. Mercer, B.A., N. Kolesnikova, J. Sonett, and J. D'Armiento, *Extracellular regulated kinase/mitogen activated protein kinase is up-regulated in pulmonary emphysema and mediates matrix metalloproteinase-1 induction by cigarette smoke.* J Biol Chem, 2004. **279**(17): p. 17690-6.

71. Hellermann, G.R., S.B. Nagy, X. Kong, R.F. Lockey, and S.S. Mohapatra, *Mechanism of cigarette smoke condensate-induced acute inflammatory response in human bronchial epithelial cells.* Respir Res, 2002. **3**(1): p. 22.

72. Zhang, Q., P. Adiseshaiah, and S.P. Reddy, *Matrix metalloproteinase/epidermal growth factor receptor/mitogen-activated protein kinase signaling regulate fra-1 induction by cigarette smoke in lung epithelial cells.* Am J Respir Cell Mol Biol, 2005. **32**(1): p. 72-81.

73. Li, J.M., T.X. Cui, T. Shiuchi, H.W. Liu, L.J. Min, M. Okumura, T. Jinno, L. Wu, M. Iwai, and M. Horiuchi, *Nicotine enhances angiotensin II-induced mitogenic response in vascular smooth muscle cells and fibroblasts.* Arterioscler Thromb Vasc Biol, 2004. **24**(1): p. 80-4.

74. Mochida-Nishimura, K., K. Surewicz, J.V. Cross, R. Hejal, D. Templeton, E.A. Rich, and Z. Toossi, *Differential activation of MAP kinase signaling pathways and nuclear factor-kappaB in bronchoalveolar cells of smokers and nonsmokers.* Mol Med, 2001. **7**(3): p. 177-85.

75. D'Armiento, J., S.S. Dalal, Y. Okada, R.A. Berg, and K. Chada, *Collagenase expression in the lungs of transgenic mice causes pulmonary emphysema.* Cell, 1992. **71**(6): p. 955-61.

76. Segura-Valdez, L., A. Pardo, M. Gaxiola, B.D. Uhal, C. Becerril, and M. Selman, *Upregulation of gelatinases A and B, collagenases 1 and 2, and increased parenchymal cell death in COPD.* Chest, 2000. **117**(3): p. 684-94.

77. Molet, S., C. Belleguic, H. Lena, N. Germain, C.P. Bertrand, S.D. Shapiro, J.M. Planquois, P. Delaval, and V. Lagente, *Increase in macrophage elastase (MMP-*

12) in lungs from patients with chronic obstructive pulmonary disease. Inflamm Res, 2005. **54**(1): p. 31-6.

78. Finlay, G.A., L.R. O'Driscoll, K.J. Russell, E.M. D'Arcy, J.B. Masterson, M.X. FitzGerald, and C.M. O'Connor, *Matrix metalloproteinase expression and production by alveolar macrophages in emphysema.* Am J Respir Crit Care Med, 1997. **156**(1): p. 240-7.

79. Russell, R.E., S.V. Culpitt, C. DeMatos, L. Donnelly, M. Smith, J. Wiggins, and P.J. Barnes, *Release and activity of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 by alveolar macrophages from patients with chronic obstructive pulmonary disease.* Am J Respir Cell Mol Biol, 2002. **26**(5): p. 602-9.

80. Kang, M.J., Y.M. Oh, J.C. Lee, D.G. Kim, M.J. Park, M.G. Lee, I.G. Hyun, S.K. Han, Y.S. Shim, and K.S. Jung, *Lung matrix metalloproteinase-9 correlates with cigarette smoking and obstruction of airflow.* J Korean Med Sci, 2003. **18**(6): p. 821-7.

81. Zheng, T., Z. Zhu, Z. Wang, R.J. Homer, B. Ma, R.J. Riese, Jr., H.A. Chapman, Jr., S.D. Shapiro, and J.A. Elias, *Inducible targeting of IL-13 to the adult lung causes matrix metalloproteinase- and cathepsin-dependent emphysema.* J Clin Invest, 2000. **106**(9): p. 1081-93.

82. Wang, Z., T. Zheng, Z. Zhu, R.J. Homer, R.J. Riese, H.A. Chapman, Jr., S.D. Shapiro, and J.A. Elias, *Interferon gamma induction of pulmonary emphysema in the adult murine lung.* J Exp Med, 2000. **192**(11): p. 1587-600.

83. Takeyabu, K., T. Betsuyaku, M. Nishimura, A. Yoshioka, M. Tanino, K. Miyamoto, and Y. Kawakami, *Cysteine proteinases and cystatin C in bronchoalveolar lavage fluid from subjects with subclinical emphysema.* Eur Respir J, 1998. **12**(5): p. 1033-9.

84. Takahashi, H., K. Ishidoh, D. Muno, A. Ohwada, T. Nukiwa, E. Kominami, and S. Kira, *Cathepsin L activity is increased in alveolar macrophages and bronchoalveolar lavage fluid of smokers.* Am Rev Respir Dis, 1993. **147**(6 Pt 1): p. 1562-8.

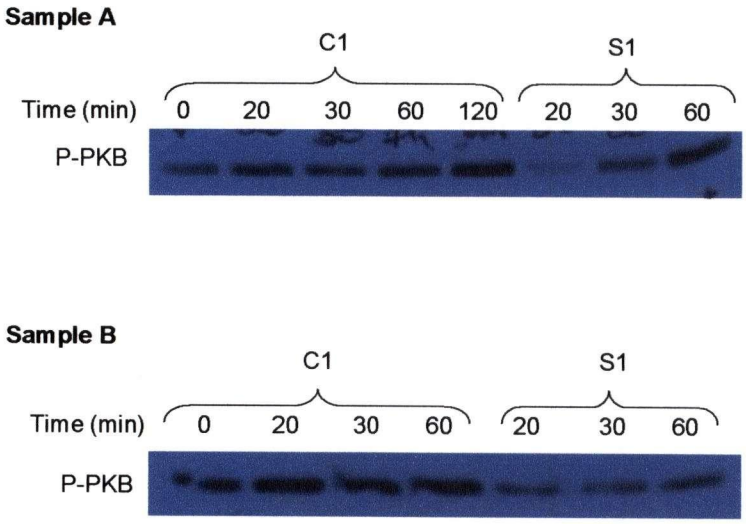
85. Lesser, M., N. Galicki, C. Cardozo, and C.G. Gariola, *Cathepsin L activity in alveolar macrophages of rats: response to cigarette smoke.* Am J Respir Cell Mol Biol, 1989. **1**(5): p. 371-6.

86. Xu, L., B.Q. Cai, and Y.J. Zhu, *Pathogenesis of cigarette smoke-induced chronic obstructive pulmonary disease and therapeutic effects of glucocorticoids and N-acetylcysteine in rats.* Chin Med J (Engl), 2004. **117**(11): p. 1611-9.

XIII. APPENDIX 1

A. Exposure of Alveolar Macrophages to Cigarette Smoke gas phase.

AM in uncovered culture plates were put into a humidified closed plastic box into which 5% CO_2 in air flowed at 1.5 L/min, and incubated at 37°C. The plastic box was used to expose the culture plates to the gaseous phase of tobacco smoke. Cigarette smoke was generated from one medium tar commercial filter cigarette. Smoke from each cigarette was generated by withdrawing a puff of 30 ml with a 60 ml syringe over 2 sec, and then emptying it over 30 sec into the tubing through which the 5% CO_2 in air flows into the exposure chamber. Each cigarette yielded about 8 puffs over a period of about 5 minutes. AM exposed to air in a similar manner served as controls. After the exposure, the whole cell lysate was collected for western blotting.



- C1: control air exposed, S1: exposure to gaseous phase of tobacco from one cigarette.