

Effect of cigarette smoke extract and electronic cigarette vapour extract on the production of cytokines and chemokines in human airway smooth muscle cells

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Abstract

Introduction: Chronic obstructive pulmonary disease (COPD) is characterised by an inflammatory response in the airways to inhaled substances like cigarette smoke. It is known that cigarette smoke extract (CSE) stimulates the production of a number of inflammatory cytokines and chemokines in human airway smooth muscle cells (HASMCs). E-cigarettes are used to aid smoking cessation, but they may also have pro-inflammatory effects. The effect of e-cigarette vapour extract (ECVE) on the production of cytokines and chemokines in HASMCs is unknown. The transcription factor NF-κB regulates immune functions and serves as a mediator of inflammatory responses. It is widely implicated in cigarette smoke-induced airway inflammation. We aim to compare the effect of CSE and ECVE on the production of inflammatory cytokines and chemokines in HASMCS, and investigate whether NF-κB is involved in mediating CSE- and ECVE-induce cytokines and chemokines.

Methods: CSE (from 2 cigarettes) and ECVE with and without nicotine (N+/N- respectively) (from 6ml e-liquid, 0.24mg/ml nicotine) were prepared by bubbling cigarette smoke or e-cigarette vapour into 20ml of cell culture medium. Confluent and growth-arrested HASMCs were treated with CSE and ECVE for 24 hours and cell viability was analysed

by MTT assay. Enzyme-linked immunosorbent assays (ELISA) were used to measure the secretion of cytokines and chemokines in to the cell culture medium. In addition, NF-κB was inhibited by using BAY11-7082 to evaluate its involvement in the production of cytokines and chemokines.

Results: 5% CSE and 10% ECVE (N+/N-) reduced HASMC cell viability, whereas CSE (1,2, and 3.5%) and ECVE (1, 2.5 and 5%) did not affect cell viability. CSE (1, 2 and 3.5%) concentration-dependently induced the production of IL-5, IL-6, IL-8, IL-13, vascular endothelial growth factor (VEGF) and CCL2, but not CXCL-10 and RANTES. ECVE (N+/N-) (1, 2.5 and 5%) only induced the production of IL-13, but not the others. BAY11-7082 (a NF-κB inhibitor) partially inhibited the production of CSE-induced IL-6 and IL-8 at the highest concentration tested (100μM), while causing complete inhibition of VEGF production at all investigated concentrations (0.1-100μM). But it had no effect on CSE-induced IL-5, MCP-1 and IL-13 and ECVE-induced IL-13.

Conclusion: Our results suggest that CSE has a wider pro-inflammatory effect in the airway than ECVE. However, the ability of ECVE without nicotine (N-) to induce cytotoxicity and IL-13 production in HASMCs in a similar manner to ECVE with nicotine (N+) suggests that e-cigarette

vapor in itself is harmful to the airway and induces pro-inflammatory response independently of the presence of nicotine. We have shown that NF-κB is involved in mediating CSE-induced IL-6, IL-8 and VEGF production but is not involved in CSE- and ECVE-induced IL-13 from HASMCs. This suggests that the signalling pathways involved in CSE- and ECVE-induced pro-inflammatory responses in HASMCs differ from one another.

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Abbreviations:

A

ASH action on smoking and health

B

BALF Bronchoalveolar lavage fluid

BAY11-7082 an inhibitor of NF-κB

BCA Bicinchoninic Acid protein Assay

BECs bronchial epithelial cells

BEAS-2B bronchial epithelial cells

BSA Bovine serum Albumin

C

CS Cigarette smoke

CSE Cigarette smoke Extract

CSC cigarette smoke condensate

COPD Chronic obstructive pulmonary disease

D

DMEM Dulbecco's Modified Eagle's Medium

DMSO dimethyl sulfoxide

E

ECVE Electronic cigarette smoke extract

ELISA Enzyme-linked immunosorbent assay

ECVC Electronic Cigarette Vapour Condensate

ENDS Electronic Nicotine Delivery Systems

F

FBS Foetal Bovine Serum

FEV_1 Forced expiratory volume-1

FVC Forced volume capacity

FDA Food and Drug Administration

G

GOLD chronic Obstructive Lung Disease

H

HASMC Human airway smooth muscle cells

H292 human bronchial epithelial cells

M

MCP-1 monocyte chemoattractant protein-1

N

NO Nitric Oxide

OD Optical density

P

PBS Phosphate-buffered saline

PG propylene glycol

PIC Protease Inhibitor Cocktail

PMSF Phenylmethylsulphonyl Fluoride

R

ROS reactive oxygen species

RANTES: Regulated upon Activation, Normal T cell Expressed, and Secreted

RIPA Radioimmunoprecipitation assay

S

SDS Sodium Dodecyl Sulphate

N

NF-κB nuclear factor kappa B

N+ with nicotine

N- without nicotine

NHBE normal human bronchial cells

T

TAD transcription activation domains

TBS Tris-buffered saline

TEDTA Trypsin–Ethylenediamine Tetraacetic Acid

Tris-HCl Trizma hydrochloride

TSLP thymic stromal lymphopoietin

TNF-α Tumour necrosis factor alpha

TH1 T helper 1

TH2 T helper 2

V

VEGF vascular endothelial growth factor

VG vegetable glycerin

U

UK United Kingdome

US United States

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Chapter 1. Introduction

1.1 Chronic Obstructive Pulmonary Disease (CPOD)

1.1.1 COPD Definition

Chronic obstructive pulmonary disease (COPD) is a ‘’preventable and treatable disease that is characterised by persistent respiratory symptoms and airflow limitation that is due to airway and/or alveolar abnormalities usually caused by significant exposure to noxious particles or gases’’ [1].

It is a severe chronic lung disease that is common in later life, mainly caused by cigarette smoking, and can be recognised by symptoms such as a chronic cough, shortness of breath (dyspnoea), frequent respiratory infections, cyanosis, fatigue, production of large amounts of phlegm, sputum and wheezing [2, 3].

As the disease progresses, there is a reduction in the lung compliance (elasticity) resulting in increased effort to exhale the air and a reduction of the expiratory air volume (FEV1). Global initiative for COPD (GOLD) have classified this disease into four stages based on the severity of the airflow limitation as predicted by spirometry. Stage 1 mild FEV1 ≥ 80% predicted, stage 2 moderate FEV1 ≤ 80% predicted, stage

3 severe ≤ 30% FEV1 < 50% predicted, and stage 4 very severe FEV1< 30% predicted' [1].

1.1.2 COPD Prevalence, Morbidity, Mortality and Impact

COPD represents a global public health challenge with nearly 251 million cases being reported worldwide in 2016 [4]. In the United Kingdom (UK) it is the 2nd most common lung disease affecting nearly 2% of the population as per the Department of Health UK report released in 2011 [5, 6] . It has been estimated that by 2030, the number of COPD cases diagnosed would rise from 952,000 to approximately 1.3 million in England and from 108,000 to 128,000 in Scotland [7] .

COPD has a significant impact on morbidity and economy worldwide [8]. With a higher number of people being diagnosed with COPD, the healthcare cost associated with the management, hospitalisations and maintenance is constantly increasing. The additional cost associated with the treatment of moderate to severe exacerbation attacks also imposes a financial burden on the countries.

According to a study on COPD burden, there were 3.17 million COPD-related deaths worldwide in 2015, representing 5% of all deaths globally [4]. Furthermore, COPD mortality is increasing in the UK and

ranks third in Europe [6]. The estimated rate of deaths associated with COPD in England in 2011 was 99,200 and is projected to rise to 129,400 by 2030 [8]. In Scotland, the estimated rate of death associated with COPD in 2011 was 9,700 and is expected to grow to 13,900 by 2030 [8].

The assessment of the monetary impact of COPD is increasing worldwide. The rising costs are primarily due to the increasing number of people who are diagnosed with COPD. In the United States alone, it costs $72 billion per year [9]. It was estimated that cost of COPD in England is projected to increase from £1.50 billion in 2011 to about £2.32 billion by 2030 [8] and from £159 million in 2011 to £207 million in Scotland by 2030 [8].

1.1.3 Pathogenesis of COPD

Chronic inflammation has been shown to play a central role in the pathogenesis of COPD. COPD causes airflow limitation and results in loss of lung elastic recoil which limits the flow to airways, obstruction of the lumen that leads to increase resistance in the airway and increase secretions [9]. The clinical manifestations of COPD include chronic bronchitis, which is a condition of large airways inflammation, and emphysema, a disease of distal airways and parenchyma that results in

destruction of the airway wall and reduces the maximal expiratory airflow by reducing the elastic recoil force [10].

Furthermore, chronic bronchitis involves the rate of coughing and production of sputum. Chronic bronchitis is characterized by a decline in lung function, development of airway obstruction and frequency of exacerbation, wheezing, cough and dyspnoea [11]. The cough with chronic bronchitis is due to hypersecretion of mucus which is resulted of mucus gland enlargement [12]. Also, airway structural changes with chronic bronchitis involve atrophy, focal squamous metaplasia, abnormalities of epithelial cilia, bronchial wall thickening goblet cell metaplasia and smooth muscle hyperplasia [12]. This chronic condition is brought by the decline in the FEV1 [10].

In the case of emphysema, the airway wall can be destroyed or constricted which causes the distal airspaces to grow in size in a disproportional and potentially damaging way [13]. Oxidative stress and reactive oxygen species (ROS) play a role in lung inflammation and increase the alveolar destruction involved in the inflammatory process in the lungs [14, 15].

1.2 Airway Inflammation in COPD

1.2.1 Inflammatory cells

COPD is an inflammatory disease involving inflammatory cells such as neutrophils, macrophages and lymphocytes. Various studies have identified neutrophil infiltration as one of the most common findings in the pulmonary tissue of COPD patients. A cohort study demonstrated an increased localization of neutrophils within the bronchial glands and epithelium of COPD patients as opposed to the bronchial samples collected from non-COPD patients [16]. Extent of neutrophil infiltration has been associated with the severity of pulmonary dysfunction in COPD patients. Hallmark features of COPD (airway obstruction and chronic expectoration) as well as increased decline in the FEV1 levels have been found to be directly proportional to the number of neutrophils within the sputum of COPD patients. This study showed that neutrophilic inflammation of the lung plays a critical role in the pathogenesis of COPD [17, 18].

Eosinophil infiltration in the small airways has been identified in the lung biopsy specimens of COPD patients [19]. Furthermore, the number of eosinophils found in the sputum and bronchial biopsies of COPD patients during exacerbations has been shown to increase dramatically

[20]. Eosinophil infiltration has also been shown to be associated with reversible airflow obstruction [21].

Macrophages are also associated with the release of pro-inflammatory cytokines, reactive oxygen species, and extracellular matrix remodelling [22]. Increased numbers of alveolar macrophages have been demonstrated in the lamina propria of the lobar bronchial biopsies of COPD patients [23].

The adaptive immunity is also known to play a significant role in the pathogenesis of COPD. Increased cytotoxic CD8+ T lymphocytes numbers are present in bronchial biopsies from COPD patients compared with healthy controls and CD8+ T lymphocytes numbers correlate with a decline in pulmonary function [24, 25].

1.2.2 Inflammatory Cytokines and Chemokines

COPD inflammation is characterised by an increased number of alveolar macrophages, neutrophils, T-lymphocytes (as discussed in section 1.2) and the release of numerous inflammatory mediators such as cytokines and chemokines [2]. Cytokines and chemokines are small (approximately 5-20kDa) cell-signalling protein molecules that are utilised in intracellular communication, offering signalling pathways that

coordinate the complex immune cell responses of the human body [26]. Also, T cells were increased in the airway with COPD patients [27]. However, the T cells classify to two types based on their cytokine production.

T helper 1 (Th1) is responsible for expression of interferon-γ (IFN-γ), interleukin-2 (IL-2) and lymphotoxin-α [26, 27]. T helper 2 (Th2) is responsible of the expression of IL-4, IL-5, and IL-13 which are associated with an increased number of eosinophils [27]. IL-6 is a Th2 pro-inflammatory cytokines produced by HASMCs, endothelial cells, epithelial cells, and fibroblasts [28]. The level of IL-6 is elevated in sputum, serum and exhaled breath of patients with COPD [29, 30]. IL-13 is a Th2 cytokine that is implicated in the pathogenesis of COPD, as evidenced by the increased of IL-13 levels in peripheral blood [31, 32].

Furthermore, IL-8 is a chemokine that belongs to the CXC family that attracts and activates neutrophils in inflammatory sites. Also, Human airway smooth muscles (HASM) can secrete the production of IL-8 that participate in airway inflammatory response [33]. IL-8 is presented in excess amount of sputum with COPD patients [34, 35].

Vascular endothelial growth factor (VEGF) is produced by various cell types, including macrophages, mast cells and epithelial cells. It

stimulates the development of blood vessels and has a role in airway remodelling in asthma cases [36, 37]. Moreover, the level of VEGF is increased not only in the sputum of smokers with COPD but also in asymptomatic smokers [38].

Monocyte chemoattractant protein (MCP-1) is a chemokine that has a major role in the recruitment of inflammatory cells in the lungs of COPD patients [39]. It is a prototypic inflammatory chemokine that targets monocytes and T lymphocytes [40]. MCP-1 can be produced by many cells types such as endothelial, fibroblasts, epithelial, smooth muscle, astrocytic monocytic and microglial cells [41]. In addition to that, MCP-1 is produced by the bronchial epithelial cells which has a role in recruitment of the inflammatory cells and pathogenesis of allergic asthma [42]. MCP-1 has a major role in allergic asthma, airway remodelling and bronchial hyperreactivity [42]. Also, MCP-1 regulates the migration and the infiltration of monocytes and macrophages [39, 43]. The MCP-1 level is considerably increased among patients with asthma and COPD [39, 42, 43].

Furthermore, regulated upon activation, normal T cell expressed, and secreted (RANTES) is a chemokine that is activated by T cells. RANTES found to be elevated in the sputum sample and bronchoalveolar

lavage among patients diagnosed with COPD [44]. CXCL-10 is another chemokine increased during airway inflammation. It is secreted by the keratinocytes, endothelial cells, neutrophils and eosinophils which are all increased in the BAL of COPD patients [45, 46].

1.3. NF-κB and the Expression of Inflammatory Cytokines and Chemokines

The nuclear factor kappa B (NF-κB) is a transcription factor that regulates immune functions and works as a mediator of inflammatory responses [47]. NF-κB plays an important role in the induction of pro-inflammatory gene expression, which leads to the synthesis of cytokine/chemokine, growth factors and enzymes [48]. The NF-κB family consists of five closely related transcription factors: p100, p105, p65 (also RelA), c-Rel, and RelB [49]. NF-κB heterodimer can bind to a range of downstream DNA targets called κB sites in order to modulate gene expression. P65, RelB and c-Rel contain transcription activation domains (TAD) in their C-terminal, through which they can recruit co-activators and target gene expression [49]. As p105 and p100 lack (TAD), their proteolytically cleaved forms p50 and p52, respectively, need to form heterodimers by binding with p65, RelB or c-Rel in order to activate transcription [49].

NF-κB activates and regulates the production of IL-8 that participates in airway inflammatory response and is presented in excess amount of sputum with COPD patients [33, 34, 50]. IL-5 and IL-13 are major Th2 cytokines that are capable of inducing inflammation, excessive mucus production alveolar remodelling and airway hype-responsiveness and are mediated and regulated by NF-κB [29, 33, 51].

The stimulation of T-cells through NF-κB regulation release chemokine RANTES [52, 53]. MCP-1 is regulated by NF-κB and is involved in migration and infiltration of the macrophages and T-lymphocytes, contributing to the inflammation of lungs in COPD [39, 43, 54]. CXCL-10 is a chemokine that secreted by endothelial cells, keratinocytes, eosinophils and neutrophils, which activated and expressed by NF-κB [55]. In addition to that, VEGF is an angiogenic factor that expressed in vascularized organs, airway microvasculature lungs, epithelial cells, and smooth muscles cells [56] which mediated and regulated by NF-κB [57, 58] (Figure 1.1).

BAY11-7082 is considered as a potent NF-κB inhibitor through its inhibitory effects on I-kappa B kinase (IKK) which is a complex signal integration for NF-κB activation [59]. IKK mediates the phosphorylation and degradation of IκB, the NF-κB inhibitor, which then enables the NF-κB dimer to enter the nucleus and activate gene expression [59]. It has been reported that cigarette smoke induced the pro-inflammatory IL-6 in bronchial epithelial cells and this effect was inhibited by the drug BAY11-7082 [60].

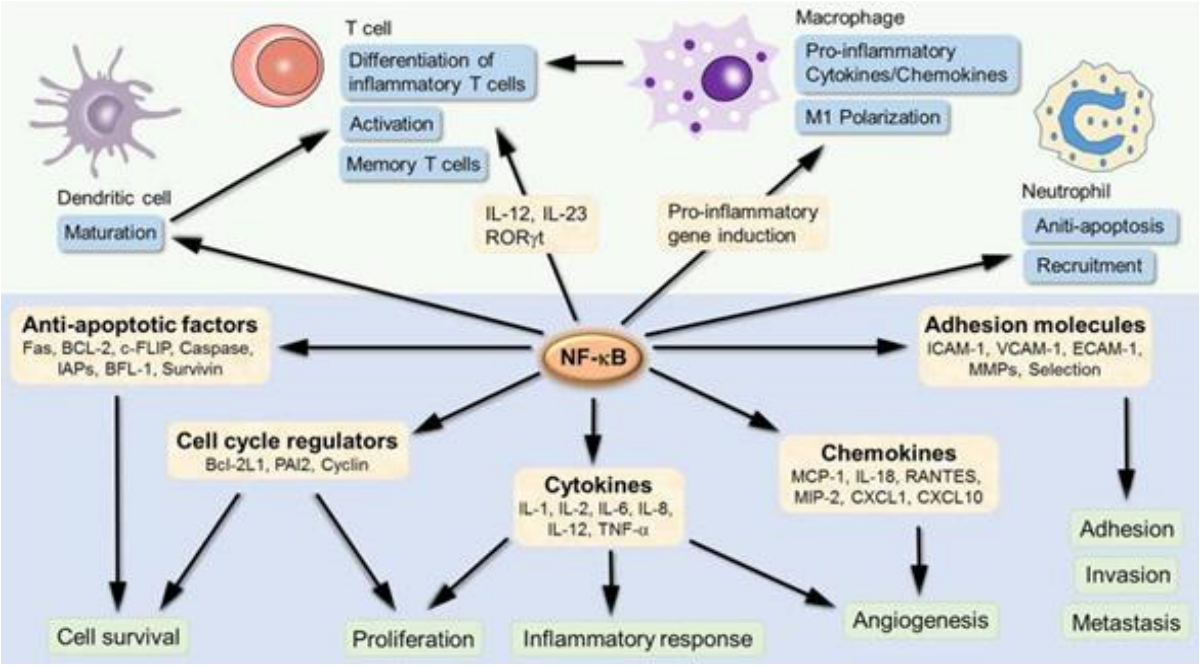


Figure 1.1 NF-κB and the Expression of Inflammatory Cytokines and Chemokines.

NF-κB plays an important role in activating the production of inflammatory cytokines and chemokines. NF-κB is an inducible transcription factor that is involved in progression and development of inflammation. NF-κB activates the production of inflammatory cytokines, chemokines adhesion molecules and regulate cell proliferation. Adopted from Liu, T., Lingyun Zhang, and Donghyun Joo., *NF-κB signaling in inflammation.* Signal Transduct Target Ther, 2017. **2**: **p**. 17023.

1.4. Role of Human Airway Smooth Muscle in Airway Inflammation

Airway smooth muscle is developed from the mesenchymal cells located around the tip of growing epithelial buds [61]. It plays an important role in the airway structure and function and also in the pathogenesis of COPD and contributes to alterations of contractility, inflammatory response, immunoreaction, phenotype and size of the airway [62].

Increased contraction of HASM has been positively associated with the development of COPD [62]. HASM, when activated by external TNF-α, can lead to increased cell-adhesiveness and attraction of immune cells within the airways, facilitating the inflammatory process [62]. Also, HASM can result in activation of the epithelial cells and release of inflammatory mediators causing secondary systemic inflammation [62]. According to a transcriptomics analytic study, HASM is associated with facilitating the production of cytokines like IL-8, IL-6, IL-5, VEGF and chemokines like RANTES, CXCL10. During the airway inflammation the cytokines and chemokines that produced by airway smooth muscle cells cases proliferation and lead to structural changes in the airway [63].

1.5. COPD Association with Cigarette Smoke

COPD is rapidly increasing and becoming a worldwide public health crisis [64]. According to the World Health Organization (WHO), cigarette smoke is the primary cause of COPD either by active cigarette smoke or second-hand smoke [65]. In the United States (US) during 2014 and 2015, more than 15.9 million have been diagnosed with COPD with 15.1% being reported as smokers [66]. A study reported that lounger smoking duration has an impact on COPD prevalence. In the study, 4135 adult smokers age ≥45 with 1-9 years, 10-19 years, and 20-29 years of smoking, respectively, they found increase years of smoking is associated with the development of COPD prevalence [67]. A research report has shown that environmental tobacco smoke exposure also contributes to increase risk of COPD [68]. Long-term exposure to second-hand smoke during childhood, such as living with a smoker for 16–18 years, is associated with COPD related mortality [69]. Similarly, adults who are exposed to second-hand smoke for more than 10 hours per week have a higher risk of developing COPD [69].

1.6. Cigarette Smoke and Airway Inflammation

Cigarette smoke (CS) is associated with chronic airway inflammation, which may eventually lead to COPD development. CS is composed of a mixture of chemicals such as acrolein, nicotine, carbon monoxide, ROS, acetaldehyde, ammonia and other dangerous chemicals that are considered to be harmful to the lungs [70]. These chemicals in CS might contribute directly or indirectly to the addiction of cigarette smoke [70]. In addition to that, cigarette smoke is linked to overproduction of the mucus with COPD patients [71]. The mucus in the airway is generally cleared by the cilia and becomes damaged with chronic inflammation which leads to the accumulation of the excessive volume of secretions [71].

An *in vitro* study on the effect of cigarette smoke on airway inflammation showed that T cells, macrophages, neutrophils and mast cells are elevated with patients whether they are current smokers or ex-smokers [72]. Cigarette smoke extract (CSE) induced the inflammatory gene in different experimental designs. In a study on human airway smooth muscles cells (HASMCs), cigarette smoke extract induced IL-8 release [73]. Furthermore, smokers with COPD have a significantly increased amount of IL-6 and IL-8 levels [74]. Additionally, a study

showed that CS can induced the release of IL-8 from the human bronchial epithelial cells [75].

Cigarette smoke increased chemotaxis of neutrophils in response to IL-8 in smokers with and without COPD. This study provides evidence that CS is associated with systemic activation of neutrophils irrespective of the degree of COPD pathology [76]. Another study showed the micro-localization of inflammatory cells neutrophils in the airway smooth muscle cells of COPD patients who were smokers. Some infiltration of neutrophils was also seen in HASMCs of smokers having normal pulmonary functioning. The study concluded that neutrophil infiltration played a significant role in the pathogenesis of airflow limitation in COPD [77].

An *in vivo* study on mouse macrophage cells showed that cigarette smoke condensate (CSC) increased the production of IL-5, IL-6, VEGF, IP-10 and MCP-1, but not IL-13 [78]. A mouse study was conducted to demonstrate the importance of CD8+ T-lymphocytes in the recruitment of inflammatory cells and lung destruction associated with cigarette smoke induced emphysema. This study showed an increase in the inflammatory cells seen in the wild-type mice exposed to CS, whereas there was no increase in macrophages, eosinophils or other immune cells in the CD8+ deficient mice exposed to CS [79].

Smoking cessation is the ultimate solution to slow down disease progression in COPD. Clinically, lung function in smokers showed significant improvement within 1 year of smoking cessation. It was observed that the FEV_1 improved and there was a reduction in the expectorant coughing in people after a year of smoking cessation [80]. Collectively, these studies indicate that CS plays a key role in airway inflammation by inducing inflammatory gene expression from different cells.

1.7. Role of NF-κB in Cigarette Smoke-Induced Inflammation

As discussed in Section 1.3, NF-κB is a transcription factor that regulates immune functions and serves as a mediator of inflammatory responses [47]. A study was done on human lymphocytes that isolated from peripheral blood showed that CS induced NF-κB in lymphocytes through the increase in oxidative stress and reduction in the intracellular glutathione levels [81]. Thus, it can be concluded that CS is associated with NF-κB activation within human lymphocytes through activation of IKK. Various studies in the past have demonstrated that NF-κB activation was directly proportional to the number of pack-years smoked [82].

A study showed that CS directly induced NF-κB along with the release of various cytokines like IL-8, IL-1β and IL-6 in human bronchial epithelial cells. In addition, an anti-inflammatory p120-catenin which known to regulate E-cadherin-mediated cell-cell adhesion that act as anti-inflammatory. The p120-catenin was used to investigate the activation of NF-κB signalling. The anti-inflammatory p120 inhibited CSE-induced the release of IL-8, IL-1β and IL-6 in human bronchial epithelial cells. However, by Knockdown p120 enhanced CSE-induced NF-κB activation and increased upregulation of IL-8, IL-1β and IL-6 after CSE [83].

1.8. Rising use of E-cigarettes

E-cigarettes are battery-powered devices that work by heating e-liquid and vaporising it by inhalation [84]. The e-liquid used in the e-cigarettes are a mixture of propylene glycol, glycerin, nicotine and flavours. Currently the e-cigarettes are classified as disposable or reusable. The disposable devises are not rechargeable and not refillable, whereas the reusable one is rechargeable and can be refillable with e-liquid. The users of e-cigarettes can control and adjust the voltage supplied to heating coil, temperature, and air flow rate of puffing [85]. E-cigarettes are used currently as alternatives to smoking or aids for smoking cessation [86]. However, their safety has been controversial,

and various deleterious effects of e-cigarettes have been identified. Currently the use of e-cigarettes is wildly popular among the consumers. According to Action on Smoking and Health (ASH) in Great Britain the number of e-cigarette vaper users was 700,000 in 2012 and is projected to 3.2 million by 2018 [87].

1.9. E-cigarette vapour and Airway inflammation

The e-liquid with and without nicotine has a flavouring substance which adds to the reason for the e-cigarettes being popular. E-liquid contains propylene glycol (PG) and vegetable glycerin (VG) and have been deemed by the Food and Drug Administration (FDA) as being toxic for lungs similar to tobacco cigarettes [88].

The exposure to e-cigarette vapour that contains propylene glycol may cause irritation to the upper airway with non-smoker [88]. When heated, the particles of PG and VG can be converted to acrolein, formaldehyde and acetaldehyde, which are known as strong irritants and stimulate inflammation in the lung [89]. A study showed that electronic vapour condensate ECVC with nicotine induced the production of IL-6, TNF-α, CXCL-8, MCP-1 and MMP-9. Also, ECVC without nicotine

induced the production of IL-6, CXCL-8 and MMP-9 but not TNF-α and MCP-1 [90].

Another study was focused on providing evidence for the effect of e-cigarette vapour exposure on the healthy human airway epithelial cells of non-smokers. It was concluded that e-cigarette vapour without nicotine induced the release of IL-6 [88]. Another study on mice showed that the exposure of e-cigarette fluid with nicotine to mice for 4 months had a significant increase in the levels and expressions of MCP-1, CXCL-10, IL-6 and CXCL-2 found in the Bronchoalveolar lavage fluid (BALF) [91]. On the other hand, the flavours used in the e-cigarettes and other vaping devices can be harmful to the lungs. The cinnamon flavour of electronic cigarette refill fluids showed the most cytotoxic effect compared to other 36 flavours tested on human embryonic stem cells and human adult pulmonary fibroblasts [92]. However, the effect of electronic cigarettes on HASMCs remains unknow. Collectively, electronic cigarettes are harmful to the lungs by producing the inflammatory mediators from different cells as mentioned in these studies.

1.10. Summary

COPD is a chronic disease that is characterised by airflow limitation and causes destruction of the alveolar wall. It is a severe chronic lung disease that is common in later life, mainly caused by cigarette smoke. Cigarette smoke can induce the production of cytokines and chemokines that contribute to airway inflammation. It is known that CSE stimulates the production of a number of inflammatory cytokines and chemokines in HASMCs. E-cigarette is commonly used as smoking cessation method, however evidence related to the safety use of the e-cigarette is limited. NF-κB plays an important role in mediating the production of cytokines and chemokines in COPD. However, the effect of ECVE and CSE induce some inflammatory cytokines and chemokines production in HASMCs remains unknown as well as the role of NF-κB in mediating ECVE- and CSE-induced cytokines and chemokines.

1.11 Hypothesis and Aims

1.11.1 Hypothesis

We hypothesis that ECVE like CSE could induce the production of inflammatory cytokines and chemokines in HASMCs and whether NF-κB is involved in mediating ECVE- and CSE-induced cytokines and chemokines.

1.11.2 Aims

- To investigate the effect of CSE/ECVE on the production of cytokines and chemokines that have been implicated in airway inflammation of COPD, including IL-5, IL-6, IL-8, IP-10, IL-13, MCP-1, RANTES, and VEGF.

- To explore the role of NF-κB in mediating CSE- and ECVE-induced cytokine and chemokine production by using NF-κB specific inhibitor (BAY11-7082).

Chapter 2

Materials and Methods

2.1 Tables of Materials

2.1.1 Drug

BAY11-7082 Mass:10mg Molecular weight:207.25 To make original stock of 0.5M, dissolve in 96µL Dimethyl sulfoxide (DMSO) IC_{50} 10µM	Sigma Aldrich

2.1.2 ELISA Kits

Human IL-5 ELISA DuoSet kit	DY205-5, R&D Systems
Human IL-6 ELISA DuoSet kit	DY206-5, R&D Systems
Human CXCL-8 (IL-8) ELISA DuoSet kit	DY208, R&D Systems
Human IL-13 ELISA DuoSet kit	DY213-5, R&D Systems
Human CXCL-10 (IP-10) ELISA DuoSet kit	DY266, R&D Systems
Human RANTES ELISA DuoSet kit	DY278-5, R&D Systems
Human CCL2/MCP-1 ELISA DuoSet kit	DY279-5, R&D Systems
Human VEGF ELISA DuoSet kit	DY293B-05, R&D Systems

2.1.3 ELISA Buffers and Reagents

Phosphate-buffered saline (PBS) tablets	Thermo Fisher Scientific
Tween 20	Sigma Aldrich
Tris-buffered saline (TBS) 20 mM Trizma hydrochloride (Tris-HCI) 150 mM NaCl	Sigma-Aldrich
Bovine serum albumin (BSA)	Thermo Fisher Scientific.
Wash buffer 0.05% (1ml) Tween in 10 tablets PBS (PH7.2-7.4)	
Block buffer, serves as reagent diluent-1 for (IL-5,IL-6,L-8,IP-10, IL-13,MCP-1,VEGF and RANTES) 1% BSA in PBS Filtered by 0.22-µm pore syringe filter (pH7.2–7.4)	
Substrate solution 1:1 mixture of Colour Reagent A (H2O2) and B (Tetramethylbenzidine)	Thermo Fisher Scientific
Radioimmunoprecipitation assay (RIPA) buffer 50mM Tris-HCL, pH8.0 0.5% Sodium Deoxycholate 150mM Sodium Chloride 0.1% Sodium Dodecyl Sulphate (SDS) After RIPA buffer preparation, the following materials were added before use: 2mM Phenylmethylsulphonyl Fluoride (PMSF) 1mM Sodium Orthovanadate 1nM Protease Inhibitor Cocktail (PIC)	

2.1.4 Materials for Cells Culture and MTT Cell Viability Assay

Dulbecco's Modified Eagle's Medium (DMEM)	Sigma Aldrich
Amphotericin-B 250mg/ml in deionised water, sterile-filtered, BioReagent suitable for cell culture	Sigma Aldrich
L-glutamine solution, 200mM solution, sterile-filtered, Bioxtra suitable for cell culture	Sigma Aldrich
Penicillin/streptomycin 5000 units of penicillin and 5mg of streptomycin/ml, sterile-filtered, BioReagent suitable for cell culture	Sigma Aldrich
Foetal bovine serum (FBS)	Thermo Fisher Scientific
Trypsin–Ethylenediamine Tetraacetic Acid (EDTA) solution, 0.25%, sterile-filtered, BioReagent suitable for cell culture, 2.5g porcine trypsin and 0.2g EDTA 4Na per litre of Hanks' Balanced Salt Solution with phenol red	Sigma Aldrich
Dimethyl sulfoxide (DMSO)	Sigma Aldrich
Thiazolyl Blue Tetrazolium Bromide	Sigma Aldrich
DMEM+ supplemented with 10%(50ml) FBS 10ml penicillin/streptomycin 10ml L-glutamine 5ml amphotericin-B	
DMEM-supplemented with 0.5%(2.5ml) FBS 10ml penicillin/streptomycin 10ml L-glutamine 5ml amphotericin-B	

2.1.5 CSE and ECVE Material

3R4F research-grade cigarettes one cigarette contains 9.4mg of tar 0.7mg of nicotine 12mg of carbon monoxide Cigarette pump	Kentucky Tobacco Research and Development Centre, University of Kentucky, USA DA7C Charles Austen Pumps
E-cigarette flavourless vegetable Glycerine (VG) propylene glycol (PG) 50:50 with and without nicotine (N+/N-) 0.24mg/ml amount of e-liquid nicotine wattage range: 6–220 W coil type (V8 Baby-Q2 from China) E-cigarette pump	Smok Alien Vape Device from USA Vapor Vapes solution from USA DA7C Charles Austen Pumps

2.2 CSE Preparation

CSE was prepared from 3R4F research-grade cigarettes (Kentucky Tobacco Research and Development Centre, University of Kentucky, USA; refer to section 2.1.5 for content and concentration). In a cell culture hood, 20ml of DMEM- (refer to section 2.1.4 for contents) was transferred to an 80ml glass bottle. Then, the bottle was attached to a vacuum pump (DA7C Charles Austen Pumps) in a fume hood. Next, two cigarettes were attached to the pump one by one, with 0.2 bar of pressure, and bubbled through 20 ml of DMEM-. The 20ml of CSE was filtered through a 0.22μm filter. A FLUO star Omega microplate reader (BMG LABTECH, UK) at

320nm was used to measure the absorbance (optical density), which equates to CSE strength (1.5=100%).

2.3 ECVE Preparation

E-cigarette liquid (e-liquid) flavourless mixture of propylene glycol and vegetable glycerine (50:50%) with and without nicotine (N+/N-), was used (refer to section 2.1.5 for device, wattage and coil information). The amount of nicotine in 6ml of e-liquid (0.24mg/ml) was equivalent to the amount of nicotine from two cigarettes. In a cell culture hood, 20ml of DMEM− was transferred to an 80ml glass bottle. Then, the 80ml glass bottle was attached to the vacuum pump in a fume hood. 6ml of e-liquid was vaporised and bubbled through 20ml of DMEM−. Each “puff” lasted approximately 3–5 seconds at 0.2 bar of pressure in the fume hood and the temperature of the e-cigarette device during the extraction was not allowed to exceed 37°C. The e-cigarette extracted medium (ECVE) was filtered through a 0.22μm filter. The vaporized medium could not be quantified using optical density, because there was no difference in the OD value between non-extracted (control) and the extracted medium. The stock solution of the extracted medium ECVE(N+/N-) was therefore considered as 100%.

2.4 Drug Preparation

The drug BAY11-7082 is an inhibitor of NF-κB which has pharmacological activities that includes anti-inflammatory effect. The drug was used to investigate whether the induction of inflammatory cytokines and chemokines in response to CSE and ECVE was mediated by NF-κB signaling. The drug was prepared according to the manufacturer's instructions. BAY11-7082 was dissolved in DMSO, and five concentrations of the drug (0.01, 0.1, 1, 10 and 100μM) were used based on the IC_{50} as described in (Section 2.1.1). 3μl of the drug and drug vehicle solution (DMSO) was added to 1.5ml of medium (Dilution factor 1:500) in control wells to achieve 0.2% final concentration of DMSO.

2.5. Cell Culture

Human airway smooth muscle cells (HASMCs) were kindly provided by Professor Dominick Shaw (Division of Respiratory Medicine, University of Nottingham). The cells were isolated from bronchial biopsy tissue obtained from a non-asthmatic donor and cultured in DMEM+ (refer to section 2.1.4 for DMEM+ details). The cells were maintained in a humidified incubator at 37°C with 21% O_2 and 5% CO_2. Culture medium was changed every 48 hours during cell growth, until the cells were

confluent. A mycoplasma test (MycoAlert mycoplasma detection kit from Lonza) was routinely performed to confirm that cell cultures were not contaminated with mycoplasma.

2.5.1 Cell Subculture

The cells were cultured to passage 6 in T-225 flasks. Every 48 hours, the medium was changed until the cells reached confluence. To trypsinise the cells, the medium was removed using an aspirator and 10ml of trypsin-EDTA solution (refer to section 2.1.4 for concentration details) was applied to the cells. The flask was then incubated at 37°C for 3 minutes. Following that, the cells were visualised under a microscope to ensure they had detached from the flask. To neutralise the trypsin, 10ml of DMEM+ was added to the flask, and the cells were centrifuged at 1100 rpm for 5 min. The supernatant was removed using an aspirator and the cells were resuspended in 10ml of fresh DMEM+. The cells were counted and resuspended in freezing solution (90%FBS + 10%DMSO). The suspension was then aliquoted at a cell concentration density of 1×10^6 cells per 1ml in the cryogenic tubes. The tubes were transferred to a Mr Frosty Freezing Container (Thermo Fisher Scientific) that contained 100% isopropyl alcohol, and the container was stored at −80°C for

24hours to achieve a cooling rate of −1°C/minute. The next day, the cells were transferred to liquid nitrogen for long term storage.

2.5.2 Cell Counting

The cells were counted using a glass haemocytometer and coverslip. To differentiate between live and dead cells, the cells were diluted 1:1 with Trypan-blue and gently mixed. The cells that were not stained blue were counted on each of the large squares and cells on the top and right-hand lines of each square were counted. The average number of cells was multiplied by the dilution factor and then multiplied by 10^4.

2.5.3 Cell Treatment

The cells were seeded in 12-well plates with a consistent density of 83333 cells per-well and the medium was changed every 48 hours until the cells were fully confluent. The cells were then serum starved with DMEM− for 24 hours prior to the treatment. They were treated with CSE and ECVE (N+/N-) in DMEM- and incubated for 24 hours. Furthermore, to test the effect of the drug (BAY11-7082), the cells were pre-treated with it for 1 hour prior to CSE and ECVE (N−) for 24 hours. At the end of the experiments, the medium was collected to analyse the cytokines and chemokines by enzyme-linked immunosorbent assay (ELISA).

2.5.4 Cell Lysate Preparation

After the medium was collected from the plates, PBS was used to wash the cells. Then, 150µl of radioimmunoprecipitation assay (RIPA) buffer (refer to section 2.1.3) was added to each well. Cell scrapers were used to collect the lysates from the wells, which were then stored at −80°C. A bicinchoninic acid (BCA) assay kit was used to quantify the protein concentration.

2.6. Bicinchoninic Acid Protein Assay

A BCA assay kit (Thermo Fisher Scientific) was used to quantify the amount of protein in the lysates. Bovine serum albumin (BSA) standards solution was prepared according to the manufacturer's instructions (0–2000µg/ml). The test samples (5µl) were transferred in duplicate to a 96-well plate. 5µl of BSA standards in duplicates were also added to the wells. The BCA A:B (1:50) reagent was prepared and mixed in a Falcon tube, and 95µl of this reagent was transferred to each well. The 96-well plate was incubated at room temperature for 30 minutes. Then, a FLUO star Omega microplate reader (BMG LABTECH) was used to measure the absorbance at 562nm. The Omega analysis software was used to obtain the background corrected absorbance values. A standard curve was plotted with the known concentrations of BSA standards on the

x-axis and corresponding absorbance values on the y-axis. Absorbance values of test samples were extrapolated from the standard curve graph to calculate the protein concentrations which were reported in µg/ml.

2.7. Enzyme-linked Immunosorbent Assay (ELISA)

DuoSet ELISA kits (R&D Systems) were used for analysing the cytokines/chemokines (IL-5, IL-6, IL-8, IP-10, IL-13, VEGF, MCP-1 and RANTES) profiles in the cell culture samples. The ELISAs were performed according to the manufacturer's instructions, which is described below.

2.7.1 Assay Procedure

A high-binding 96-well plate was used for the ELISAs. The capture antibody was diluted in PBS, and 100µl was applied to each well. The plate was incubated at room temperature overnight. Then, the wells were washed three times with 400µl of wash buffer then blocked by incubating with 300µl of block buffer for 1hour at room temperature. The plate was rewashed, and 100µl of diluted standard was applied in duplicates to the wells. 100µl of each sample was then transferred to each well and incubated for 2 hours on a shaker set to 300–400rpm. The plate was washed again and 100µl of diluted biotinylated goat anti-human detection

antibody was applied to each well followed by incubation for 2 hours. The plate was rewashed, and 100µl of streptavidin horseradish peroxidase (HRP) was applied to each well and the plate was incubated for 20 minutes. Finally, the plate was rewashed and incubated with 100µl per well of substrate solution for 20 minutes. Then, the reaction was stopped with 50µl of stop solution (sulphuric acid). The FLUO star Omega microplate (BMG LABTECH) was used to measure the optical density for each well at 450nm, and Omega software was used to obtain the background corrected absorbance values. The concentrations of cytokines/chemokines were calculated by extrapolating the absorbance values using the standard curve. The normalisation of the concentrations to total amount of protein was completed as follow:

The cytokines and chemokines concentration were multiplied by 1.5 ml to achieve the total cytokine/chemokine in ng. The mean cytokine and chemokine were divided by total amount of protein present in the cell lysate (see section 2.6. Bicinchoninic Acid Protein Assay) to give concentration of secreted cytokine/chemokine per mg of cell protein

$$\text{Normalised concentration} = \frac{\text{cytokines/chemokines (ng/ml)}}{\text{protein concntration (mg/ml) X 0.15 ml total of cell lysates}}$$

(ng/mg protein)

2.8. Cell Viability Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay was used to evaluate the cytotoxicity of CSE and ECVE on HASMCs. The cells were cultured in 12-well plates and treated with different concentrations of CSE (1,2,3.5 and 5%) or ECVE (N+/N-; 1, 2.5,5 and10%) and CSE, ECVE (N–) along with increasing concentrations of BAY11-7082 for 24 hours. Then, the medium was removed and replaced with 500µl of MTT solution at a concentration of 1mg/ml in warm medium. After 30 minutes of incubation at 37°C, the MTT solution was removed, and the plate was allowed to dry for 6 hours at room temperature. Following this, 500µl of DMSO was added to the wells, and the solutions were transferred to a 96-well plate. A FLUO star Omega microplate reader (BMG LABTECH) was used to measure the absorbance at 550nm. Viability data were presented as fold change over control.

2.9. Statistical Analysis

The data were presented using the GraphPad Prism software. One-way ANOVA followed by a *t*-test was used to determine the significance of the difference between the control and the treated cells. A *p*-value of less than 0.05 was accepted as statistically significant.

Chapter 3 Results

3.1. Effect of CSE and ECVE (N+/N-) on Cell Viability

To evaluate the cytotoxicity of CSE (1, 2, 3.5 and 5%) and ECVE (N+/N-; 1, 2.5, 5 and 10%) on HASMCs an MTT assay was used as shown in figure 3.1. The assay was performed after the cells were incubated with CSE and ECVE (N+/N-) for 24 hours as described in section 2.8. 5% CSE and 10% ECVE (N+/N-) significantly reduced HASMCs with 50% reduction in cell viability ($p<0.0001$). CSE with concentrations up to 3.5% and ECVE (N+/N-) up to 5% were tolerated by the cells as no decrease in cell viability was observed. Therefore, these concentrations of CSE and ECVE (N+/N-) were used in further experiments to study the effect on the production of cytokines and chemokines by HASMCs. In addition, the similarity in cytotoxic effect of ECVE (N+/N-) suggests that the cytotoxicity is not caused by nicotine.

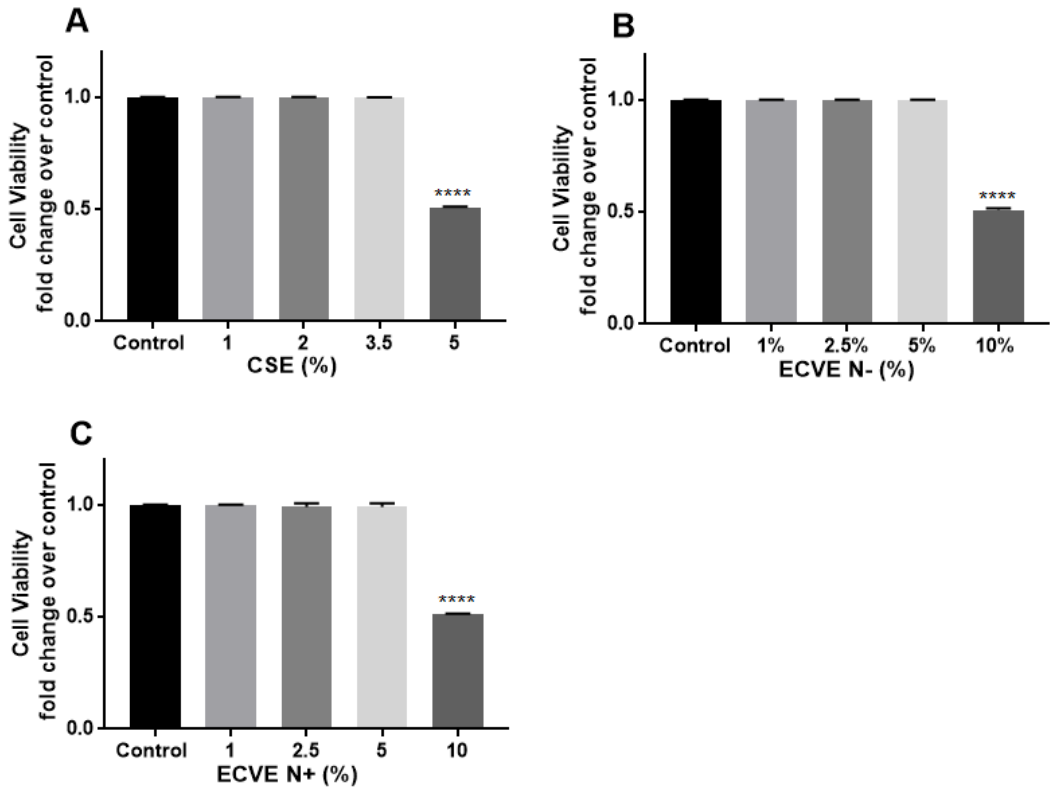


Figure 3.1. Effect of CSE and ECVE (N+/N-) on Cell Viability

Confluent and growth-arrested HASMCs were treated with (A) CSE (1, 2, 3.5, and 5%) and (B-C) ECVE (N+/N-;1, 2.5, 5% and 10%) for 24 hours. Cell viability was analysed by MTT cell viability assay. These data represent mean ± SEM of one experiment carried out in triplicate. ****$p<$ 0.0001 versus control.

3.2. Effect of CSE and ECVE (N+/N-) on CXCL-10 and RANTES Production

To assess the effect of CSE and ECVE on CXCL-10 and RANTES production, confluent and growth-arrested HASMCs were treated with CSE (1, 2 and 3.5%) and ECVE (N+/N-;1, 2.5 and 5%) for 24 hours. The medium was then collected, CXCL-10 and RANTES levels were measured by ELISA. CXCL-10 and RANTES were undetectable in HASMCs either at baseline or after CSE and ECVE (N+/N-) treatment. Figure 3.2 represents the raw absorbance values of the standards and tested samples of CXCL-10 (A) and RANTES (B). The results suggest that CXCL-10 and RANTES are undetectable in the medium from unstimulated cells, and CSE and ECVE had no effect on the production of CXCL-10 and RANTES in HASMCs.

A

		1	2	3	4	5	6	7	8	9	10	11	12
STANDARD	A	2025.013	1007.445	414.776	264.322	86.87	69.182	39.644					
	B	2028.309	1023.506	398.626	423.829	133.238	59.981	22.414					
	C	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range
	D	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range
SAMPLES	E	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range
	F	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range
	G	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range
	H	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range

B

		1	2	3	4	5	6	7	8	9	10	11	12
STANDARD	A	991.3	459.681	221.999	90.016	46.346	27.382	<< Y range					
	B	1052.44	490.8	339.163	115.333	64.686	64.686	43.475					
	C	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	23.605	<< Y range	<< Y range	<< Y range	<< Y range		
	D	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range		
SAMPLES	E	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range		
	F	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range		
	G	9.28	25.529	17.257	23.605	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range		
	H	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	14.861	<< Y range	<< Y range	<< Y range	std range		

Figure 3.2. Effect of CSE and ECVE (N+/N-) on CXCL-10 and RANTES Production

Confluent and growth-arrested HASMCs were treated with CSE (1, 2 and 3.5%) and ECVE (N+/N-;1, 2.5 and 5%) for 24 hours. The medium was collected, CXCL-10 (A) and RANTES (B) were measured by ELISA. Raw absorbance values of standard and test samples are represented in blue and red font respectively.

3.3. Effect of CSE and ECVE (N+/N-) on IL-5 Production

To study the effect of CSE and ECVE on IL-5 production, confluent and growth-arrested HASMCs were treated with CSE and ECVE for 24 hours. The cell culture medium was subjected to an ELISA to measure the concentration of IL-5. We found that IL-5 was basally produced at a low level in unstimulated cells (control). CSE treatment increased the production of IL-5 in a concentration-dependent manner (Figure 3.3 A). 3.5% CSE significantly induced IL-5 secretion (2ng/mg protein) compared with untreated cells control (2 vs 1ng/mg respectively) (p<0.01). On the other hand, ECVE (N+/N-) had no significant effect on IL-5 production (Figure 3.3 B). These findings suggest that CSE, but not ECVE, increase IL-5 production in HASMCs and may contribute to eosinophilic airway inflammation.

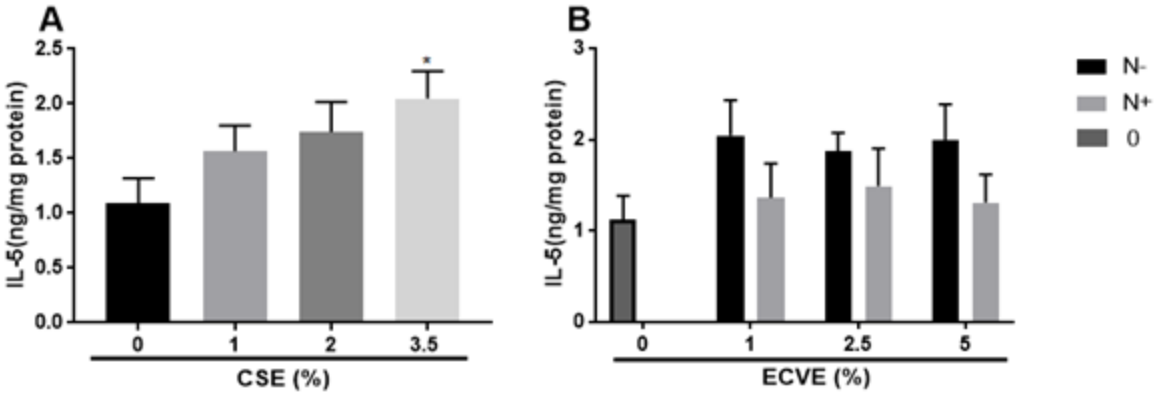


Figure 3.3. Effect of CSE and ECVE (N+/N-) on IL-5 production

Confluent and growth-arrested HASMCs were treated with (A) CSE (1, 2 and 3.5%) and (B) ECVE (N+/N-; 1, 2.5 and 5%) for 24 hours. The medium was collected and IL-5 was measured by ELISA. The data were normalised to total cellular protein. Each data point represents mean ± SEM of one experiment carried out in triplicate. *p<0.05 versus control (0).

3.4. Effect of CSE and ECVE (N+/N-) on IL-6 Production

To investigate whether CSE and ECVE can induce the production of IL-6 from HASMCs, confluent and growth-arrested HASMCs were treated with CSE and ECVE for 24 hours and the cell culture medium was collected to measure IL-6 concentration by ELISA. IL-6 was basally produced at a low level in unstimulated cells. CSE treatment increase the production of IL-6 in a concentration-dependent manner (Figure 3.4 A). 2% CSE induced IL-6 production in HASMCs (10.6 ng/mg protein vs 1.3 ng/mg protein in control cells) (p<0.01). A further increase was observed with 3.5% CSE (13 ng/mg protein) compared with untreated control (1.3 ng/mg protein) (p<0.001). ECVE (N+/N-) had no significant effect on IL-6 production (Figure 3.4 B). Our findings suggest that CSE, but not ECVE, induces IL-6 production in HASMCs and may contribute to eosinophilic airway inflammation.

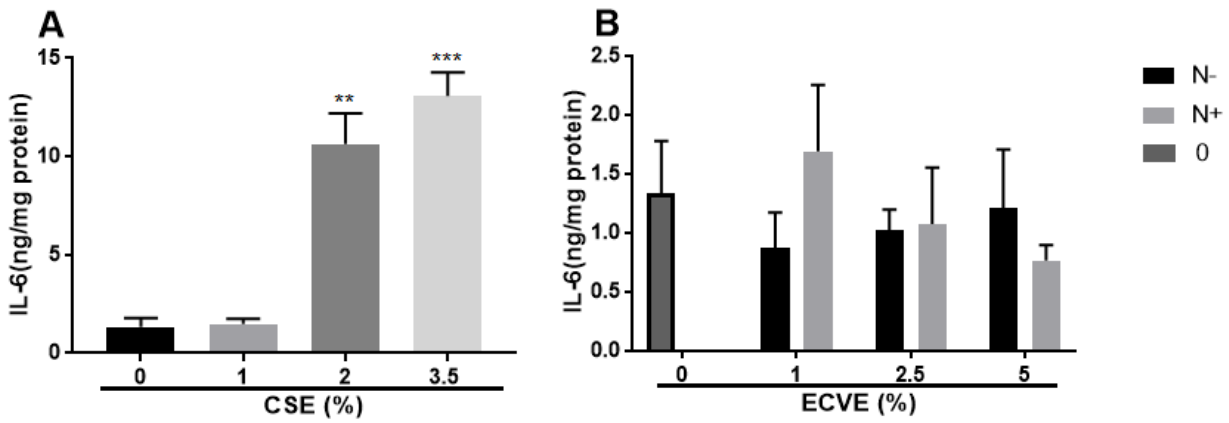


Figure 3.4. Effect of CSE and ECVE (N+/N-) on IL-6 Production

Confluent and growth-arrested HASMCs were treated with (A) CSE (1, 2 and 3.5%) and (B) ECVE (N−/N+; 1, 2.5 and 5%) for 24 hours. The medium was collected and IL-8 was measured by ELISA. The data were normalised to total protein. Each data point represents mean ± SEM of one experiment carried out in triplicate. ***p*<0.01, and ****p*<0.001 versus control (0).

3.5. Effect of CSE and ECVE (N+/N-) on IL-8 Production

To investigate the effect of CSE/ECVE on IL-8 production, confluent and growth-arrested HASMCs were treated and incubated for 24 hours with CSE and ECVE. ELISA was used to quantify IL-8 in cell culture supernatant after 24 hours with different concentrations of CSE and ECVE (N+/N-). 2% CSE significantly increased IL-8 secretion (0.66 ng/mg protein) ($p<0.05$) and a further increase was achieved by 3.5% CSE (1.88 ng/mg protein) compared with untreated control cells (0.32 ng/mg protein) ($p<0.0001$) (Figure 3.5 A). ECVE (N+/N-) had no significant effect on IL-8 production (Figure 3.5 B). The results suggest that CSE, but not ECVE, induces IL-8 production in HASMCs and may contribute to neutrophilic airway inflammation.

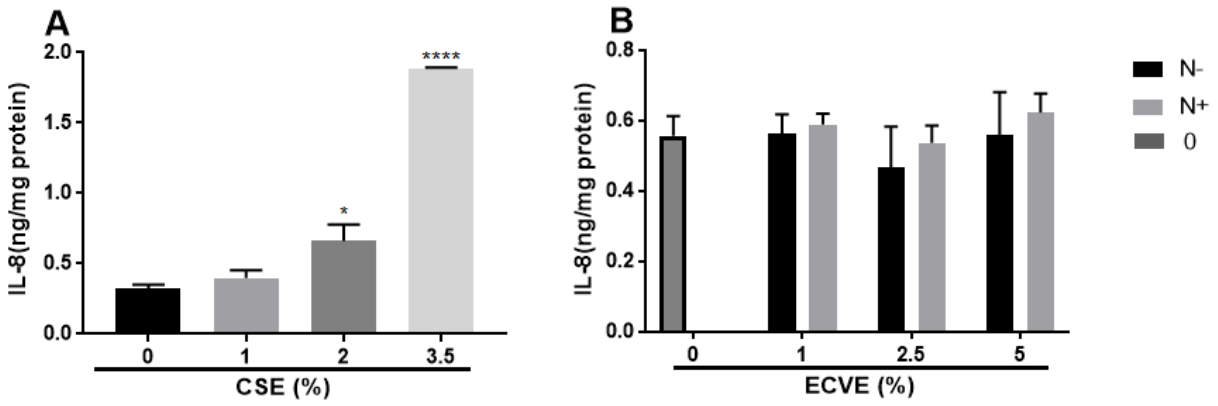


Figure 3.5. Effect of CSE and ECVE (N+/N-) on IL-8 Production

Confluent and growth-arrested HASMCs were treated with (A) CSE (1, 2 and 3.5%) and (B) ECVE (N+/N-;1, 2.5 and 5%) for 24 hours. The medium was collected and IL-8 was measured by ELISA. The data were normalised to total protein. Each data point represents mean ± SEM of one experiment carried out in triplicate. *p<0.05, and ****p<0.0001 versus control (0).

3.6. Effect of CSE and ECVE (N+/N-) on IL-13 Production

To investigate the effect of CSE and ECVE on IL-13 production, confluent and growth-arrested HASMCs were treated with CSE and ECVE for 24 hours. The cell culture supernatant was collected and IL-13 concentration was measured by ELISA. CSE treatment induced the production of IL-13 in a concentration-dependent manner similar to IL-5, IL-6 and IL-8 (Figure 3.6 A). 3.5% CSE significantly induced IL-13 production (23.7 ng/mg protein) compared with control (9.7 ng/mg protein) (p<0.01) (Figure 3.6 A). ECVE (N+) induced the production of IL-13 in HASMCs at 1, 2.5 and 5% with 28.7, 42.9 and 30.8 ng/mg protein compared with control (10.6 ng/mg protein) (p<0.05, p<0.01 and p<0.05, respectively) (Figure 3.6 B). ECVE (N−) induced the production of IL-13 in HASMCs at 1, 2.5 and 5% with 32.1, 33 and 41 ng/mg protein compared with control (10.6 ng/mg protein) (p<0.05, p<0.01 and p<0.05, respectively) (Figure 3.6 B). The data suggest that CSE and ECVE induce IL-13 production in HASMCs and the effect of ECVE (N+/N-) on IL-13 suggests that e-cigarette vapor, not the nicotine in e-cigarette liquid, is harmful to the airway and may contribute to eosinophilic airway inflammation and mucus secretion.

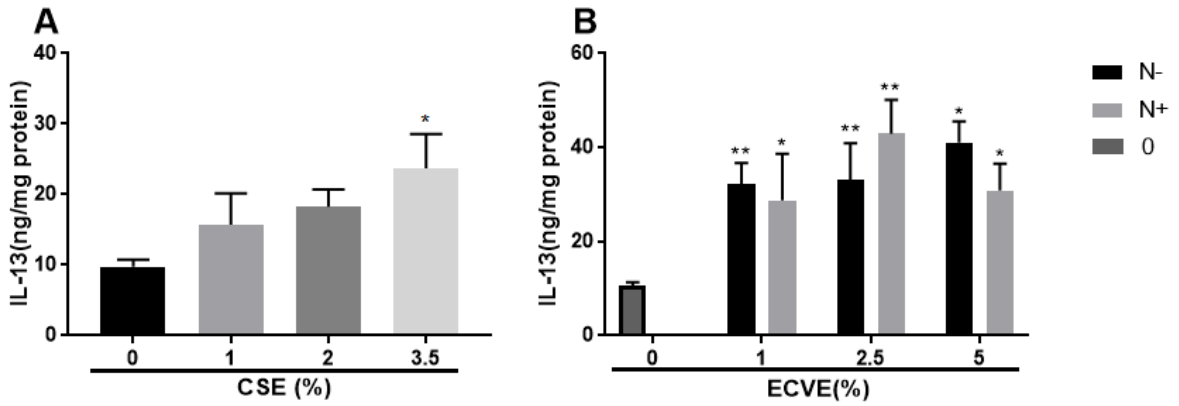


Figure 3.6. Effect of CSE and ECVE (N+/N-) on IL-13 Production

Confluent and growth-arrested HASMCs were treated with (A) CSE (1, 2 and 3.5%) and (B) ECVE (N+/N-;1, 2.5 and 5%) for 24 hours. The medium was collected and IL-13 was measured by ELISA. The data were normalised to total protein. Each data point represents mean ± SEM of one experiment carried out in triplicate. *p<0.05, and **p<0.01 versus control (0).

3.7. Effect of CSE and ECVE (N+/N-) on VEGF Production

To investigate the effect of CSE/ECVE on VEGF production, confluent and growth-arrested HASMCs were treated with CSE and ECVE (N+/N-) for 24 hours and ELISA was performed on the cell culture supernatants. CSE treatment induced the production of VEGF in a concentration-dependent manner compared with untreated control. 1% and 2% CSE induced VEGF production with 1 ng/mg protein compared with control (0.57 ng/mg protein) ($p<0.01$ and $p<0.05$, respectively). A further increase was achieved by 3.5% with 1.37 ng/mg protein increase compared with control (0.57 ng/mg protein) ($p<0.01$) (Figure 3.7 A). ECVE (N+/N-) had no significant effect on VEGF production (Figure 3.7 B). The results suggest that ECVE has no effect on the production of VEGF, whereas CSE induces VEGF production in HASMCs and may contribute to airway remodelling.

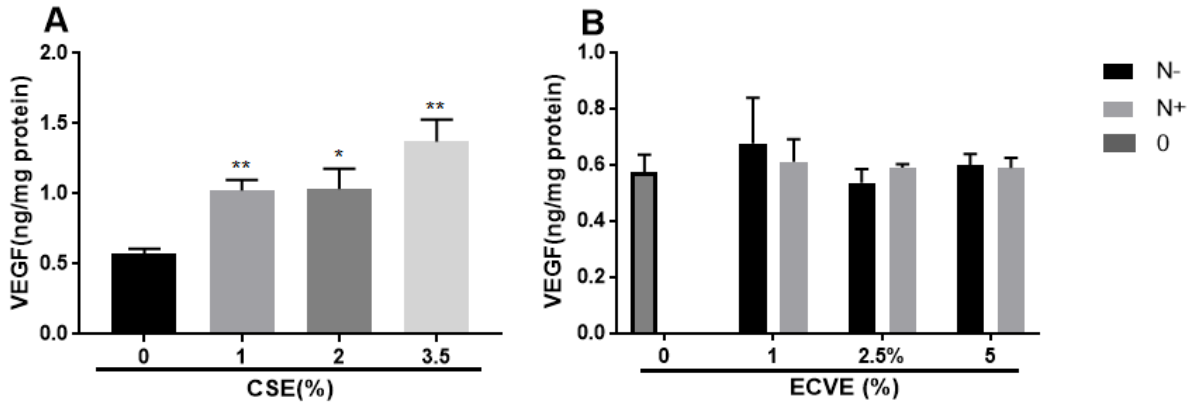


Figure 3.7. Effect of CSE and ECVE (N+/N-) on VEGF Production

Confluent and growth-arrested HASMCs were treated with (A) CSE (1, 2 and 3.5%) and (B) ECVE (N+/N-;1, 2.5 and 5%) for 24 hours. The medium was collected and VEGF was measured by ELISA. The data were normalised to total protein. Each data point represents mean ± SEM of one experiment carried out in triplicate. *p<0.05, and **p<0.01 versus control (0).

3.8. Effect of CSE and ECVE (N+/N-) on MCP-1 Production

To identify whether CSE and ECVE can increase the production of MCP-1, confluent and growth-arrested HASMCs were treated with CSE and ECVE (N+/N-) for 24 hours. The cell culture medium was collected, and MCP-1 concentration was measured by ELISA. 3.5% CSE induced the production of MCP-1 (11.83 ng/mg protein) compared with control (6.61 ng/mg protein) (p<0.05) (Figure 3.8 A). ECVE (N+/N-) had no significant effect on MCP-1 production (Figure 3.8 B). The data suggest that ECVE has no effect on MCP-1, but CSE induces MCP-1 production in HASMCs and may contribute to lung allergy and hyperreactivity in asthma.

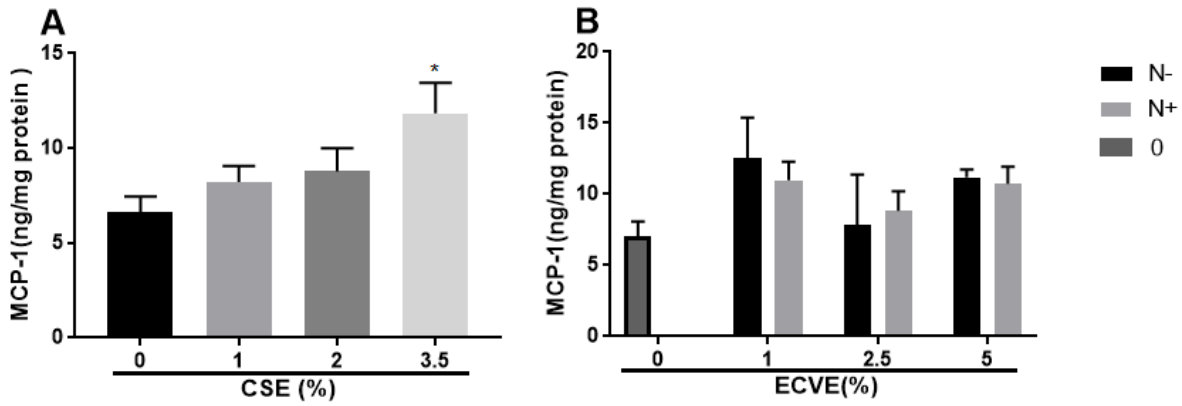


Figure 3.8. Effect of CSE and ECVE (N+/N-) on MCP-1 Production

Confluent and growth-arrested HASMCs were treated with (A) CSE (1, 2 and 3.5%) and (B) ECVE (N+/N-;1, 2.5 and 5%) for 24 hours. The medium was collected and MCP-1 was measured by ELISA. The data were normalised to total protein. Each data point represents mean ± SEM of one experiment carried out in triplicate. *p<0.05 versus control (0).

3.9 Effect of BAY11-7082 on Cell Viability

In our experiments, we found that CSE (3.5%) induced cytokine and chemokine production from HASMCs. ECVE had a nicotine-independent effect on IL-13 secretion. We therefore used the concentration of CSE (3.5%) and ECVE (N-;5%) to assess the effect of the NF-κB inhibitor BAY11-7082. MTT assay was used to assess the cytotoxicity of BAY11-7082 along with (A) CSE (3.5%) and (B) ECVE (N−;5%). Confluent and growth-arrested HASMCs were pre-treated with BAY11-7082 (0.01, 0.1,1, 10 and 100 μM) for 1 hour; following that, CSE (3.5%) and ECVE (N−;5%) were added to the cells and incubated for 24 hours as described in section 2.8. There was no significant difference in cell viability when cells were treated BAY11-7082 with or without CSE or ECVE compared with untreated control. The results suggest that BAY11-7082 at all tested concentrations are not toxic to HASMCs (Figure 3.9 A-B). Experiments were therefore carried out to explore the role of NF-κB in mediating CSE/ECVE-induced cytokine and chemokine production in HASMCs.

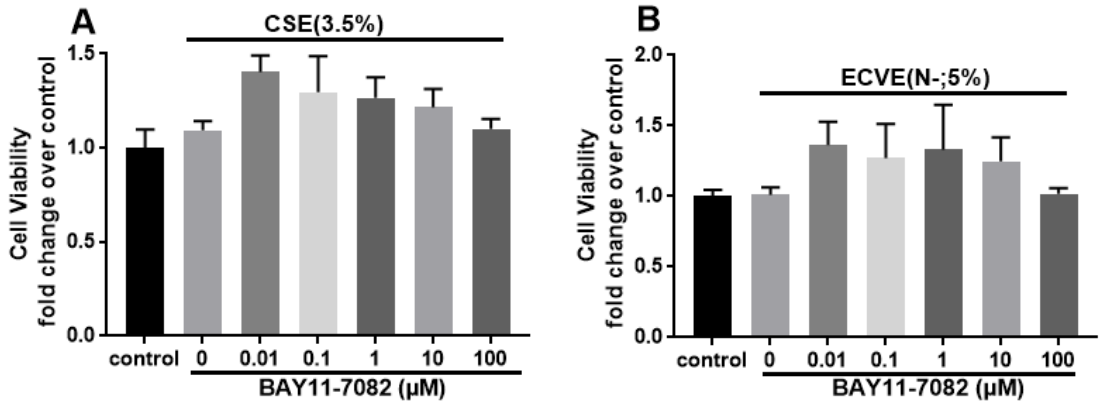


Figure 3.9 Effect of BAY11-7082 on Cell Viability

Confluent and growth arrested HASMCs were treated with BAY11-7082 (0.0,1 0.1, 10 and 100 μM) for 1 hour. Then the cells were treated with (A) CSE (3.5%) and (B) ECVE (N−;5%) for 24 hours. The cell viability was analysed by MTT cell viability assay. Each data represents mean ± SEM of one experiment carried out in triplicate.

3.10 Effect of BAY11-7082 on CSE-induced IL-6 and IL-8 Production

To investigate the role of NF-κB in mediating CSE-induced IL-6 and IL-8 production in HASMCs using an NF-κB specific inhibitor BAY11-7082, confluent and growth-arrested HASMCs were pre-treated with BAY11-7082 (0.01-100μM) for 1 hour. 3.5% CSE was then added to the cells and incubated for 24 hours. ELISA was used to measure the concentrations of IL-6 and IL-8 in collected cell culture medium. CSE (3.5%) significantly induced IL-6 (7.4 ng/mg protein) (p<0.001) (Figure 3.10. A) and IL-8 (24.2 ng/mg protein) (p<0.0001) compared with untreated control (0.69 and 1.3 ng/mg protein, respectively) (Figure 3.10. B). BAY11-7082 treatment resulted in partial inhibition of IL-6 in a concentration-dependent manner, with a significant inhibition at 100 μM (4 ng/mg protein) compared with CSE alone (7.4 ng/mg protein) (p<0.01) (Figure 3.10 A). BAY11-7082 also inhibited IL-8 production in a concentration dependent manner which was maximal at 100 μM (16 ng/mg protein vs 24.2 ng/mg protein in CSE treated cells) (p<0.01) (Figure 3.10 B). These findings suggest that NF-κB may play a partial role in mediating CSE-induced IL-6 and IL-8 production in HASMCs.

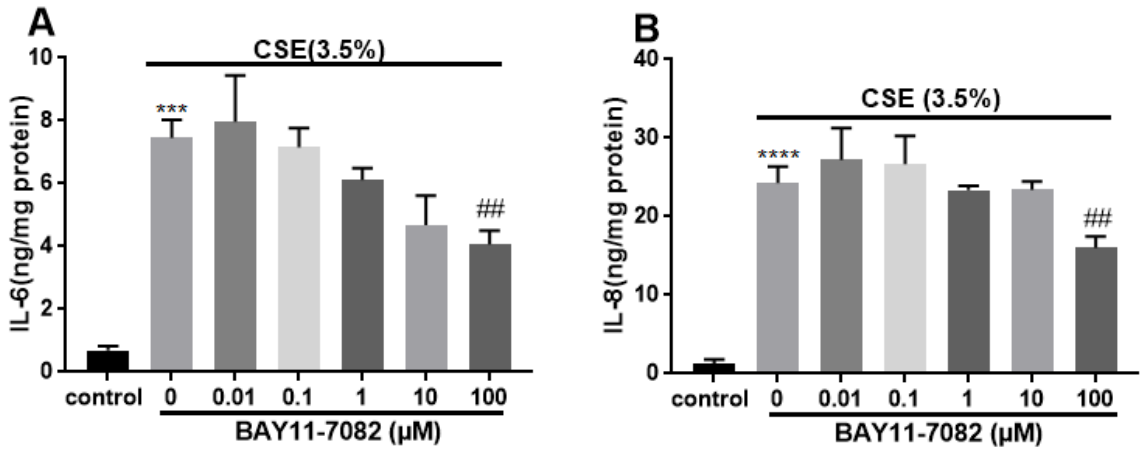


Figure 3.10 Effect of BAY11-7082 on CSE-induced IL-6 and IL-8 Production

Confluent and growth-arrested HASMCs were pre-treated with BAY11-7082 (0.01-100 μM) for 1 hour. They were then treated with CSE (3.5%) and incubated for 24 hours. The medium was collected then (A) IL-6 and (B) IL-8 were measured by ELISA. The data were normalised to total protein. Each data point represents mean ± SEM of one experiment carried out in triplicate. ***p<0.001, ****p<0.0001 versus control and $^{\#\#}p$< 0.01 versus CSE alone.

3.11 Effect of BAY11-7082 on CSE- and ECVE-induced IL-13 Production

To explore the role of NF-κB in mediating CSE- and ECVE-induced IL-13 production in HASMCs the NF-κB specific inhibitor BAY11-7082 was used. Confluent and growth-arrested HASMCs were pre-treated with BAY11-7082 (0.01-100 μM) for 1 hour then 3.5% CSE or 5% ECVE (N−) were added to the cells and incubated for 24 hours. The cell culture medium was collected, and IL-13 concentration was measured by ELISA. 3.5% CSE increased IL-13 release compared with control (10.40 ng/mg protein vs 2.15 ng/mg protein respectively) (p<0.001) (Figure 3.11 A). 5% ECVE (N−) induced the production of IL-13 compared with control (4.96 ng/pm protein vs 2.57 ng/mg protein) (Figure 3.11 B). Bay11-7082 had no effect on CSE- and ECVE-induced IL-13 production at all tested concentrations. The results suggest that NF-κB may not play a role in mediating CSE- and ECVE-induced IL-13 production in HASMCs.

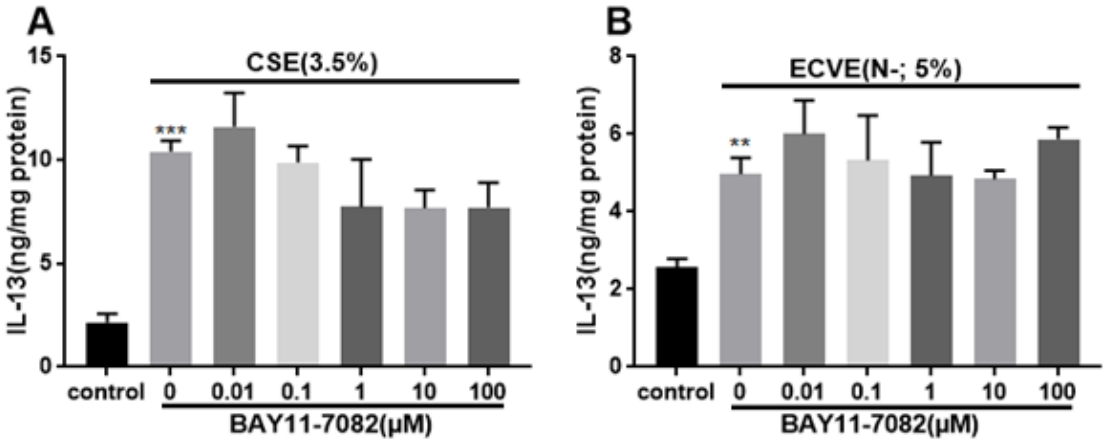


Figure 3.11 Effect of BAY11-7082 on CSE- and ECVE-induced IL-13 Production

Confluent and growth-arrested HASMCs were pre-treated with BAY11-7082 (0.01-100 μM) for 1 hour. They were then treated with (A) CSE (3.5%) and (B) ECVE (N−;5%) for 24 hours. The medium was collected then IL-13 was measured by ELISA. The data were normalised to total protein. Each data point represents mean ± SEM of one experiment carried out in triplicate. ** p<0.01 and *** p<0.001 versus control.

3.12 Effect of BAY11-7082 on CSE-induced IL-5 and MCP-1 Production

To investigate the inhibitory effect of BAY11-7082 on CSE-induced IL-5 and MCP-1, confluent and growth-arrested HASMCs were pre-treated with BAY11-7082 (0.01-100 μM) for 1 hour. Following that CSE (3.5%) was added and incubated for 24 hours. IL-5 and MCP-1 concentrations were measured by ELISA in the cell culture supernatants. 3.5% CSE treatment significantly induced the production of IL-5 compared with control (0.27 ng/mg protein vs 0.089 ng/mg protein) (p<0.001) (Figure 3.12 A). Also 3.5% CSE induced MCP-1 production compared with untreated control (10.41 ng/mg protein vs 1.8 ng/mg protein respectively) (p<0.01) (Figure 3.12 B). BAY11-7082 had no effect on CSE-induced IL-5 and MCP-1 production at any tested concentration. The results suggest that NF-κB may not play a role in mediating CSE-induced IL-5 and MCP-1 production in HASMCs.

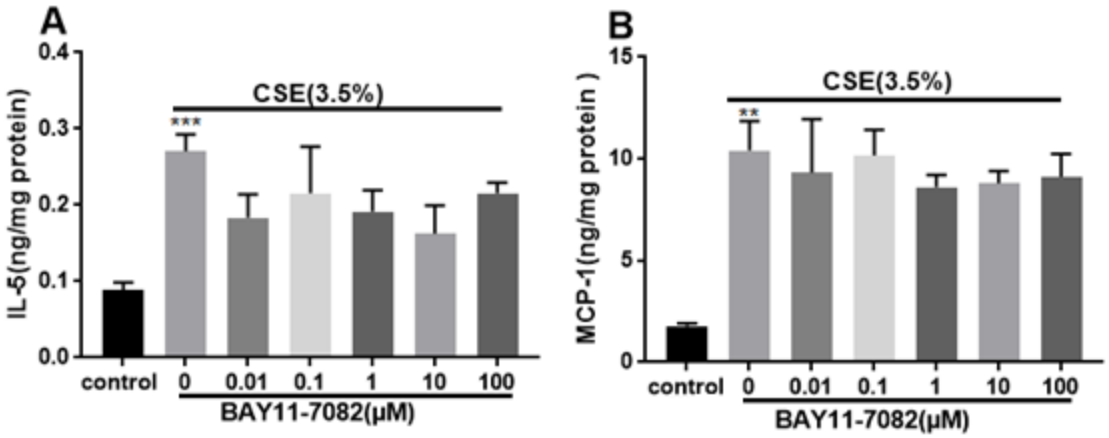


Figure 3.12 Effect of BAY11-7082 on CSE-induced IL-5 and MCP-1 Production

Confluent and growth-arrested HASMCs were pre-treated with BAY11-7082 (0.01-100 μM) for 1 hour. They were then treated with CSE (3.5%) and incubated for 24 hours. The medium was collected then (A) IL-5 and (B) MCP-1 were measured by ELISA. The data were normalised to total protein. Each data point represents mean ± SEM of one experiment carried out in triplicate. ** $p<0.01$ and *** $p<0.001$ versus control.

3.13 Effect of BAY11-7082 on CSE-induced VEGF Production

To assess the effect of BAY11-7082 on CSE-induced VEGF, confluent and growth-arrested HASMCs were treated with BAY11-7082 (0.01-100 μM) for 1 hour prior to the addition of CSE (3.5%) for 24 hours. After the cell culture medium was collected, ELISA was used to measure the concentrations of VEGF. CSE (3.5%) treatment induced VEGF production compared with control (5.83 ng/mg protein vs 0.28 ng/mg protein) (p<0.001) (Figure 3.13). This increase was inhibited by BAY11-7082 in a concentration-dependent manner, with a significant inhibition at all 4 tested concentrations (0.1, 1, 10 and 100 μM) with 3.19, 2.70, 1.36 and 1.18 ng/mg protein compared wtih CSE alone (5.83 ng/mg protein) (p<0.01) respectively (Figure 3.13). These findings suggest that NF-κB plays a crucial role in mediating CSE-induced VEGF production in HASMCs.

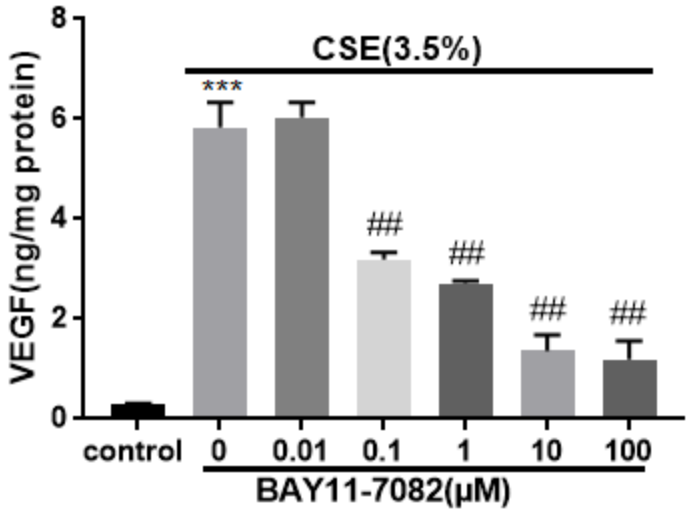


Figure 3.13 Effect of BAY11-7082 on CSE-induced VEGF Production

Confluent and growth-arrested HASMCs were pre-treated with BAY11-7082 (0.01-100 μM) for 1 hour. The cells were then treated with CSE (3.5%) and incubated for 24 hours. The medium was collected then VEGF was measured by ELISA. The data were normalised to total protein. Each data point represents mean ± SEM of one experiment carried out in triplicate. ***p<0.001 versus control and $^{\#\#}p$<0.01 versus CSE alone.

Chapter 4. Discussion

COPD is a respiratory condition associated with irreversible airways limitation and damage to the lung parenchyma. In relation, various studies [2, 3] have shown that cigarette smoke is the leading cause of the development of COPD. Currently the use of e-cigarettes are widely increased and used to aid smoking cessation. The effect of e-cigarette vapour extract (ECVE) on the production of cytokines and chemokines in HASMCs is unknown.

According to a transcriptomics analytic study, HASM was shown to be associated with facilitating the production of cytokines like IL8, IL6, IL5, VEGF and chemokines like RANTES, CXCL10 [10]. Moreover, the nuclear factor NF-κB is a transcription factor that mediates the expression of the inflammatory mediators, including cytokines, chemokines, adhesion molecules and growth factors.

NF-κB possesses an essential role in chronic inflammatory diseases like COPD [93]. Therefore, we aimed to understand whether cigarette smoke extract (CSE) and e-cigarette vapour extract (ECVE) could induce the production of inflammatory cytokines and chemokines in HASMCs and whether NF-κB is involved in mediating CSE- and ECVE-induce cytokines and chemokines.

4.1 CSE/ECVE and Cytokine/Chemokine Production

To investigate the impact of CSE/ECVE on the inflammatory responses of HASMCs, we assessed the effect of CSE/ECVE on the production of cytokines and chemokines that are involved in COPD, such as IL-5, IL-6, IL-8, IL-13, MCP-1, CXCL-10, RANTES and VEGF. Our results showed that CXCL-10 and RANTES were undetectable with HASMCs after CSE and ECVE treatment. To support our findings, CSE induced the release of IL-8 from HASMCs, whereas the level of eotaxin and RANTES were undetectable in cell culture supernatant [73].

It has been demonstrated that the CSE induced the release of CXCL-8 and CXCL-1 from HASMCs, but RANTES and CXCL-10 were undetectable after CSE. In addition to that, MCP-1 was produced by HASMCs cells but was not induced by CSE [94]. However, in our study we were able to see the effect of CSE on IL-8 and MCP-1, but in their case CSE did not induce MCP-1. In addition to that, our result share similarity with their finding that CSE did not induce CXCL-10 and RANTES from HASMCs. For instance, MCP-1 in their study was basally expressed with untreated control, but CSE has no effect on the production of MCP-1 from HASMCs. In our study, CSE stimulated the production of MCP-1 from HASMCs. However, in their study, 1 cigarette (Marlboro

Red) was used, while in our study 2 cigarettes (3R4F) research cigarettes were used. Also, the CSE concentrations were regarded as 100% in their study, while in our study, the optical density at 320 nm was used to measure the CSE strength (1.5=100%) to ensure the desired concentrations were obtained. Additionally, in their study CSE (0.05 to 10%) were applied to the cells for 72 hours, but in our study, CSE (1,2 and 3.5%) were applied to the cells for 24 hours. Therefore, the difference in CSE concentration are not directly comparable. In their study, CSE concentration used up to 10% which seemed to be much higher than the CSE concentration we used up to 3.5%. However, there are differences in the experiment design, methodology, cigarettes type, CSE concentrations and the longer incubation time for 72 hours in their study vs 24 hours in our case. This could explain the possible differences in cells passages, cell culture and growth-arrest.

Some studies showed that CSE can induce cytokines and chemokines in other cells. CSE induced IL-6 and IL-8 from bronchial epithelial cells (BEAS-2B) [95]. *In vivo* study, cigarette smoke condensate (CSC) in mouse macrophage cells induced the proinflammatory cytokines IL-5, IL-6, VEGF and chemokines IP-10 and MCP-1 but not IL-13 [78].

Cigarette smoke extract with different concentration activates the production of IL-8 from HASMCs, which is an important mediator of neutrophilic inflammation in COPD. Interestingly no study has investigated the effect of CSE on Th2 cytokines such as IL-5, IL-6 and IL-13 from HASMCs. In our study, CSE induced IL-5, IL-6 and IL-13 production in HASMCs that may contribute to eosinophilic airway inflammation. Also, we sought to investigate the effect of CSE on the production of VEGF, and we found that CSE induced the production of VEGF, which has a role in airway remodelling. Additionally, we aimed to assess the effect of CSE on MCP-1, and we found that CSE induced the production of MCP-1, which is known to play a role in allergic asthma. Therefore, our results suggest that cigarette smoke contributes to airway inflammation by the production of cytokines and chemokines from HASMCs.

Moreover, in our study, we investigated the effect of ECVE (N+/N-) flavourless on the production of the cytokines and chemokines from HASMCs. We found that ECVE (N+/N-) did not stimulate the production of RANTES and CXCL-10 from HASMCs. However, other findings investigated the exposure of aerosol electronic nicotine delivery systems (ENDS) on human bronchial epithelial cells (H292) with five flavours, pina colada, menthol, coffee, and strawberry, and it was revealed that the

strawberry flavoured ENDS significantly increased the release of CXCL-10 [96]. This study supported the fact that flavours have a significant and differential effect on cytotoxicity, as well as contribute to airway inflammation.

We found that ECVE (N+/N-) has no significant effect on IL-5, IL-6, IL-8, MCP-1 and VEGF, but significantly induced IL-13 production from HASMCs. A study showed that (ECVE) had no effect on the release of IL-6 and IL-8 from normal bronchial epithelial cells (BECs), but increased the release of IL-6 and IL-8 from human lung cancer cells (Calu-3) [97]. Therefore, our finding of ECVE was consistent with their study, showing no effect of ECVE on IL-6 and IL-8 production from normal bronchial epithelial cells [97].

A study focused on providing evidence on the effect of e-cigarette vapour exposure on the healthy human airway epithelial cells from non-smokers. It was concluded that e-cigarette vapour with and without nicotine induced the release of IL-6 from airway epithelial cells [88]. Another study on isolated neutrophils from peripheral blood revealed that ECVE increased the release of CXCL-8 [98].

A study of electronic vapour condensate (ECVC) showed a direct effect on alveolar macrophages from eight never smokers with normal

spirometry and no history of asthma or COPD. ECVC 0.5% with nicotine significantly induced the production of IL-6, TNF-α, CXCL-8, MCP-1 and MMP-9 compared with untreated control. Also, ECVC without nicotine induced the production of IL-6, CXCL-8 and MMP-9 but not TNF-α and MCP-1 compared with untreated control [90]. The effect of ECVE on the production of cytokines and chemokines has not been explored before in HASMCs. It has been demonstrated that ECVE has a different effect on other cells. However, by compering to our study, there is a significant difference in the experiment design and method in their study [90]. In their study, they were able to measure the OD value while in our case we tried to measure the OD value, but there was no difference in the OD value between non-extracted control and the extracted medium. Therefore, the difference in concentration are not directly comparable. In their study ECVC resulted in cytotoxicity, apoptosis and necrosis with lower concentration 0.8%, while in our study, 5% ECVE did not lead to significant cytotoxicity but 10% ECVE resulted in cytotoxicity and then 10% ECVE was removed from our study. This is inconsistent with our finding and could be explained by the differences in cells, and method.

In vivo study, the e-cigarette fluid with nicotine were exposed to mice for 4 months. It was observed that there was a significant increase in the levels and expressions of MCP-1, CXCl-2, IL-6 and CXCL-10 in the

BALF [78]. Also, in mice the Long-term inhalation of e-cigarette stimulated the production of Th2 cytokine IL-4, IL-5 and IL-13 in the BALF [99]. In our study, ECVE induced the production of IL-13 from HASMCs. These findings suggest that ECVE can contribute to airway inflammation by induce the production of cytokines and chemokines.

4.2 Role of NF-κB in CSE- and ECVE-induced cytokine / chemokine production

In our study, we have used BAY11-7082 NF-κB inhibitor BAY11-7082 to investigate the role of NF-κB in mediating CSE- and ECVE-induced cytokine and chemokine production from HASMCs. NF-κB is a transcription factor that mediates the expression of the inflammatory mediators, such as cytokines, chemokines, adhesion molecules and growth factors [47]. NF-κB has a role in the development of chronic inflammatory diseases, such as COPD.

In human bronchial epithelial cells (HBEs) the activation of NF-κB by CSE- increased the pro-inflammatory level of IL-6, and by blocking NF-κB with BAY11-7082, the drug inhibited CSE-induced IL-6 [100]. Our results share similarity with this study, showing that BAY11-7082 inhibit CSE-induce IL-6 from HASMCs.

To support our findings, a study showed that cigarette smoke directly induced NF-κB along with the release of various cytokines like IL-8, IL-1β and IL-6 in human bronchial epithelial cells. In their study, p120-catenin which known to regulate E-cadherin-mediated cell-cell adhesion that act as anti-inflammatory was used to investigate the activation of NF-κB signalling. The anti-inflammatory p120 inhibited CSE-induced the release of IL-8, IL-1β and IL-6 in human bronchial epithelial cells. However, by Knockdown p120 enhanced CSE-induced NF-κB activation and increased upregulation of IL-8, IL-1β and IL-6 after CSE [83].

Another finding on bronchial epithelial cells (16-HBE) showed that CSE stimulates the release of IL-8, but the inhibitor BAY11-7082 did not reduce the release of IL-8 [101]. They used a commercial cigarette from (Marlboro), and it is likely different from the research cigarettes (3R4F) that was used in our study. In their study, 3 cigarettes were bubbled through 25 ml phosphate-buffered saline (PBS) while in our case 2 cigarettes were bubbled through 20 ml of DMEM. In their study, they were able to calculate the concentration of CSE by measuring the OD value as well as in our case. They used BAY11-7082 concentration (50 μM) and applied it to the cells for 30 min; following that the cells incubated with CSE 10% for 18 hours. However, in our study, we used BAY11-7082 with five concentrations (0.01, 0.1, 1, 10 and 100 μM). The cells were pre-

treated with BAY11-7082 for 1 hour; following that the cells incubated with 3.5% CSE for 24 hours. The level of IL-8 was measured by ELISA in their study as well as in our case. This inconsistency could be due to the differences in methodology, cigarette types, time of incubation and cell line.

In our study, the activation of NF-κB by CSE induced IL-6, IL-8 and VEGF production from HASMCs. Bay11-7082 inhibited the increase of IL-6, IL-8 and VEGF, but not the other cytokines. In our study, NF-κB signal pathway was involved in CSE-induced the production of IL-6, IL-8 and VEGF from HASMCs.

Chapter 5. Future Directions and Conclusion

5.1 Future Directions

In our study, we have a number of limitations that need to be pointed out for the future study.

Firstly, we compared the effect of CSE and ECVE on human airway smooth muscles cells. we have only used HASMCs, and it may have a different effect from other airway cells. Also, our study used only one cell line of HASMCs from one donor, and it will be more useful to use different cell lines to confirm our findings.

Secondly, we could not quantify the vaporized medium after the exposure to ECVE (N+/N-) because there was no difference in the OD value between non-extracted (control) and the extracted medium. In the future study, the use e-liquid with flavour in order to quantify the vaporized medium could solve this issue.

Thirdly, the NF-κB specific inhibitor drug BAY11-7082 was used to explore the role of NF-κB in mediating CSE/ECVE-induced cytokine and chemokine production. However, it is necessary for the future study to knock down the expression NF-κB to determine whether the effect is the same as inhibiting its actions.

5.2 Conclusion

Our study conclude that cigarette smoke has a wider pro-inflammatory effect in the airway than e-cigarette vapor. However, the ability of ECVE (N-) to induce cytotoxicity and IL-13 production in HASMCs suggest that e-cigarette vapor in itself is harmful to the airway and induces pro-inflammatory response even in the absence of nicotine. The toxicity caused by ECVE in HASMCs raises concerns over the safety of e-cigarette use. NF-κB has a role in mediating CSE-induce IL-6, IL-8 and VEGF production, but it has no effect on ECVE-induce IL-13 from HASMCs. This suggests that the signalling pathways involved in CSE- and ECVE-induced pro-inflammatory responses in HASMCs differ from one another.

References

1. Global Initiative for Chronic Obstructive Lung Disease-. *Global Strategy for Prevetion, diagnosis, and management of COPD* 2018 [01-01-2019]; Available from: https://goldcopd.org/archived-reports/.
2. Barnes, P.J., *Inflammatory mechanisms in patients with chronic obstructive pulmonary disease.* J Allergy Clin Immunol, 2016. **138**(1): p. 16-27.
3. Qureshi, H., A. Sharafkhaneh, and N.A. Hanania, *Chronic obstructive pulmonary disease exacerbations: latest evidence and clinical implications.* Ther Adv Chronic Dis, 2014. **5**(5): p. 212-27.
4. Mathers, C.D. and D. Loncar, *Projections of global mortality and burden of disease from 2002 to 2030.* PLoS medicine, 2006. **3**(11): p. e442-e442.
5. Chronic obstructive pulmonary disease (COPD) statistics *British Lung Foundation Statistics*. 2011 [01-01-2019]; Available from: https://statistics.blf.org.uk/copd.
6. Snell, N., Strachan, and Gruffydd-Jones, K, , *S32 Epidemiology of chronic obstructive pulmonary disease (COPD) in the uk: findings from the british lung foundation's 'respiratory health of the nation' project.* Thorax, 2016. **71**(Suppl 3): p. A20-A20.
7. McLean, S., and Martine, *Projecting the COPD population and costs in England and Scotland: 2011 to 2030.* Sci Rep, 2016. **6**: p. 31893-31893.
8. Quaderi, S.A. and J.R. Hurst, *The unmet global burden of COPD.* GHEG, 2018. **3**: p. e4.
9. Papandrinopoulou, D., V., and G. Tsoukalas, *Lung compliance and chronic obstructive pulmonary disease.* Pulm Med, 2012. **2012**: p. 542769.
10. MacNee, W., *Pathogenesis of chronic obstructive pulmonary disease.* Proceedings of the ATS, 2005. **2**(4): p. 258-291.
11. Kim, V. and G.J. Criner, *Chronic Bronchitis and Chronic Obstructive Pulmonary Disease.* Am J Respir Crit Care Med, 2013. **187**(3): p. 228-237.
12. Sturm, R., *Theoretical models for the simulation of particle deposition and tracheobronchial clearance in lungs of patients with chronic bronchitis.* Ann Transl Med, 2013. **1**(1): p. 3.
13. Kaczka, a., Kenneth, and Z. Hantos, *Emergent behavior of regional heterogeneity in the lung and its effects on respiratory impedance.* Am J Physiol Lung Cell Mol Physiol 2011. **110**(5): p. 1473-1481.
14. Sharafkhaneh, A., a. Hanania, and Kim, *Pathogenesis of emphysema: from the bench to the bedside.* Proc Am Thorac Soc, 2008. **5**(4): p. 475-7.
15. van Eeden, S.F. and D.D. Sin, *Oxidative stress in chronic obstructive pulmonary disease: a lung and systemic process.* Can Respir J, 2013. **20**(1): p. 27-9.
16. MARINA SAETTA, G.T., and FABRIZIO M. , *Inflammatory Cells in the Bronchial Glands of Smokers with Chronic Bronchitis.* Am J Respir Crit Care Med, 1997. **156**(5): p. 1633-1639.
17. O'Donnell, P., C., and Ward, J. A., *Relationship between peripheral airway dysfunction, airway obstruction, and neutrophilic inflammation in COPD.* Thorax, 2004. **59**(10): p. 837-42.
18. Stanescu, D., Sanna, A., and Veriter, C., *Airways obstruction, chronic expectoration, and rapid decline of FEV1 in smokers are associated with increased levels of sputum neutrophils.* Thorax, 1996. **51**(3): p. 267-71.

19. Lams, B., Sousa, A., and Rees, P., *Immunopathology of the small-airway submucosa in smokers with and without chronic obstructive pulmonary disease.* Am J Respir Crit Care Med, 1998. **158**(5 Pt 1): p. 1518-23.
20. M Saetta, A.D.S., and P Maestrelli., *Airway eosinophilia in chronic bronchitis during exacerbations.* Am J Respir Crit Care Med, 1994. **150**(6): p. 1646-1652.
21. Fujimoto, K., Kubo Keishi, and Yamamoto, Hiroshi, *Eosinophilic Inflammation in the Airway Is Related to Glucocorticoid Reversibility in Patients With Pulmonary Emphysema.* Chest, 1999. **115**(3): p. 697-702.
22. Barnes., P., and J. , *Alveolar Macrophages as Orchestrators of COPD.* COPD: Journal of COPD, 2004. **1**(1): p. 59-70.
23. Marina Saetta, A.D.S., and Piero Maestrelli., *Activated T-Lymphocytes and Macrophages in Bronchial Mucosa of Subjects with Chronic Bronchitis.* Am Rev Respir Dis, 1993. **147**(2): p. 301-306.
24. Kirkham., S., and Rahman, Irfan., *Macrophage phagocytosis of apoptotic neutrophils is compromised by matrix proteins modified by cigarette smoke and lipid peroxidation products.* Biochem Biophys Res Commun, 2004. **318**(1): p. 32-37.
25. T C O'Shaughnessy., T.W.A., and N C Barne, *Inflammation in bronchial biopsies of subjects with chronic bronchitis: inverse relationship of CD8+ T lymphocytes with FEV1.* Am J Respir Crit Care Med, 1997. **155**(3): p. 852-857.
26. Robinson., H., and Buhl, R., *Revisiting Type 2-high and Type 2-low airway inflammation in asthma: current knowledge and therapeutic implications.* Clin Exp Allergy, 2017. **47**(2): p. 161-175.
27. Barnes, P.J. and M.G. Cosio, *Characterization of T lymphocytes in chronic obstructive pulmonary disease.* PLoS Med, 2004. **1**(1): p. e20.
28. Hirano, T., *Interleukin 6 and its receptor: ten years later.* Int Rev Immunol, 1998. **16**(3-4): p. 249-84.
29. Barnes, P., and J., *The cytokine network in asthma and chronic obstructive pulmonary disease.* J Clin Invest, 2008. **118**(11): p. 3546-56.
30. Grubek-Jaworska, P., and Hermanowicz-Salamon., *IL-6 and IL-13 in induced sputum of COPD and asthma patients: correlation with respiratory tests.* Respiration, 2012. **84**(2): p. 101-7.
31. Brightling, S., and Hollins, F., *Interleukin-13: prospects for new treatments.* Clin Exp Allergy, 2010. **40**(1): p. 42-9.
32. Liao, N., Zhao, H, and Chen. , *Association of the IL-13 polymorphisms rs1800925 and rs20541 with chronic obstructive pulmonary disease risk: An updated meta-analysis.* Medicine, Balt., 2017. **96**(47): p. e8556.
33. John, M., Au, B., and Jose, P., *Expression and release of interleukin-8 by human airway smooth muscle cells: inhibition by Th-2 cytokines and corticosteroids.* Am J Respir Cell Mol Biol, 1998. **18**(1): p. 84-90.
34. Yamamoto, Y., T., and Yoshikawa, M., *Airway inflammation in COPD assessed by sputum levels of interleukin-8.* Chest, 1997. **112**(2): p. 505-10.
35. Aldonyte, J., and Piitulainen., *Circulating monocytes from healthy individuals and COPD patients.* Respir Res, 2003. **4**: p. 11.
36. Faffe, D., Flynt, and Mellema, M., *Oncostatin M causes VEGF release from human airway smooth muscle: synergy with IL-1beta.* Am J Physiol Lung Cell Mol Physiol, 2005. **288**(6): p. L1040-8.

37. Volpi, F., and Moretto., *Cigarette smoke and alpha,beta-unsaturated aldehydes elicit VEGF release through the p38 MAPK pathway in human airway smooth muscle cells and lung fibroblasts.* Br J Pharmacol, 2011. **163**(3): p. 649-61.
38. Yuksel, H., Yilmaz, and Karaman, M., *Role of vascular endothelial growth factor antagonism on airway remodeling in asthma.* Ann Allergy Asthma Immunol, 2013. **110**(3): p. 150-5.
39. Bai, J., Song, and Cai. , *The association of monocyte chemotactic protein-1 and CC chemokine receptor 2 gene variants with chronic obstructive pulmonary disease.* DNA Cell Biol, 2012. **31**(6): p. 1058-63.
40. Marra, F., *Renaming cytokines: MCP-1, major chemokine in pancreatitis.* Gut, 2005. **54**(12): p. 1679-81.
41. Deshmane., K., and Amini., *Monocyte chemoattractant protein-1 (MCP-1): an overview.* J Interferon Cytokine Res, 2009. **29**(6): p. 313-26.
42. Ip, W.K., C.K. Wong, and C.W. Lam, *Interleukin (IL)-4 and IL-13 up-regulate monocyte chemoattractant protein-1 expression in human bronchial epithelial cells: involvement of p38 mitogen-activated protein kinase, extracellular signal-regulated kinase 1/2 and Janus kinase-2 but not c-Jun NH2-terminal kinase 1/2 signalling pathways.* Clin Exp Immunol, 2006. **145**(1): p. 162-72.
43. Traves, S.C., and Russell., *Increased levels of the chemokines GROalpha and MCP-1 in sputum samples from patients with COPD.* Thorax, 2002. **57**(7): p. 590-5.
44. Andrea M. Guerrero, M.D.S., and Brett Masters., *Evaluation of Systemic RANTES/CCL5 as a Possible Biomarker in Stable COPD Subjects.* Am J Respir Crit Care Med, 2017: p. A5251-A5251.
45. Yeruva, S., Ramadori, and D. Raddatz, *NF-kappaB-dependent synergistic regulation of CXCL10 gene expression by IL-1beta and IFN-gamma in human intestinal epithelial cell lines.* Int J Colorectal Dis, 2008. **23**(3): p. 305-17.
46. Ghebre, M., Pang, P. H., and Diver, S., *Biological exacerbation clusters demonstrate asthma and chronic obstructive pulmonary disease overlap with distinct mediator and microbiome profiles.* J Allergy Clin Immunol, 2018. **141**(6): p. 2027-2036.e12.
47. Liu, T., Lingyun Zhang, and Donghyun Joo., *NF-κB signaling in inflammation.* Signal Transduction And Targeted Therapy, 2017. **2**: p. 17023.
48. Wright, J.G. and J.W. Christman, *The Role of Nuclear Factor Kappa B in the Pathogenesis of Pulmonary Diseases: Implications for Therapy.* Am J of Res Med, 2003. **2**(3): p. 211-219.
49. Oeckinghaus, A. and S. Ghosh, *The NF-kappaB family of transcription factors and its regulation.* Cold Spring Harb Perspect Biol, 2009. **1**(4): p. a000034.
50. Schuliga, M., *NF-kappaB Signaling in Chronic Inflammatory Airway Disease.* Biomolecules, 2015. **5**(3): p. 1266-83.
51. Chapoval, S., Al-Garawi, A., and Elias, J. A., *Inhibition of NF-kappaB activation reduces the tissue effects of transgenic IL-13.* J Immunol, 2007. **179**(10): p. 7030-41.
52. Mori, N., Krensky, A. M., and Yamamoto, N., *Helicobacter pylori induces RANTES through activation of NF-kappa B.* Infect Immun, 2003. **71**(7): p. 3748-56.
53. Genin, P., Algarte, M., and Hiscott, J., *Regulation of RANTES chemokine gene expression requires cooperativity between NF-kappa B and IFN-regulatory factor transcription factors.* J Immunol, 2000. **164**(10): p. 5352-61.
54. Wang, J.C., Zhao, Y., and Long, H. B., *AOPPs induce MCP-1 expression by increasing ROS-mediated activation of the NF-kappaB pathway in rat mesangial cells: inhibition by sesquiterpene lactones.* Cell Physiol Biochem, 2013. **32**(6): p. 1867-77.

55. Yeruva, S., G. Ramadori, and D. Raddatz, *NF-kappaB-dependent synergistic regulation of CXCL10 gene expression by IL-1beta and IFN-gamma in human intestinal epithelial cell lines.* Int J Colorectal Dis, 2008. **23**(3): p. 305-17.
56. Volpi, G., F. Facchinetti, and R. Patacchini, *Cigarette smoke and α,β-unsaturated aldehydes elicit VEGF release through the p38 MAPK pathway in human airway smooth muscle cells and lung fibroblasts.* Br J Pharmacol, 2011. **163**(3): p. 649-61.
57. Alvira, C.M., *Nuclear factor-kappa-B signaling in lung development and disease: one pathway, numerous functions.* Birth Defects Res A Clin Mol Teratol, 2014. **100**(3): p. 202-16.
58. DeNiro, M., Al-Mohanna, F. H., and Al-Mohanna, F. A., *The nexus between VEGF and NFkappaB orchestrates a hypoxia-independent neovasculogenesis.* PLoS One, 2013. **8**(3): p. e59021.
59. Hinz, M. and C. Scheidereit, *The IkappaB kinase complex in NF-kappaB regulation and beyond.* EMBO Rep, 2014. **15**(1): p. 46-61.
60. Zhao, Y., Xu, Y., and Liu, Q., *NF-kappaB-mediated inflammation leading to EMT via miR-200c is involved in cell transformation induced by cigarette smoke extract.* Toxicol Sci, 2013. **135**(2): p. 265-76.
61. Badri, K.R., Y. Zhou, and L. Schuger, *Embryological origin of airway smooth muscle.* Proc Am Thorac Soc, 2008. **5**(1): p. 4-10.
62. Yan, F., Gao, H., and Zeng, Y., *Roles of airway smooth muscle dysfunction in chronic obstructive pulmonary disease.* J Transl Med, 2018. **16**(1): p. 262.
63. Dileepan, M., A.E. Sarver, and M.S. Kannan, *MicroRNA Mediated Chemokine Responses in Human Airway Smooth Muscle Cells.* PLoS One, 2016. **11**(3): p. e0150842.
64. Laniado-Laborín, R., *Smoking and chronic obstructive pulmonary disease (COPD). Parallel epidemics of the 21 century.* Int J Environ Res Public Health, 2009. **6**(1): p. 209-24.
65. World Health Organization-. *Causes of Chronic Obstructive Lung Disease*. 2013 [01-01-2019]; Available from: https://www.who.int/respiratory/copd/causes/en/.
66. Journal of the Chronic Obstructive Pulmonary Diseases Foundation. *National and State Estimates of COPD Morbidity and Mortality in United States*. 2014 [01-01-2019]; Available from: http://doi.org/10.15326/jcopdf.5.4.2018.0157
67. Liu, Y., Pleasants, R. A., and Strange, C., *Smoking duration, respiratory symptoms, and COPD in adults aged ≥45 years with a smoking history.* Int J Chron Obstruct Pulmon Dis, 2015. **10**: p. 1409-16.
68. Lee, P., Forey, BA, and Thornton, AJ, *Epidemiological evidence relating environmental smoke to COPD in lifelong non-smokers: a systematic review [version 1; peer review: 1 approved with reservations].* F1000Research, 2018. **7**(146).
69. Diver, W.R., E.J. Jacobs, and S.M. Gapstur, *Secondhand Smoke Exposure in Childhood and Adulthood in Relation to Adult Mortality Among Never Smokers.* Am J Prev Med 2018. **55**(3): p. 345-352.
70. Lee, J., V. Taneja, and R. Vassallo, *Cigarette smoking and inflammation: cellular and molecular mechanisms.* J Dent Res, 2012. **91**(2): p. 142-9.
71. Osadnik, C.R., C.F. McDonald, and A.E. Holland, *Clinical issues of mucus accumulation in COPD.* Int J Chron Obstruct Pulmon Dis, 2014. **9**: p. 301-2.
72. Tamimi, A., D. Serdarevic, and N.A. Hanania, *The effects of cigarette smoke on airway inflammation in asthma and COPD: therapeutic implications.* Respir Med, 2012. **106**(3): p. 319-28.
73. Oltmanns, U., et al., *Cigarette smoke induces IL-8, but inhibits eotaxin and RANTES release from airway smooth muscle.* Respir Res, 2005. **6**: p. 74.

74. de Moraes, M.R., da Costa, A. C., and Rabahi, M. F., *Interleukin-6 and interleukin-8 blood levels' poor association with the severity and clinical profile of ex-smokers with COPD.* Int J Chron Obstruct Pulmon Dis, 2014. **9**: p. 735-43.
75. Mio, T., Romberger, D. J., and Rennard, S. I., *Cigarette smoke induces interleukin-8 release from human bronchial epithelial cells.* Am J Respir Crit Care Med, 1997. **155**(5): p. 1770-6.
76. Blidberg, K., Palmberg, L., and Larsson, K., *Increased neutrophil migration in smokers with or without chronic obstructive pulmonary disease.* Respirology, 2012. **17**(5): p. 854-60.
77. Baraldo, S., Turato, G, and Saetta, M, *Neutrophilic infiltration within the airway smooth muscle in patients with COPD.* Thorax, 2004. **59**(4): p. 308-312.
78. Zhao, J., Li, Xiang, and Xie, Jianping, *Immunomodulatory effects of cigarette smoke condensate in mouse macrophage cell line.* Int J Immunopathol Pharmacol 2017. **30**(3): p. 315-321.
79. Maeno, T., Houghton, A. M., and Shapiro, S. D., *CD8+ T Cells are required for inflammation and destruction in cigarette smoke-induced emphysema in mice.* J Immunol, 2007. **178**(12): p. 8090-6.
80. Paul D. Scandlon, J.E., and Donlald P.Tashkin. , *Smoking Cessation and Lung Function in Mild-to-Moderate Chronic Obstructive Pulmonary Disease.* Am J Respir Crit Care Med., 2000. **161**(2): p. 381-390.
81. Hasnis, E., Bar-Shai, M., and Reznick, A. Z., *Cigarette smoke-induced NF-kappaB activation in human lymphocytes: the effect of low and high exposure to gas phase of cigarette smoke.* J Physiol Pharmacol, 2007. **58 Suppl 5**(Pt 1): p. 263-74.
82. Di Stefano, A., Caramori, G., and Adcock, I. M., *Increased expression of nuclear factor-kappaB in bronchial biopsies from smokers and patients with COPD.* Eur Respir J, 2002. **20**(3): p. 556-63.
83. Zhang, C., Qin, S., and Wang, X., *Cigarette smoke extract-induced p120-mediated NF-κB activation in human epithelial cells is dependent on the RhoA/ROCK pathway.* Sci Rep, 2016. **6**: p. 23131.
84. Zhang, G., Wang, Zhangli, and Liu, Enqi, *Safety Assessment of Electronic Cigarettes and Their Relationship with Cardiovascular Disease.* Int J Environ Res Public Health, 2018. **15**.
85. Brown, C.J. and J.M. Cheng, *Electronic cigarettes: product characterisation and design considerations.* Tobacco Control, 2014. **23**(suppl 2): p. ii4-ii10.
86. Meier, E., A.P. Tackett, and T.L. Wagener, *Effectiveness of electronic aids for smoking cessation.* Curr Cardiovasc Risk Rep, 2013. **7**(6).
87. Action on Smoking and Health. *Use of e-Cigarettes among Adults in Great Britain*. [01-01-2019]; Available from: ash.org.uk/information-and-resources/fact-sheets/use-of-e-cigarettes-among-adults-in-great-britain-2017.
88. Wu, Q., D. Jiang, and H.W. Chu, *Electronic cigarette liquid increases inflammation and virus infection in primary human airway epithelial cells.* PLoS One, 2014. **9**(9): p. e108342.
89. Shields, P.G., Berman, Micah, and Wewers, Mark D., *A Review of Pulmonary Toxicity of Electronic Cigarettes in the Context of Smoking: A Focus on Inflammation.* Cancer Epidemiol Biomarkers Prev, 2017. **26**(8): p. 1175-1191.
90. Scott, A., S.T. Lugg, and D. Thickett, *Pro-inflammatory effects of e-cigarette vapour condensate on human alveolar macrophages.* Thorax, 2018. **73**(12): p. 1161-1169.
91. Garcia-Arcos, I., Geraghty, Patrick, and Foronjy, Robert, *Chronic electronic cigarette exposure in mice induces features of COPD in a nicotine-dependent manner.* Thorax, 2016. **71**(12): p. 1119-1129.
92. Behar, R.Z., Davis, B. and Talbot, P., *Identification of toxicants in cinnamon-flavored electronic cigarette refill fluids.* Toxicol in Vitro, 2014. **28**(2): p. 198-208.

93. Zaynagetdinov, R., Sherrill, T. P., and Blackwell, T. S., *Chronic NF-kappaB activation links COPD and lung cancer through generation of an immunosuppressive microenvironment in the lungs.* Oncotarget, 2016. **7**(5): p. 5470-82.
94. Chen, L., Ge, Q. and Oliver, B. G., *Effects of cigarette smoke extract on human airway smooth muscle cells in COPD.* Eur Respir J, 2014. **44**(3): p. 634-46.
95. Xue, H. and M.X. Li, *MicroRNA-150 protects against cigarette smoke-induced lung inflammation and airway epithelial cell apoptosis through repressing p53: MicroRNA-150 in CS-induced lung inflammation.* Hum Exp Toxicol, 2018. **37**(9): p. 920-928.
96. Leigh, N.J., Lawton, R. I.m, and Goniewicz, M. L., *Flavourings significantly affect inhalation toxicity of aerosol generated from electronic nicotine delivery systems (ENDS).* Tob Control, 2016. **25**(Suppl 2): p. ii81-ii87.
97. Higham, A., Bostock, D., and Singh, D., *The effect of electronic cigarette and tobacco smoke exposure on COPD bronchial epithelial cell inflammatory responses.* Int J Chron Obstruct Pulmon Dis, 2018. **13**: p. 989-1000.
98. Higham, A., N.J. Rattray, and D. Singh, *Electronic cigarette exposure triggers neutrophil inflammatory responses.* Respir Res, 2016. **17**(1): p. 56.
99. Lim, H.B. and S.H. Kim, *Inhallation of e-Cigarette Cartridge Solution Aggravates Allergen-induced Airway Inflammation and Hyper-responsiveness in Mice.* Toxicol Res, 2014. **30**(1): p. 13-8.
100. Zhao, Y., X. Wang, and Q. Liu, *NF-κB-Mediated Inflammation Leading to EMT via miR-200c Is Involved in Cell Transformation Induced By Cigarette Smoke Extract.* Toxicol Sci, 2013. **135**(2): p. 265-276.
101. Pace, E., M. Ferraro, and M. Gjomarkaj, *Cigarette smoke increases Toll-like receptor 4 and modifies lipopolysaccharide-mediated responses in airway epithelial cells.* Immunology, 2008. **124**(3): p. 401-11.