Effect of cigarette smoke extract on inflammatory cytokine production and corticosteroid insensitivity in human airway smooth muscle cells

Mushabbab Ali Mushabbab Alahmari RRT, RRT-ACCS, RRT-NPS, RRT-SDS, RPFT

Division of Respiratory Medicine

School of Medicine

The University of Nottingham

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Abbreviation

A

AA	Arachidonic acid
AC	Adenylyl cyclase
AHR	Airway hyperresponsiveness
AChE	Acetylcholinesterase
AERD	Aspirin-Exacerbated Respiratory Disease

B

BALF	Bronchoalveolar lavage fluid
β2M	βeta-2-Microglobulin
BCA	Bicinchoninic acid
BSA	Bovine serum albumin

C

cAMP	Cyclic adenosine monophosphate
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
cPGES	Cytosolic prostaglandin E synthase.
CS	Cigarette smoke

CSE	Cigarette smoke extract
C_T	Cycle threshold
cDNA	Complementary DNA

D

DAN	2, 3-diaminonaphthalene
DMEM	Dulbecco's modified eagles medium
DMEM-	DMEM with 0.5% FBS
DMEM+	DMEM with 20% FBS
DMSO	Dimethyl sulfoxide
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
dsDNA	Double-stranded DNA

E

ECL	Enhanced chemiluminescence
ERS	European Respiratory Society
ELISA	Enzyme-linked immunosorbent assay

F

FBS	Foetal bovine serum
FEV1	Forced expiratory volume (in one second)
FVC	Forced vital capacity
FW	Forward

G

GWAS	Genome-wide association studies
GSH	Glutathione
GR-β	Glucocorticoid receptor-β

H

HASMCs	Human airway smooth muscle cells
HRP	Horseradish peroxidase

I

IC_{50}	Half-maximal inhibitory concentration
IL-1β	Interleukin-1β
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-13	Interleukin-13
IP-10	Interferon gamma-induced protein 10
ICS	Inhaled corticosteroids (ICS)

J

JAK	Janus kinase

L

LABA	Long-acting β_2-agonist
LTRA	Leukotriene receptor antagonist

LPS	Lipopolysaccharide

M

MCh	Methacholine
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MDB	Membrane desalting buffer
mPGES-1	Microsomal prostaglandin E synthase-1
mPGES-2	Microsomal prostaglandin E synthase-2
mRNA	Messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

N

Non-T2	Non-type 2 asthme
NAChR	Nicotinic acetylcholine receptor
NSAID	Nonsteroidal anti-inflammatory drug

O

OVA	Ovalbumin

P

PBS	Phosphate buffered saline
PAECs	Pulmonary artery endothelial cells
PASMCs	Pulmonary artery smooth muscle cells
PGs	Prostaglandins

PGE_2	Prostaglandin E_2
PGD_2	Prostaglandin D_2
PGDS	Prostaglandin D synthase
PGH_2	Prostaglandin H_2
PGI_2	Prostaglandin I_2
PVDF	Polyvinylidene fluoride

R

ROS	Reactive oxygen species
RIPA	Radio-immunoprecipitation assay
RT	Reverse transcription
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
RV	Reverse

S

SABA	Short-acting β_2-adrenoreceptor agonists
SEM	Standard error of the mean
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
STAT	Signal transducer and activator of transcription

T

T2 asthma	Type 2 asthma
TNFα	Tumour necrosis factor alpha

TSLP	Thymic stromal lymphopoietin
TBS-T	Tris-buffered saline plus tween 20
TGF-β1	Transforming growth factor-β1
TXA_2	Thromboxane A_2
TXAS	Thromboxane synthase
TXB_2	Thromboxane B_2

V

VEGF	Vascular endothelial growth factor

Abstract

Background: Asthma is a chronic lung disease characterised by the presence of one or more respiratory symptoms, such as wheezing, chest tightness, breathlessness and coughing, which are attributed to airflow obstruction, airway hyperresponsiveness and airway inflammation [1]. Airway inflammation in asthma is categorised into endotypes—namely Type 2 (T2, eosinophilic and T2-high) asthma, which has a good response to corticosteroid treatment [2], and non-Type 2 (non-T2, neutrophilic and T2-low) asthma, which has a poor response to corticosteroid treatment. Human airway smooth muscles (HASMCs) are crucial components of the airway structural wall because they display an inflammatory response by secreting cytokines and chemokines in response to inflammatory stimuli [3, 4]. Cigarette smoke (CS) is a known risk factor in developing airway inflammation, which can lead to asthma. Clinical studies have shown that asthmatic smokers tend to have low eosinophil counts and high neutrophil counts and display a poor response to corticosteroid therapy. But whether CS can modulate T2 and non-T2 inflammatory responses and induce corticosteroid insensitivity *in vitro* is largely unknown. It has been suggested that various mechanisms such as oxidative stress and cyclooxygenase-2 (COX-2) induction may play a key role in mediating the CS effect. However, the mechanisms underlying the proinflammatory effect of CS in asthma remain to be explored. We hypothesised that CS extract (CSE) may inhibit the production of T2 inflammatory cytokines (Th2 cytokines and eosinophil chemokines) and induce non-T2 inflammatory cytokines (IL-6, IL-8, and VEGF) in

HASMCs via oxidative stress and COX-2 induction, thereby contributing to the development of corticosteroid-insensitive non-T2 endotype in asthma smokers.

Methods: CSE was prepared from the smoke of two cigarettes (3R4F research-grade cigarettes) in 20 ml of cell culture medium. Confluent and serum-starved HASMCs were used at passage 6. TNFα was used to mimic airway inflammation. A Bio-plex Pro^{TM} Human Cytokine Assay and ELISA kits were used to detect the concentration of inflammatory cytokines and the prostaglandin E_2 production, respectively, in the cell culture supernatants. RT-qPCR and Western blot were used to assess the mRNA and protein expression of the genes. GraphPad prism was used to calculate the IC_{50} of fluticasone.

Results: CSE inhibited the production of Th2 cytokines IL-4 and IL-13 and eosinophil chemokines (eotaxin, IP-10 and RANTES), but induced the production and mRNA expression of non-T2 inflammatory cytokines (IL-6, IL-8 and VEGF) in HASMCs. TNFα alone induced the production of T2 inflammatory cytokines and non-T2 inflammatory cytokines. CSE inhibited TNFα-induced production of Th2 cytokines IL-4 and IL-13 and eosinophil chemokines, but it caused an additive effect on TNFα-induced IL-8 production compared with TNFα alone. CSE-induced IL-6 and IL-8 was inhibited by the oxidative stress inhibitor glutathione (GSH). In addition, the COX-2 inhibitor NS-398 inhibited CSE-induced IL-6, IL-8 and VEGF. CSE induced the protein and mRNA expression of COX-2, microsomal prostaglandin E synthase-1 (mPGES-1) and PGE_2 production in HASMC, and GSH inhibited CSE-induced the protein expression of COX-2 and PGE_2 production. Moreover, exogenous PGE_2 increased the production of IL-6, IL-8 and VEGF, and an additive effect on the

production of these cytokines was observed when both exogenous PGE_2 and CSE were used to stimulate HASMCs. In addition, the EP_2 receptor antagonist PF 04418948 and the EP_4 receptor antagonist L-161,982 inhibited CSE-induced IL-6, IL-8 and VEGF. The cAMP activator forskolin induced the production of IL-6, IL-8 and VEGF, and further induction of IL-6 and VEGF, but not IL-8, was observed when HASMCs were treated with both forskolin and CSE. Interestingly, the β_2-agonist salbutamol also significantly induced the production of IL-6, IL-8 and VEGF in HASMCs. The corticosteroid fluticasone inhibited CSE- and TNFα-induced the production of IL-8 in a concentration-dependent manner and the inhibitory effect of fluticasone on TNFα-induced production of IL-8 was not affected by CSE in HASMCs.

Conclusion: Our results show that CSE can modify the inflammatory responses in HASMCs, inducing a shift from T2 airway inflammation to non-T2 airway inflammation by suppressing T2 inflammatory response and promoting non-T2 inflammatory response, largely through COX-2/mPGES-1/PGE_2/EP_2/EP_4/cAMP pathway via oxidative stress, thereby contributing to the development of corticosteroid-insensitive non-T2 endotype in asthma smokers. Our results also suggest that targeting the COX-2 pathway may provide potentially novel therapeutics for controlling non-T2 airway inflammation in asthma smokers

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Chapter 1. Introduction

1.1 Asthma

1.1.1 Definition

British guidelines have defined asthma as the presence of one or more respiratory symptoms such as wheezing, chest tightness, breathlessness and coughing that can be attributed to airflow obstruction, airway hyperresponsiveness and inflammation of the airways [1]. The presence of isolated symptoms of coughing, breathlessness or chest tightness is not sufficient for a diagnosis of asthma; rather, the combination of these signs and symptoms establishes a diagnosis in the clinical setting [1]. The causes and natural history of asthma differ from patient to patient [5]. Asthma is associated with recurrent bouts of reversible bronchoconstriction that resolve either with medication or spontaneously [1]. A strong indicator of asthma is the reversibility of airflow obstruction with the use of bronchodilators or corticosteroids, as measured by a 12% improvement in forced expiratory volume in 1 sec (FEV_1) or increase in FEV_1 volume to 400 ml [1]. Chronic inflammation of bronchi is promoted by cells such as mast cells, eosinophils, T-lymphocytes, macrophages, neutrophils, epithelial cells [6] and airway smooth muscle cells [7] and the various inflammatory mediators they release. This inflammation is responsible for narrowing the airways, resulting in airflow obstruction and increasing bronchial responsiveness to various stimuli, which can trigger an asthma attack [6, 7].

1.1.2 Epidemiology of asthma: prevalence and impact

Asthma is one of the most common chronic respiratory diseases globally and affects individuals of all ages. It has a significant impact on the quality of life of these individuals. Asthma affects around 8 million people in the UK, accounting for approximately 12% of the population. However, some people may have grown out of the disease, and 5.4 million people are being treated for asthma. Each year, over 160,000 persons in the UK are diagnosed with asthma; however, incidence rates decreased by approximately 10% between 2008 and 2012 [8].

Asthma imposes a huge burden on the UK economy, as evident in a recent study. According to the results, asthma imposed an excess of £1.1 billion on healthcare in the UK. Most of these costs were attributed to prescriptions, inpatient exacerbation attacks and deaths because of asthma [9, 10]. In addition to health service utilisation, asthma imposes financial and societal burdens by increasing morbidity and mortality rates in patients. The societal burden arises because of school or work absenteeism, disability living allowances, care-at-home services and asthma-related premature deaths [10].

1.1.3 Risk factors

Asthma has multiple triggers, including infections, genetic and environmental factors [11, 12]. Viral infections are a common trigger of asthma, and human rhinovirus (HRV) and respiratory syncytial virus (RSV) have been reported to be associated with triggering an asthmatic attack [13, 14].

Genetics also play an essential role in the development of asthma [15]. Over the past decade, several genome-wide association studies (GWAS) have identified numerous genes contributing to an individual's susceptibility to developing asthma [16]. A meta-analysis of genetic studies reported hundreds of different genes as being associated with asthma [17]. Genetic polymorphisms in the genes encoding interleukin (IL)-4 and IL-13 have been linked to increased susceptibility to asthma, as demonstrated in a recent meta-analysis [18]. Another study showed that abnormal genetic interactions and environmental triggers can determine asthma development [19].

In addition, cigarette smoke (CS), one of the environmental factors which has been reported to play a role in airway inflammatory responses [20]. Asthma and CS interplay to induce adverse effects on therapeutic outcomes [21]. Several reviews and studies have found strong evidence linking parental smoking to the development of asthma in children [22-24]. Furthermore, there is a correlation between exposure to passive smoke and the development of asthma in both adults and adolescents [25]. Interestingly, passive smoke exposure during childhood has been linked to an increased risk of developing asthma as an adult [26]. In addition, *Strzelak et al*. showed that CS increases the release of IL-17A, a potent pro-inflammatory cytokine associated with the pathogenesis of asthma [20]. Furthermore, it has been reported that CS increases the total immunoglobulin E (IgE) concentration which plays a role in the pathogenesis of asthma [27]. These observations suggest that CS is a potential risk factor for the development of asthma in all ages.

1.2 Treatment for asthma

The underlying goal of asthma management is to reduce asthma symptoms, morbidity and exacerbations [28]. Bronchodilators short-acting beta-agonists (SABAs) and long-acting beta-agonists (LABAs), inhaled corticosteroids (ICSs), anti-leukotrienes [29] and antibodies are the most common forms of asthma treatment [30-32].

1.2.1 Bronchodilators

SABAs mainly treat acute episodes and relieve bronchospasms, while LABAs control asthma's chronic symptoms [28]. Treatment with SABAs is the first step in managing asthma; these drugs are taken as needed by the patient to control asthma symptoms. SABAs specifically target β_2 adrenergic receptors, which are present in human airway smooth muscle cells (HASMCs) and, upon activation, cause bronchial smooth muscle relaxation, leading to bronchodilation. Salbutamol, levalbuterol and metaproterenol are some drugs categorised as SABA bronchodilators [28, 33]. SABAs are considered quick relievers, causing instant bronchodilation lasting for four to six hours. They are delivered via inhalers and begin the bronchodilation process within minutes; thus, they are an excellent choice of therapy for managing acute exacerbations. However, it is essential to note that these are prescribed for short-term use and only as required. Increased use of SABAs in a patient should encourage the physician to review the patient's asthma management plan [34].

Although SABAs provide instant relief, one of the disadvantages of SABAs is that they are ineffective in controlling nocturnal symptoms because of their short

duration of action. Therefore, the use of a long-acting bronchodilator has shown promising results in the management of asthma [33].

It has been reported that an improvement in asthma outcomes was observed when LABAs were used with ICSs; however, as a type of monotherapy, LABAs have shown to increase the risk of mortality in asthma patients [35]. This could be because of the constant bronchodilation masking of the inflammatory effect in asthma. However, when used with ICSs, LABAs exhibit a steroid-sparing effect, thus preventing the side effects of steroids in patients taking high doses of ICS [35]. Studies have also shown enhancement of the anti-inflammatory action of ICSs when used with LABAs such as salmeterol or formoterol [36].

1.2.2 Inhaled corticosteroids in asthma

ICSs are anti-inflammatory drugs used to control asthmatic patients; they function by reducing airway inflammation, exacerbation, emergency visits or hospitalisations and airway hyper-responsiveness (AHR) incidents [37]. The regular use of ICSs reduces the responsiveness of the bronchi to asthma stimuli [38]. Using inhalation as the route of administration drastically reduces the adverse effects associated with steroid use [38, 39]. ICSs function by decreasing the transcription of genes related to inflammatory cytokine production [40].

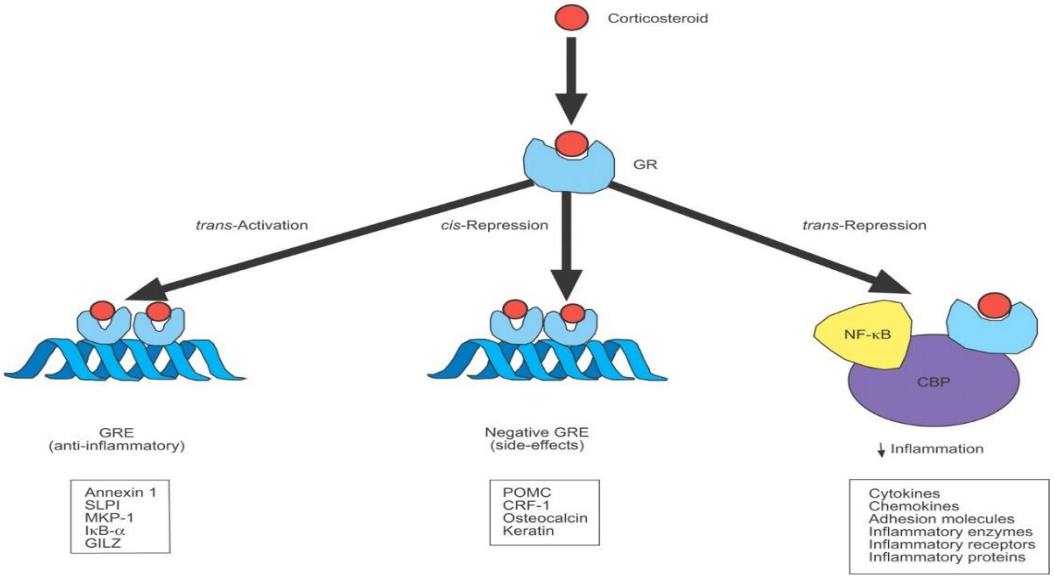

Figure 1.1. Corticosteroid binding with GRα

This Figure depicts the interaction of corticosteroids with glucocorticoid receptors (GRα) in the cytoplasm and translocation of this complex into the nucleus. These corticosteroid-GRα complexes interact with the glucocorticoid response element (GRE) on steroid-sensitive genes, producing anti-inflammatory proteins. Sometimes, these corticosteroid-GRα complexes interact with a negative GRE, resulting in the development of side effects of corticosteroids. Within the nucleus, the corticosteroid-GRα complex also interacts and switches off the cAMP–response element-binding protein (CBP), a transcription factor for inflammatory genes activated by NF-κB. Adapted from Barnes [41].

Glucocorticoids (GCs) bind to glucocorticoid receptor α (GRα; present in the cytoplasm) and activate and translocate these receptors into the nucleus [40]. Glucocorticoid receptor β (GRβ) is a spliced form of GR that does not bind glucocorticoids but instead interacts with DNA and acts as a potent inhibitor of the GC effect [40]. In the nucleus, the GR homodimer binds to glucocorticoid response elements (GREs), thereby activating gene transcription or less commonly switching off gene transcription [40]. Altered gene transcription may result in the suppression of inflammatory mediators such as TNF-α and IL-8, as well as mitogen-activated protein kinase phosphatase-1, which is a potent inhibitor of the mitogen-activated protein kinase (MAPK) pathway [40]. The GR homodimer may bind to NF-κB and deactivate it in a process called trans-repression, which contributes to the anti-inflammatory effect of GCs [42] (Figure 1). Although GCs have a broad spectrum of gene targets associated with the anti-inflammatory process [40], studies have shown that responsiveness to ICS therapy among asthmatic patients is not the same and that some patients require high doses of ICS [43]. Fluticasone propionate (FP) and budesonide (BUD) are two types of ICSs that have become the therapy of choice for chronic asthma [44].

1.2.2.1 Fluticasone propionate

FP has been widely used either as a monotherapy or combined with LABAs [45]. FP controls asthma in a dose-dependent manner, with increased control of the symptoms observed in patients treated with higher doses [45]. A randomised control trial found that FP at a dosage of 200 µg twice daily improved lung function and reduced asthma exacerbation to a greater extent than lower dosages of FP [45]. FP is

extremely lipophilic, which increases its ability to bind rapidly and strongly to GR, hence leading to prolonged retention of the drug [46]. FP has a higher potency than BUD, which is hydrophilic and does not have a rapid and strong binding affinity for GR. The therapeutic efficacy of FP is better than that of BUD because of its greater GR binding affinity, which increases the time it is bound to the GR, thereby increasing the duration of its anti-inflammatory action [47].

1.3 Inflammatory cells and asthma

Airway inflammation is the underlying cause of limited airflow and exerts its effects through bronchoconstriction, airway oedema, airway remodelling and AHR in asthma [48-51]. Inflammatory cells include the adaptive immune system, such as T-helper (Th1, Th2 and Th17) cells, and cells from the innate immune system, such as eosinophils and neutrophils, play a critical role in airway inflammation in asthma [52]. These inflammatory cells, along with some structural cells of the airways, such as epithelial cells and airway smooth muscle cells, are involved in the tissue injury and repair processes that occur during asthma [53]. Moreover, inflammatory mediators, such as cytokines, chemokines and growth factors, are released from the inflammatory cells and play a significant role in the airway inflammatory process [54]. It has been reported that the severity of asthma is determined by the level of cytokines released because they can modulate airway inflammation in asthma [55, 56].

Th1 cells have been associated with antagonising the effect of Th2 receptors; recent findings suggest that they play a significant role in airway inflammation. This is attributed to the presence of high levels of interferon (IFN)-γ, a Th1 cytokine, in the

airways, which is known to promote neutrophilic inflammation [57, 58]. IFN-γ is associated with increased inflammatory infiltrates, corticosteroid resistance and airway resistance, causing AHR. Therefore, Th1 cells have been related to the non-T2 asthma endotype [59].

Th2 cells play a significant role in the pathogenesis of T2 inflammatory processes in asthmatic patients by releasing cytokines such as IL-4, IL-5 and IL-13, which are known as Th2 cytokines; these cytokines have been associated with eosinophilic infiltration, mucus hypersecretion, IgE production, bronchial hyperresponsiveness (BHR) and asthma exacerbations [58]. Asthmatic patients have been shown to display increased Th2 cell counts in the bronchial mucosa and bronchoalveolar lavage (BAL) fluid [57]. Th2 effector cells migrate to the airways and compel airway cells to recognise and react to environmental allergens. Thus, Th2 cells induce various immunopathologic features of allergic asthma and are associated with T2 airway inflammation [59, 60].

Th17 cells play a significant role in developing severe asthma phenotypes that are insensitive to corticosteroid therapies [61]. The cytokines released by Th17 cells include IL-17A, IL-17F, IL-22 and IL-21, and they play a significant role in the pathogenesis of non-T2 asthma [61]. For example, IL-17A induces the production of IL-8, which recruits neutrophils and promotes neutrophilic airway inflammation in asthma [59, 62]. These cytokines cause increased mucus production through mucus cell metaplasia, increased neutrophilic infiltration and ASMC proliferation-migration, hence leading to increased airway remodelling. Moreover, these cytokines increase

airway reactivity, leading to BHR, and are known to induce corticosteroid resistance [59, 62].

Neutrophils are associated with corticosteroid insensitivity in asthma, as well as asthma exacerbation. Th17-mediated asthma is most strongly associated with neutrophilic infiltration; however, it may also play a role during certain episodes of allergic asthma exacerbations [63, 64]. IL-17, which is secreted from Th17 cells, promotes the differentiation of CD34+ progenitor cells into neutrophils and is also associated with inducing the production of IL-8, a known chemoattractant for neutrophils, within inflamed airways. Therefore, IL-17 promotes the infiltration and adhesion of neutrophils, while neutrophil recruitment and migration into the airways are mediated by IL-8, collectively leading to the development of a non-T2 asthma endotype [63].

Eosinophils are a trademark feature of T2 asthma, which have IL-4, IL-5, IL-13, IL-33, TSLP and transforming growth factor β (TGF-β) receptors on their cell surfaces [65]. These cells also express chemokine receptor (CCR) 3 and CCR1, which play a significant role in airway inflammation and become activated in the presence of eosinophil chemokines (eotaxin, IP-10) and regulated upon activation; normal T cell expressed and secreted (RANTES). It also has receptors for leukotrienes, platelet-derived growth factors and prostaglandins, all of which have a significant role in the pathophysiology of asthma [65].

1.4 Asthma endotypes

Asthma is a complex disease with a biological network of different inflammatory pathways. Asthma can be classified based on the variable clinical presentation, phenotype, and mechanistic pathway, endotypes [66]. The differentiation between asthma endotypes is predominantly based on the response from CD4+ T cells. CD4+ T cells have distinct subsets, mainly T helper type 1 and 2 (Th1 and Th2), of which Th2 cells are significantly associated with eosinophilic airway inflammatory responses in asthma patients. Airway inflammation in asthma is categorised into endotypes—namely Type 2 (T2, eosinophilic and T2-high) asthma and non-Type 2 (non-T2, neutrophilic and T2-low) asthma (Table 1) [59]. The two endotypes of asthma can be identified based on the presence or absence of eosinophilia in the airways [59].

Table 1.1. Difference between T2 and non-T2 asthma endotypes.

	T2 Asthma	**Non-T2 Asthma**
Common phenotypes	• Atopic	• Non-atopic • Smoking
Inflammatory mediators	• IL-4 • IL-5 • IL-13 • Eosinophil chemokines	• IL-17 • IL-8 • IL-6
Biomarkers	• Eosinophils	• Neutrophils
Treatment	• Corticosteroids • Biologics: - Mepolizuma - Reslizumab - Benralizumab - Dupilumab - Omalizumab	• Corticosteroids (poor responsiveness) • Macrolides

1.4.1 T2 inflammatory cytokines in asthma

T2 asthma is characterised by eosinophilic airway inflammation and is responsive to corticosteroid therapy [67]. Th2 cells play a significant role in the pathogenesis of T2 inflammatory processes in asthmatic patients by releasing cytokines like IL-4, IL-5 and IL-13, which are known as Th2 cytokines. These cytokines associated with eosinophilic infiltration, mucus hypersecretion, IgE production, BHR and asthma exacerbations [58]. Around 50% of asthmatic patients show increased Th2 cell counts in the bronchial mucosa and BAL fluid [57, 68]. Furthermore, there is a correlation between the level of eosinophils, production of Th2 cytokines (IL-4, IL-5 and IL-13) and eosinophil chemokines (eotaxin, IP-10 and RANTES) in asthma [69, 70]; in addition, the synergy between Th2 cytokines and eosinophil chemokines plays a significant role in eosinophil recruitment *in vivo* [71].

IL-4 is a cytokine released from Th2 cells, eosinophils, basophils, mast cells and macrophages. IL-4 is responsible for augmenting the polarisation of T-cell differentiation towards Th2 cells and increasing IgE synthesis by B cells [72]. Many cell types produced IL-4 which binds to IL-4 receptor α (IL4Rα), which is expressed on T-lymphocytes, B-lymphocytes, eosinophils, mononuclear phagocytes, endothelial cells, lung fibroblasts, bronchial epithelial cells, and smooth muscle cells. IL4Rα functions through two receptor complexes (Types I and II). Type I is specific to IL-4 signalling, while the Type II complex is involved in both IL-4 and IL-13 signalling. This could be the underlying cause behind the overlapping functions of IL-4 and IL-13 in the pathogenesis of T2 asthma [72]. IL-4 is also associated with increased mucus production through stimulating mucosal gland cells, thus participating in airway

remodelling. Therefore, IL-4 plays a significant role in the pathogenesis of T2 asthma [72].

IL-5 is an essential cytokine in asthma released by Th2 cells, mast cells and eosinophils [32]. IL-5 promotes the maturation, proliferation, migration and survival of eosinophils [73]. *Pelaia et al*. showed that allergen inhalation by allergic asthmatic patients caused an increase in eosinophil production and increase in IL-5 levels [32]. Eosinophils are known to express the highly specific IL-5 receptor (IL-5Rα) on their surface, promoting the activation of eosinophils and aiding in eosinophil-induced AHR [74], which eventually promotes airway inflammation in asthma [75].

IL-13 is a Th2 cytokine associated with the augmentation of AHR, mucus hypersecretion, airway remodelling and inflammation in asthma [76]. As explained above, IL-13 attaches to the type II IL4Rα receptor complex; this causes activation of the Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway, leading to the release of Th2 cytokines including IL-13 through the regulation of gene transcription [77]. Several studies have demonstrated that IL-13 plays a significant role in goblet cell hyperplasia and increases MUC5AC expression in the bronchial epithelium, in turn increasing mucus production in asthma. Bronchial biopsies have revealed increased levels of IL-13 mRNA in atopic and non-atopic asthmatic patients [76]. IL-13 contributes to eosinophilia by augmenting the production of various eosinophil chemokines, including RANTES and eotaxin, by inflammatory cells. IL-13 has been associated with eosinophil migration, survival and airway activation; thus, it is associated with the T2 asthma endotype [77].

Eotaxin is a potent eosinophil chemoattractant that plays a vital role in the pathogenesis of T2 airway inflammation in asthma. Th2 cytokines, such as IL-13, induced eotaxin, leading to recruit eosinophils in the airway, thereby contributing to T2 airway inflammation in asthma [78]. Studies have shown that IL-4- and IL-13-induced eosinophil recruitment *in vitro* was also associated with an increase in the mRNA and protein expression of eotaxin. In addition, eotaxin mRNA expression was shown to be inhibited by an anti-IL-4 antibody, which suggests eotaxin plays an important role in development of T2 airway inflammation [78].

RANTES is a potent chemoattractant for eosinophils, thus playing an essential role in the pathogenesis of T2 airway inflammation in asthma; it is released from epithelial cells, macrophages and T cells. Higher levels of RANTES have been identified in the BAL of patients with allergic asthma, with the number of eosinophils within the airways correlating with the amount of RANTES. In addition to its effect on eosinophils, it is a chemoattractant for monocytes, basophils, memory T cells and mast cells [79].

IP-10 is another eosinophil chemoattractant that plays a critical role in the pathogenesis of T2 airway inflammation in asthma. Asthmatic patients have shown an increase in IP-10 levels in their bronchial alveolar lavage fluid (BALF) compared with controls [80]. An increase in IP-10 levels in the airways has been associated with increased AHR; therefore, it is related with the T2 asthma endotype [81].

1.4.2 Non-T2 inflammatory cytokines in asthma

Non-T2 asthma is associated with an increase in non-T2 inflammatory cytokines, such as the neutrophil chemoattractant IL-8 and other cytokines (e.g., IL-6 and VEGF) in the airways [59, 82, 83]. Lung injury in non-T2 asthma is triggered by stimuli such as CS, which promotes neutrophilic inflammation and corticosteroid resistance [59]. The underlying features are a lack of response to corticosteroid therapy and the activation of Th1 and/or Th17 cells. These cells are associated with a release of neutrophil cytokines and chemokines, such as IL-8, which recruit neutrophils to the airways. Upon activation, Th17 cells secrete cytokines such as IL-17A and IL-17F. IL-17 family cytokines promote corticosteroid insensitivity and neutrophil infiltration [84]. Non-T2 asthmatic patients have a higher risk of asthma exacerbation, poor corticosteroid response and increased association with smoking [59]. Several studies have demonstrated that IL-17A is a potent stimulant in inducing the production of different cytokines and growth factors, such as IL-6, IL-8, VEGF and TNF-α in fibroblasts, macrophages and mast cells [85, 86]. The levels of IL-17 have been shown to be increased in severe asthmatic patients [87]. Therefore, these cytokines could contribute to the development of non-T2 airway inflammation in asthma patients.

IL-6 is a pro-inflammatory cytokine released mainly from mast cells, dendritic cells, macrophages, neutrophils, fibroblasts, epithelial cells, endothelial cells and, to a lesser extent, T-helper cells [73]. The stimuli that lead to cell damage, such as CS, reactive oxygen species (ROS), viruses and some pro-inflammatory cytokines, are associated with the induction of IL-6 production [88]. IL-6 also suppresses the differentiation of naïve T cells into regulatory T cells, which are protective cells that

inhibit cytokine production and T-cell proliferation [89]. IL-6 binding to IL-6 receptors activates the MAPK pathways and STAT family transcription factors. Recent studies have shown increased levels of both IL-6 and soluble IL-6 receptors in the BAL and sputum of asthmatic patients [90]. Neutrophils are known to express most of the soluble IL-6 receptors at the site of airway inflammation in asthma. IL-6 plays a significant role in T-cell differentiation, promoting Th17 cell differentiation together with IL-1β. Therefore, IL-6 has been associated with non-T2 airway inflammation in asthma [90].

IL-8 is a potent chemokine secreted by various cells, including bronchial epithelial cells, HASMCs, macrophages, neutrophils and fibroblasts. IL-8 is associated with the progression of asthma via fixed obstructive ventilation dysfunction, which constitutes uncontrolled asthma [91]. IL-8 is involved in the recruitment and activation of neutrophils, promoting their accumulation within the airways. IL-8 levels are inversely proportional to pulmonary function and directly proportional to the severity of asthma [56]. IL-8 is associated with neutrophilic airway inflammation in asthma [56, 91]. *Zhang and Bai* conducted an observational case control study that measured IL-8 serum levels in patients with uncontrolled asthma and healthy controls receiving corticosteroids, here in accordance with the short-term step-up strategy by the Global Initiative of Asthma. The study showed that patients with uncontrolled asthma had increased IL-8 levels and a direct correlation between IL-8 levels and blood neutrophil counts [91]. Neutrophilic asthma was found to be associated with corticosteroid insensitivity; thus, IL-8 levels would be a useful biomarker to assess/predict the corticosteroid response in patients with non-T2 asthma [91].

VEGF is a potent angiogenic factor that plays an essential role in airway remodelling in different airway diseases, such as pulmonary hypertension, lung cancer, COPD and asthma [92]. One of the most significant functions of VEGF is its role in increasing angiogenesis in the medium and smaller airways, thus leading to thickened and oedematous airway walls that contribute to increased airway narrowing and facilitate the development of asthmatic symptoms. VEGF activates the endothelial cells of blood vessels, leading to increased migration of endothelial cells and the formation of the capillary lumen, augmenting angiogenesis within the airways. According to an observational study, the amount of VEGF was found to be elevated in stable asthmatics and even more elevated in those presenting with asthma exacerbation compared with non-asthmatic subjects [93].

1.5 HASMCs as a source of cytokines and chemokines in asthma

HASMCs are airway structural cells that play an important role in the pathogenesis of asthma [94] (Figure 2). There is an increase in the mass of HASMCs in asthma, which influences its contractility, leading to airway narrowing; this is associated with AHR, inflammation and remodelling in asthmatic patients [94, 95]. As HASMCs contract, the airway luminal diameter is reduced, which contributes to airflow restriction, wheezing and shortness of breath, all of which are clinical signs of asthma patients [7]. It has been shown that an increase in the HASMCs mass is linked to the severity of asthma [96]. The contraction of HASMCs in asthma is largely increased in response to several stimuli [7].

HASMCs produce numerous cytokines, chemokines and growth factors upon stimulation with TNF-α, IL-1β and IL-13 which contribute to airway inflammation in asthma [97]. Th2 cytokines and eosinophil chemokines have been shown to be produced by HASMCs in asthmatic patients [98-100]. IL-5 is induced from HASMCs in cultures when exposed to serum from asthmatic patients [98]. In addition, IL-13 and IL-5 have been found to be expressed by HASMCs *in vivo* [99]. IL-13, TNF-α and IL-1β can induce the expression of eosinophil chemokines eotaxin in HASMCs [7, 101]. Moreover, an *in vitro* study showed that the mRNA expression and production of RANTES was significantly increased by TNF-α in HASMCs [100]. These findings suggest that HASMCs play an important role in T2 airway inflammation.

HASMCs have also been shown to produce non-T2 inflammatory cytokines such as IL-6, IL-8 and VEGF [102]. In cultured HASMCs from asthmatic patients, the production of the neutrophilic chemoattractant IL-8 was found to be significantly higher compared with control (healthy subjects) [103]. Evidence has indicated elevated levels of IL-6 in HASMCs upon stimulation with IL-17 and Oncostatin M (OSM), which is a member of the IL-6 family [104, 105], suggesting that HASMCs respond to Th17 to produce these cytokines, which play an important role in non-T2 airway inflammation in asthma.

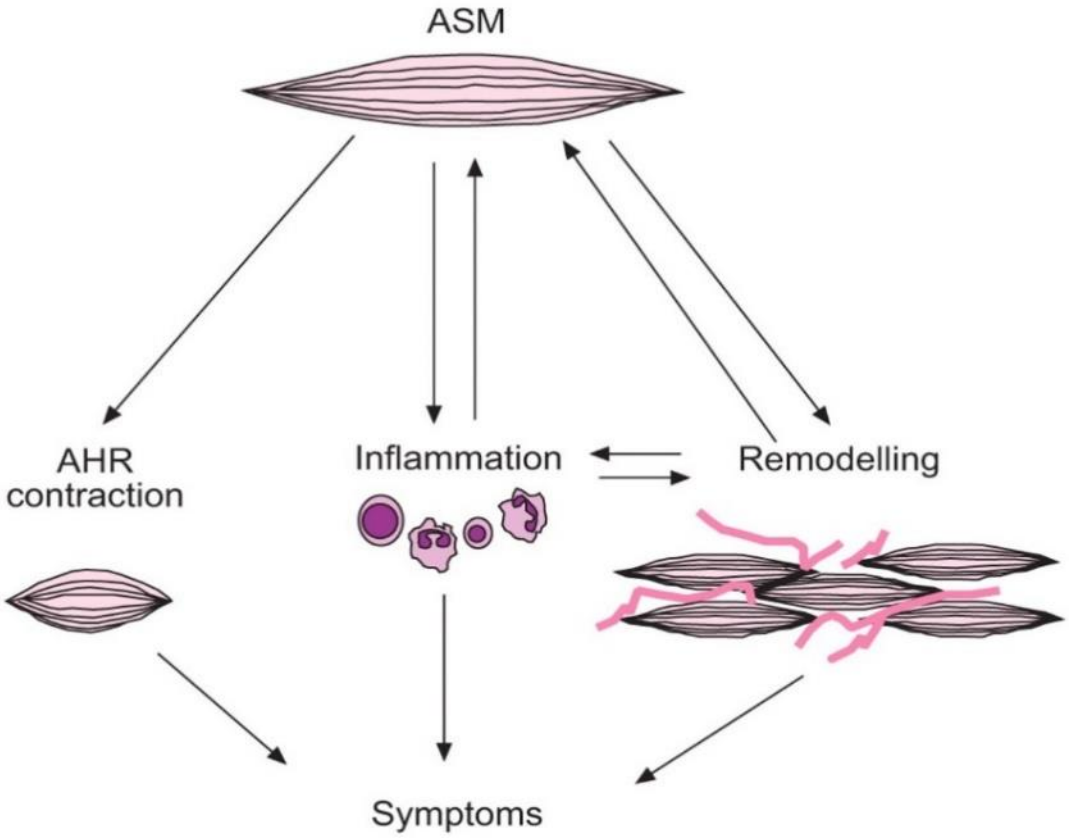

Figure 1.2. Role of HASMCs in asthma.

This Figure demonstrates the role HASMs in the pathogenesis of asthma. HASM contraction is responsible for increased AHR. HASM proliferation leads to airway remodelling, and HASM releases cytokines that promote airway inflammation. Adapted from Zuyderduyn [106].

1.6 Cigarette smoke and airway inflammation in asthma

CS is one of the risk factors associated with reduced lung function, increased asthma severity and corticosteroid insensitivity, which contribute to airway inflammation in asthma [107] (Figure 3).

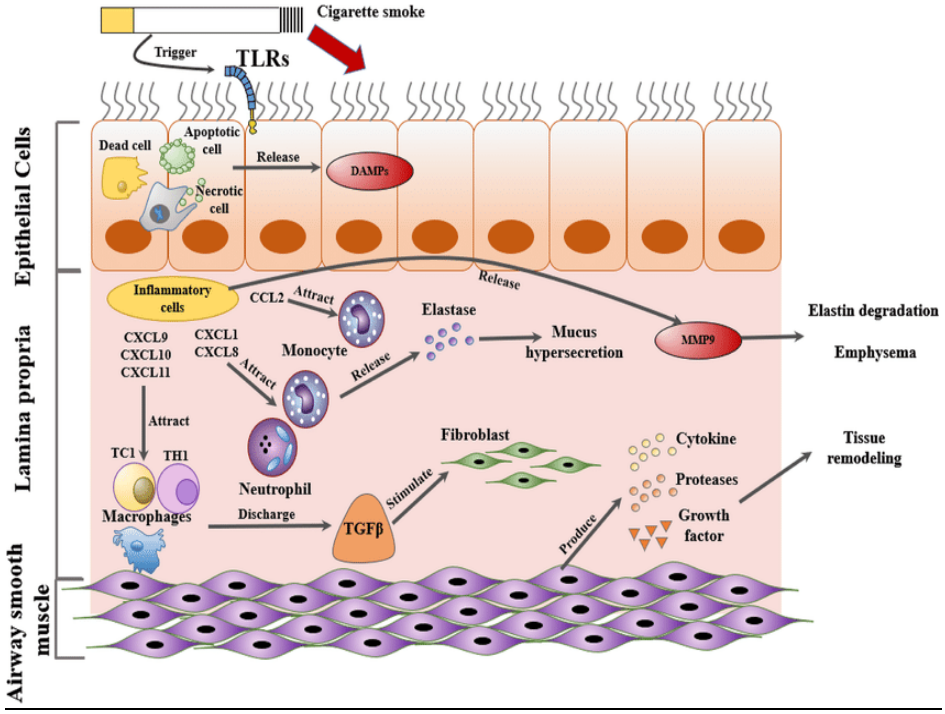

Figure 1.3. Effect of CS on the inflammatory response.

CS trigger Toll-like receptor (TLR), and necrotic, apoptotic, and dead cells start to produce damage-associated molecular patterns (DAMPs). Thereafter, neutrophils and monocyte were recruited by the activated inflammatory cells to injured region (Resolution). HASMCs produces the inflammatory cytokines and growth factors thereby contributed to airway inflammation in asthma. Adapted from Parya Aghasafari [108].

1.6.1 Cigarette smoke and T2 airway inflammation in asthma

Studies have shown a variety of responses to CS in different experimental models and cell types. In CS-treated mice, T cells were reportedly induced to express IL-5 [109]. In addition, CS increased the production of IL-4 in ovalbumin (OVA)-treated rats (asthmatic group) [110]. In another *in vivo* study, mice exposed to CS showed increased IL-5 in BALF, but their IL-4 and IL-13 levels did not change [111]. An *in vivo* study showed that OVA-treated mice exposed to CS demonstrated significant inhibition of eosinophil levels in their BALF compared with control [112]. In addition, *Oltmanns et al*. [113] and *Eddleston et al.* [114] examined the effect of CSE on the production of eosinophil chemokines RANTES and eotaxin in HASMCs [113] and human bronchial epithelial cells (16-HBE and BEAS-2B) [114, 115], demonstrated that CSE can suppress the production of RANTES and eotaxin, which suggests an association between CSE and eosinophil chemokines inhibition in airway structural cells in asthma. However, no studies have been conducted to investigate the effect of CSE on other T2 inflammatory cytokines, particularly Th2 cytokines such as IL4, IL-5 and IL-13, in HASMCs. These observations suggest that *in vivo*, exposure to CSE results in a large number of different cell types being exposed to CS making it difficult to discern whether the effect described is due to HASMs or another cell type.

1.6.2 Cigarette smoke and non-T2 airway inflammation in asthma

Clinical studies have shown that asthmatic smokers tend to have neutrophilic airway inflammation [116]. CS leads to increased neutrophilic infiltration because of increased production of IL-8 and IL-17A, as shown by the positive correlation between these cytokines and the number of cigarettes smoked as measured in pack years [117]. Various clinical studies have reported that CS induced corticosteroid insensitivity, making it difficult to control the symptoms of asthma [116]. Clinical studies have also provided statistically significant data showing improved asthma control with smoking cessation [20]. These observations suggest that CS play an important role in promoting non-T2 airway inflammation which contribute to corticosteroid insensitivity in asthmatic smokers.

Studies showed that 5% CSE, with or without methacholine chloride (MCh), induced the production of IL-6 in HASMCs [118]. This study was supported by another *in vitro* study using bronchial epithelial cells (HBECs), which reported that chronic exposure to CSE induced the production of IL-6 via the activation of nuclear factor-κB (NF-κB) [119]. In addition, CSE triggered the activation of the p38 MAPK signalling pathways, which may increase the production of VEGF and IL-8 in lung fibroblasts and HASMCs [120]. CSE at different concentrations 1%, 5% and 15% has been shown to induce the production of VEGF in HASMCs via NF-κB [121]. Furthermore, increased production of VEGF and IL-8 in the airways has been associated with angiogenesis, inflammation and airway remodelling [122]. These observations suggest that CSE may contribute to non-T2 airway inflammation in asthmatic smokers through IL-6, IL-8 and VEGF.

Neutrophilic asthma is associated with corticosteroid insensitivity; thus, IL-8 levels would be a useful biomarker in assessing the corticosteroid response in patients with uncontrolled asthma [91]. CSE can induce the production of IL-8 in HASMCs, which recruits neutrophils in the airway [113]. Moreover, CSE and TNFα have been shown to have a synergistic effect in increasing IL-8 production in HASMCs [107]. These findings indicate an association between CSE and neutrophilic airway inflammation in asthmatic smokers. However, the underlying mechanism of the effect of CSE on inflammatory cytokines is unclear.

1.7 Role of oxidative stress in airway inflammation in asthmatic smokers

Oxygen species, particularly free radicals, are induced during inflammation, contributing to airway inflammation in asthma [123]. Oxidative stress can occur when there is an imbalance between free radicals and antioxidants, resulting in an increase in reactive oxygen species (ROS) [124], which play a crucial role in airway inflammation in asthma [125].

There is extensive evidence linking oxidative stress to CSE-induced airway inflammation. It is widely recognised that oxidative stress plays a crucial role in the development of lung damage in smokers and ROS-induced infiltration of neutrophils in the airways [126]. Evidence has indicated that CSE increases the intracellular levels of ROS and results in an increased production of IL-6 and IL-8 levels in bronchial epithelial cells [127]. An *in vitro* study by *Oltmanns et al.* [128] demonstrated that a 30 minutes pre-treatment with an oxidative stress inhibitor glutathione (GSH), which is a potent antioxidant [129], showed an inhibition of CSE-induced IL-8 production in

HASMCs, suggesting that oxidative stress could play a role in the CSE-induced production of IL-8 [128].

1.8 Role of COX-2, PGE_2 and Cyclic adenosine monophosphate (cAMP) in airway inflammation in asthma

Arachidonic acid (AA) and its metabolite pathway play an important role in the pathogenies of airway inflammation in asthma [130]. AA is a polyunsaturated fatty acid (PUFA) that is found embedded in the cell membrane phospholipids [131], which can be metabolised by different pathways, including the cyclooxygenase (COX) pathway (Figure 1.4) [132].

1.8.1 COX-2

COX exists in two enzymes: COX-1 and COX-2. COX-1 is constitutively expressed in most cell types [133]. In contrast, COX-2 is an inducible enzyme upon stimulation with different stimuli like TNFα and IL-1 [134].

Different studies have examined the role of COX-2 expression in the pathogenesis of asthma. One study found a significant increase in COX-2 expression in the asthmatic bronchial epithelium compared with the non-asthmatic [135]. Another study showed that in asthmatic patients, the mRNA expression of COX-2 was higher in the asthmatic airway epithelium compared with non-asthmatic [136]. Several studies have examined the expression of COX-2 upon stimulation by different cytokines in asthma. For example, Th2 cytokines, namely IL-4 and IL-13, inhibit the expression of COX-2 induce by lipopolysaccharide (LPS) in bronchial epithelial cells [137]. In

contrast, TNFα-induced the expression of COX-2, leading to an increase in the production of PGE_2 in HASMCs [138]. CSE is a potent stimulant for inflammatory genes like COX-2 [139]. A recent study showed that CSE induced the expression of COX-2 in airway epithelial cells [140]. Another study conducted by *Lin et al.* showed that CSE induce the expression of COX-2 in human tracheal smooth muscle cells (HTSMCs) [141]. In an *in vivo* study, mice exposed to acute CS (1 h exposure) were found to have increased expression of COX-2 in their lung tissue, particularly in airway epitheliums [142]. In addition, our recent work showed that CSE induced the expression of COX-2 in human pulmonary artery smooth muscle cells (PASMCs) and pulmonary artery endothelial cells (PAECs), contributing to pulmonary vascular remodelling [143]. These findings suggest that the induction of COX-2 expression upon stimulation with CSE in HASMCs play a vital role in the pathogenesis of airway inflammation in asthma.

1.8.2 PGE_2

PGE_2 is a pro-inflammatory meditator that plays an essential role in airway inflammation [144]. AA is converted to PGH_2 via COX pathway, which can be further metabolised to PGE_2 via three different enzymes [144]: mPGES-1, mPGES-2 and cystosolic PGES (cPGES). Both mPGES-2 and cPGES are largely constitutively expressed in most cell types [145]. In contrast, mPGES-1 is an inducible enzyme [132, 146] that acts downstream of COX enzymes to produce PGE_2 [147]. Among the three distinct PGES enzymes, several studies have reported that mPGES-1 plays an important

role in the inflammatory process (144) and abolition of mPGES-1 could be a potential therapeutic target for the anti-inflammatory drug in airway inflammation [147].

An increase in the production of PGE_2 is one of the hallmarks of airway inflammation [146]. PGE_2 acts through four specific G protein–coupled prostaglandin receptors (GPCPR): EP_1, EP_2, EP_3 and EP_4. EP_1 and EP_3 receptors signalling cause smooth muscle contraction by increasing cell Ca^{2+} and reducing cAMP activity, respectively. In contrast, EP_2 and EP_4 receptors cause smooth muscle relaxation by increasing cAMP activity [148]. Therefore, the effect of PGE_2 signalling could contribute to either relaxing or constricting smooth muscles based on the activated receptors.

PGE_2 is synthesised by different cell types, such as HASMCs [149] and human epithelial cells [150]. Evidence has shown that, in asthmatic subjects, PGE_2 levels were inversely correlated to eosinophil levels in the sputum [151] and that T2 inflammatory cytokines IL-4, IL-5, and IL-13 decreased the production of PGE_2 in ASMCs [152]. Another study also showed that the production of PGE_2 was inhibited by IL-4 and IL-13 in lung fibroblast [153]. In contrast, PGE_2 was found to induce the production of non-T2 inflammatory cytokines, such as IL-6 and IL-8, in fibroblast cells [154]. This observation was supported by other studies conducted on HASMCs to explore the effect of exogenous PGE_2 on the production of non-T2 inflammatory cytokines. These studies found that exogenous PGE_2 (1.0 μM) increased the production of IL-8 [155] and VEGF [156], while a higher concentration (10 μM) of PGE_2 increased IL-6 [141] in HASMCs. Recently, several studies reported an association between CSE and PGE_2 production in airway cells. *Ren et al.* reported that CSE induced the production of PGE_2 in a human

bronchial epithelial cell line (HBE16) [157]. Another study also showed that treatment with CSE for 24 h increased the production of PGE_2 in endothelial cells [158, 159]. *Lin et al.* also showed that CSE treatment increased the production of PGE_2 in a time-dependent manner in HASMCs [141]. *Yang et al.* showed that the COX-2 inhibitor NS-398 inhibited CSE-induced PGE_2 production in HASMCs [139]. Together, these findings showed that the CSE and PGE_2 are potent stimulants for inflammatory cytokines suggesting that both CSE and PGE_2 could play an important role in the pathogenesis of airway inflammation in asthma. However, no study has investigated the role of PGE_2 in the effect of CSE on the inflammatory cytokines in airway structural cells.

1.8.3 cAMP

In most cells, the generation of cAMP occurs via the G protein–coupled receptor (GPCR) transmembrane signalling paradigm. The cAMP pathway is largely initiated upon the binding of specific ligands to GPCRs of the Gs family. Several studies identified numerous Gs-coupled receptors in ASMs, including EP_2 and EP_4 [160]. The activation of EP_2 and EP_4 receptors will cause an increase in cAMP activity [161]. A study showed that cAMP plays an important role in the regulation of inflammatory response in asthma [162]. Agents like PGE_2 and β_2 agonists, which activate receptors coupled to cAMP, regulated the expression of cytokines [163]. For example, Increased intracellular cAMP by PGE_2 and fenoterol inhibited the production of the T2 inflammatory cytokines IL-4 and IL-5 from isolated T cells [163]. In contrast, agents like PGE_2, β_2 agonists, and forskolin (FSK), an adenylyl cyclase (AC) activator,

increased cAMP activity which induced the production of non-T2 inflammatory cytokines IL-8 [164], IL-6 [165] and VEGF in HASMCs [166]. These observations suggest that increased intracellular cAMP modify airway inflammatory responses in asthma by suppressing T2 inflammatory response and promoting T2 inflammatory response.

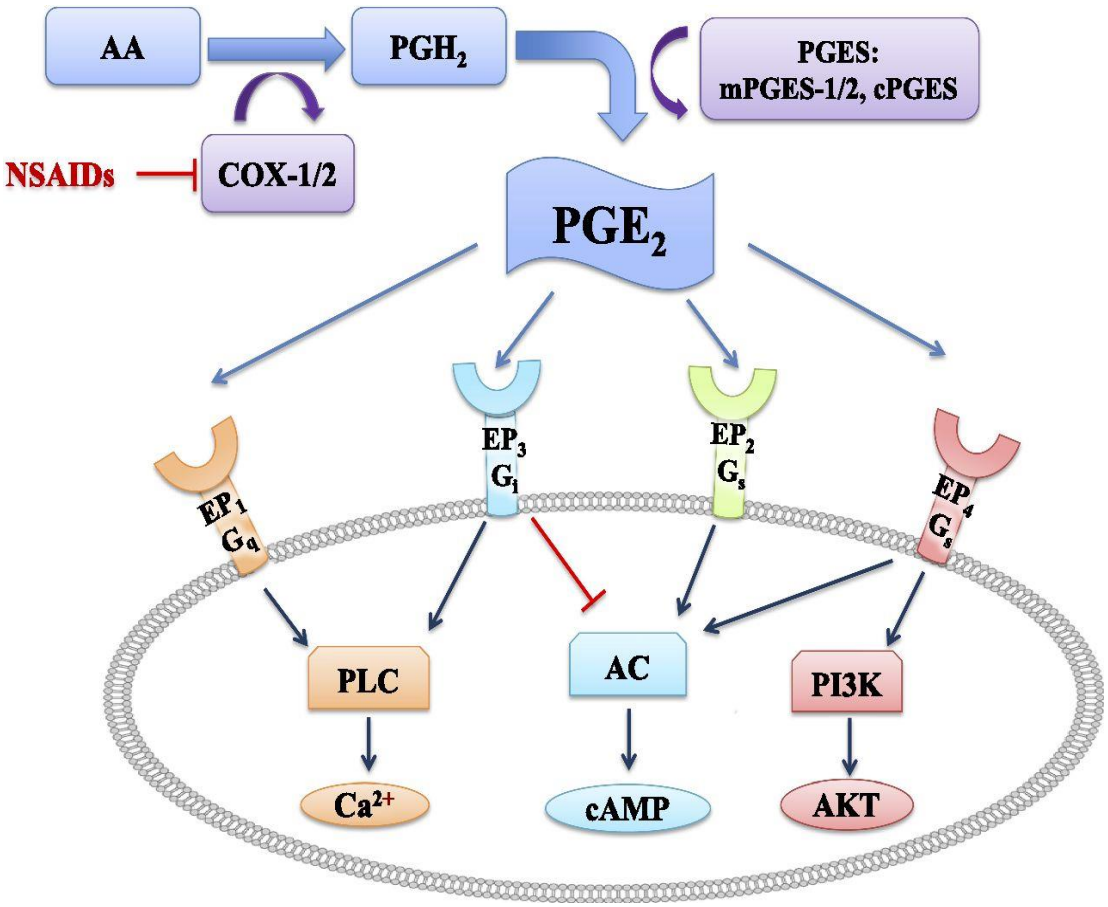

Figure 1.4. COX-2 pathway and PGE_2 effect.

Arachidonic acid (AA) converted to prostaglandin H_2 (PGH_2) via cyclooxygenase (COX) pathway, which can be further metabolised to prostaglandin E_2 (PGE_2) via three different

enzymes mPGES-1, mPGES-2 and cystosolic PGES (cPGES), which acts through G protein–coupled prostaglandin receptors (GPCPR): EP_1, EP_2, EP_3 and EP_4. Adapted from [167].

1.9 Asthma and corticosteroids

1.9.1 Corticosteroid insensitivity

Corticosteroids are the most effective treatment for controlling exacerbation in asthmatic patients [168]. It has been reported that asthmatic patients with T2 airway inflammation respond well to corticosteroid therapy [169]. Evidence has shown that corticosteroid prednisolone can significantly reduce eosinophil levels in BAL in asthmatic patients after two weeks of treatment [170]. In addition, an *in vivo* study proved that corticosteroids effectively reduce eosinophil levels; the study showed that treatment with dexamethasone (2.5 mg/kg) attenuated the production of Th2 cytokines IL-4 and IL-5 in the lung homogenate of mice exposed to OVA [171]. These observations demonstrated that the production of T2 inflammatory cytokines could be inhibited by corticosteroids, suggesting that T2 airway inflammation is sensitive to corticosteroid therapy.

On the other hand, the severity of asthma is assessed based on the dosage of corticosteroids required to control asthma symptoms [172]. Several studies have shown that severe asthmatics are less sensitive to corticosteroids and have more neutrophils in their airways [172]. Therefore, it is essential to understand whether corticosteroid insensitivity is an underlying mechanism of developing severe asthma [173]. It is important to note that corticosteroid insensitivity is restricted to their anti-inflammatory effects; the patients still demonstrate the typical side effects of systemic corticosteroids [174].

It has been reported that neutrophils express GRβ in large quantities and that IL-17A is associated with increased expression of GRβ on bronchial epithelial cells; therefore, neutrophilic airway inflammation is associated with corticosteroid insensitivity asthma [174].

1.9.2 Cigarette smoke–related corticosteroid insensitivity

A clinical study showed that asthmatic smokers showed no improvement in forced expiratory volume at the first second (FEV1) compared with asthmatic non-smokers after treatment with BUD for 11 months [175]. In another study, 39 mild asthmatic smokers and 44 asthmatic non-smokers were assessed by using corticosteroid therapy beclomethasone twice daily. In both groups, corticosteroids were able to reduce sputum eosinophils but significantly increased FEV1 in asthmatic non-smokers compared with asthmatic smokers, suggesting that asthmatic smokers had a poor response to corticosteroid therapy compared with non-smokers [176].

Several mechanisms could be initiated by CS, which may explain the development of corticosteroid insensitivity in asthmatic smokers. One such mechanism is the reduction of histone deacetylase 2 (HDAC2), which is essential for corticosteroids to suppress pro-inflammatory cytokines. Oxidative stress caused by CS (because of the high nitric oxide levels) causes a reduction in HDAC2 activity, rendering inflammatory cells unresponsive to corticosteroids [40]. Another mechanism that could contribute to corticosteroid insensitivity in smokers is the increased expression of GRβ, which inhibits the activity and number of GRα, thus limiting the ability of corticosteroids to bind to GRα and implement their anti-inflammatory effects.

CS has been associated with an increase in the production of the neutrophilic chemoattractant IL-8 and the pro-inflammatory TNFα, which are all responsible for increasing GRβ expression and may explain the poor response to ICS therapy [177, 178].

1.9.3 Cigarette smoke extract and corticosteroid insensitivity

As mentioned in section 1.10.2, several studies have provided evidence that CS could play a role in mediating corticosteroid insensitivity in asthmatic smokers. These observations were supported by an *in vitro* study showing that pre-treatment with CSE for six hours reduced the inhibitory effect of corticosteroids dexamethasone on TNFα-induced by the production of IL-8 in transformed human bronchial epithelial cells (BEAS-2B) [179].

Another *in vitro* study showed that CSE increased corticosteroid insensitivity in 16HBE cells and primary bronchial epithelial cells. Here, it was observed that CSE-induced oxidative stress in HBECs induced corticosteroid insensitivity and epithelial barrier dysfunction in asthmatics. It was also observed that oxidative stress caused phosphorylation of GR and inactivation of HDAC2, rendering HBECs unresponsive to corticosteroid therapy [180]. In addition, it has been reported that fluticasone inhibited CSE- or TNFα-induced the production of IL-8 in HASMCs. A complete inhibition by fluticasone has been observed on CSE-induced IL-8 production but only partially on CSE- and TNFα-induced IL-8 production [181]. However, whether CSE can induce corticosteroid insensitivity in HASMCs still unclear.

1.10 Summary

Airway inflammation in asthma is categorised into endotypes—namely T2 and non-T2 asthma. HASMCs are airway structural cells that play an important role in the pathogenesis of asthma which secrete numerous of cytokines and chemokines in response to inflammatory stimuli. CS is one of the risk factors associated with reduced eosinophil counts, increased neutrophil counts and corticosteroid insensitivity, which contribute to airway inflammation in asthma. But whether CS can modify asthma endotypes by modulating T2 and non-T2 inflammatory response and induce corticosteroid insensitivity *in vitro* is largely unknown. Different studies suggest that several mechanisms like oxidative stress and COX-2 induction may play a role in mediating the CS effect. However, the exact mechanisms underlying the proinflammatory effect of CS in asthma is unclear.

1.11 Hypothesis and Aims

1.11.1 Hypothesis

1. CSE affect the production of T2 and non-T2 inflammatory cytokines in HASMCs, contributing to the development of corticosteroid insensitive non-T2 asthma endotype.
2. CSE affect non-T2 inflammatory response via COX-2 and PGE_2 pathway through oxidative stress.
3. CSE influence the anti-inflammatory effect of corticosteroids on TNFα-induced IL-8 production in HASMCs.

1.11.2 Aims

1. To determine the effect of CSE on the production of T2 and non-T2 inflammatory cytokines with and without TNFα stimulation in HASMCs.
2. To assess the effect of CSE on the mRNA expression of non-T2 inflammatory cytokines in HASMCs.
3. To examine the role of nicotine, oxidative stress, COX-2/ PGE_2 and cAMP pathway in CSE-induced the production of non-T2 inflammatory cytokines in HASMCs.
4. To explore the influence of CSE on the anti-inflammatory effect of corticosteroids on TNFα-induced IL-8 production in HASMCs.

Chapter 2. Materials and Methods

2.1 Introduction

This chapter describes all the general methods that have been used in this thesis. All materials, kits, buffers, media recipes, and reagents are described in the Appendix.

2.2 Cell culture

2.2.1 Culture conditions

All supplements and culture media were prepared and provided as per the guidelines supplied by the manufacturer (Appendix, section 7.1). To provide a suitable environment for the cultured cells to grow, cells were maintained in a humidified incubator with supplied gas of 5% CO_2 at 37 °C. During culture, the medium was changed every 48 h until the cells were more than 90% confluent.

2.2.2 Maintenance of cultured cells

2.2.2.1 Human airway smooth muscle cells

HASMCs from five different healthy donors were provided by Professor Dominick Shaw (Division of Respiratory Medicine, University of Nottingham) (Table 2.1). The use of lung tissue samples has been approved by National Research Ethics Service (NRES) Committee East Midlands-Nottingham. The cells were seeded in 24 wells culture plate at a density of 41,666 cells per cm^2 and maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% of foetal bovine serum (FBS), 0.02% antibiotics (penicillin/streptomycin), 0.02% L-glutamine, and 0.01% amphotericin-B (DMEM+) (Appendix, section 7.1).

Table 2.1. list of human airway smooth muscles cells.

Cell ID	Age	Gender	Ethnicity	Diagnosis	Inhaled corticosteroid	long-acting beta agonists	Short-acting beta agonists	Smoking
AZAC05	26	F	White	Healthy	No	No	No	Never
AZAC07	39	F	Asian	Healthy	No	No	No	Never
MMP-1H10	46	M	White	Healthy	No	No	No	Never

2.2.3 Cell sub-culture

2.2.3.1 Human airway smooth muscle cells

HASMCs at early passage (passage 4) were cultured in T-225 cm^2 flask to passage 6. During passages, the medium was changed every 48 hours until the cells exhibited a typical hill-and-valley appearance and became confluent. When the cells were confluent, the medium was removed by an aspirator, and the cells were washed with 10 ml of phosphate-buffered saline (PBS) (Appendix, section 7.4). Next, 10 ml of trypsin-EDTA solution was added to the cells and incubated for 3 minutes at 37 °C in a 21% O_2 and 5% CO_2 in a humidified incubator to detach the cells from the flask. After incubation and to neutralize trypsinisation, DMEM+ with a 1:1 ratio was added to the cells. Then the cells were collected and centrifuged for 5 min at 1100 rpm. After centrifugation, the cells formed a pellet which was re-suspended in fresh DMEM+. Then, the cells were counted and frozen in a cell freezing solution.

2.2.4 Cell counting

Cells were counted using a glass haemocytometer and a coverslip. To differentiate between live and dead cells, the cell suspension was mixed with Trypan blue (1:1 ratio). Thereafter, 10 µl of the mixture was placed between the haemocytometer and a coverslip. The four haemocytometer squares were assessed under the microscope, and the number of cells in each square was counted. After counting, the average of the four squares was multiplied by the dilution factor and then multiplied by 10^4 to determine the cell count.

2.2.5 Cryopreservation of cells

Following cell counting, the cell suspension was centrifuged for 5 min at 1100 rpm, and the formed pellet of the cells was resuspended in cell freezing solution (containing 10% DMSO and 90% FBS). Thereafter, 1 ml of the suspended cells was aliquoted in Nunc Cryogenic tubes (1 million per ml) and placed in the freezing container (Appendix, section 7.7), containing 100% isopropyl alcohol to freeze the cells at a controlled freezing rate of -1C/minute and stored at -80°C freezer overnight. Finally, the cells were transferred to liquid nitrogen until needed.

2.3 Cigarette smoke extract preparation

3R4F research-grade cigarettes (Appendix, section 7.2) were used in our project. The cigarette smoke extraction process was initiated by adding 20 ml of DMEM- (DMEM with 0.5% FBS, 0.02% antibiotics (penicillin/streptomycin), 0.02%

L-glutamine, and 0.01% amphotericin B) (Appendix, section 7.2) in an 80 ml glass bottle. The vacuum pump (DA7C Charles Austen Pumps) was connected to the bottle, and the pressure was adjusted to 0.2 bar to ensure sufficient bubbling in the DMEM-. Two cigarettes were connected to the other side of the pump one by one, and their smoke was passed through into the bottle. After the two cigarettes were burnt and their smoke was absorbed into the DMEM-, the medium was filtered into a sterilised 50 ml Falcon® using a 0.22 μm pore size filter. The absorbance was used to measure CSE strength (1.5 = 100%) using a FLUO star Omega microplate reader (BMG LABTECH, UK) at 320 nm before the preparation of the desired concentration of CSE. Before each experiment, a fresh aqueous CSE was prepared to be used immediately.

2.4 Drug preparation

2.4.1 Recombinant human tumour necrosis factor (TNFα)

TNFα is a potent cytokine that plays a significant role in airway inflammation and asthma development. Recombinant TNFα was obtained from R&D Systems and prepared based on the instructions of the manufacturer (Appendix, section 7.3). TNFα was dissolved in 1 ml of PBS (0.1% BSA) to make a concentration of 20,000 ng/ml (original stock), diluted in DMEM-, and then added to the cells already cultured to obtain a concentration of 1 ng/ml.

2.4.2 (-)-Nicotine

Nicotine is one of the major substances in tobacco. Nicotine was obtained from Sigma-Aldrich and prepared based on the manufacturer's instructions (Appendix, section 7.3). Since nicotine is liquid-soluble, it was diluted in ethanol to make the original stock of 2 mM, then diluted in DMEM-, and then added to the cells already cultured to obtain a concentration of 500 μM.

2.4.3 MG 624

MG 624 is a selective antagonist for α7 neuronal nicotinic receptor. MG 624 was obtained from Sigma-Aldrich and prepared based on the instructions of the manufacturer (Appendix, section 7.3). The drug was dissolved in dimethyl sulfoxide (DMSO) at 0.05 M (original stock). A concentration of 1 μM was used to treat the cells. In addition, DMSO was also added to both the control and treated groups and maintained the final concertation of DMSO at 0.2%.

2.4.4 L-glutathione (GSH)

GSH, an endogenous antioxidant, plays a crucial role in reducing reactive oxygen species (ROS). GSH was purchased from Sigma-Aldrich and prepared as per the guidelines provided by the manufacturer (Appendix, section 7.3). The drug was dissolved in deionized water at 0.05 M (original stock). A concentration of 100 μM was used to treat the cells. In addition, deionized water was also added to both the control and treated groups and maintained the final concertation of deionized water at 0.2%.

2.4.5 NS-398

NS-398, a nonsteroidal anti-inflammatory drug (NSAID), is a selective inhibitor of cyclooxygenase 2 (COX-2) with a half-maximal inhibitory concentration (IC_{50}) value of 1.77 μM. NS-398 was purchased from Cayman Chemical and prepared as per the guidelines provided by the manufacturer (Appendix, section 7.3). The drug was dissolved in DMSO at 0.05 M (original stock). A concentration of 10 μM was used to treat the cells. In addition, DMSO was also added to both the control and treated groups and maintained the final concertation of DMSO at 0.2%.

2.4.6 Prostaglandin E_2 (PGE_2)

PGE_2, a potent inflammatory mediator, is formed via the COX-2 pathway from arachidonic acid. PGE_2 was purchased from Cayman Chemical and prepared as per the instructions of the manufacturer (Appendix, section 7.3). The drug was dissolved in DMSO to make original stock at 0.05 M, then a serial dilution was made in DMSO, a concentration of 10 μM was used to treat the cells. In addition, DMSO was also added to both the control and treated groups and maintained the final concertation of DMSO at 0.2%.

2.4.7 PF 04418948

PF 04418948, a prostaglandin E receptor $_2$ antagonist, is a potent inhibitor of PGE_2 with a half-maximal inhibitory concentration (IC_{50}) value of 16 nM for EP_2 receptor. The drug was obtained from Tocris Bioscience and prepared based on the

manufacturer's instructions (Appendix, section 7.3). The original stock at 0.05 M was obtained after the drug was dissolved in DMSO. Then, a serial dilution was made in DMSO and a concentration of 10 μM was used to treat the cells. In addition, DMSO was also added to both the control and treated groups and maintained the final concertation of DMSO at 0.2%.

2.4.8 L-161,982

L-161,982 is a prostaglandin E receptor $_4$ antagonist. The drug was obtained from Tocris Bioscience and prepared according to the manufacturer's instructions (Appendix, section 7.3). The drug was dissolved in DMSO at 0.05 M (original stock). A concentration of 1 μM was used to treat the cells. In addition, DMSO was also added to both the control and treated groups and maintained the final concertation of DMSO at 0.2%.

2.4.9 Forskolin

Forskolin, a potent and rapid activator of adenylyl cyclase (AC), converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). Forskolin was purchased from R&D Systems and prepared based on the instructions of the manufacturer (Appendix, section 7.3). The drug was dissolved in DMSO at 0.05 M (original stock). A concentration of 10 μM was used to treat the cells. In addition, DMSO was also added to both the control and treated groups and maintained the final concertation of DMSO at 0.2%.

2.4.10 Salbutamol

Salbutamol, a β_2 adrenoceptor agonist, is a short-acting bronchodilator and an effective treatment in asthma. Salbutamol was obtained from Sigma-Aldrich and prepared according to the manufacturer's instructions (Appendix, section 7.3). Salbutamol was dissolved in methanol at 0.05 M (original stock), and a concentration of 10 µM was used to treat the cells. In addition, methanol was also added to the both the control and treated groups and maintained the final concertation of methanol at 0.2%.

2.4.11 Fluticasone propionate

Fluticasone propionate (FP) is a steroid anti-inflammatory drug used to control asthma. FP was purchased from Sigma-Aldrich and prepared as per the guidelines provided by the manufacturer (Appendix, section 7.3). FP was dissolved in DMSO at 0.5 M (original stock). Six concentrations of FP were used from 10^{-11} to 10^{-6} M. In addition, DMSO was also added to both the control and treated groups and maintained the final concertation of methanol at 0.2%.

2.5 Cell treatment

For each experiment, the cells stored in liquid nitrogen were thawed and suspended in fresh DMEM+ then centrifuged for 5 minutes at 1100 rpm. After centrifugation, the cells formed a pellet which was re-suspended in fresh DMEM+. Then, the cells were seeded in 24-well plates at a density of 41,666 per cm^2. After cell seeding, the DMEM+ was changed every 48 hours. When the cells became confluent, cells were serum-starved with DMEM- for 24 hours before each experiment. To examine the effect of CSE and TNFα on the production of inflammatory cytokines in HASMCs, cells were pre-treated with or without CSE (3.5%) for 24 hours followed by treatment with CSE, TNFα (1ng/ml), or CSE+ TNFα for 24 hours. To explore the effect of nicotine, confluent HASMCs were serum-starved with DMEM- for 24 hours followed by treatment with nicotine (500 µM) and with or without CSE for 24 hours. To assess the effect of α7 neuronal nicotinic receptor antagonist, COX-2, and oxidative stress inhibitors on the effect of CSE, confluent HASMCs were serum-starved with DMEM- for 24 hours followed by treatment with or without MG 624 (1 µM), NS-398 (10 µM), and L-glutathione (100 µM), respectively for 1 hour before incubation with CSE for 24 hours. To explore the effect of PGE_2, salbutamol, forskolin, PGE receptor $_2$ antagonist, and PGE receptor $_4$ antagonist, confluent HASMCs were serum-starved with DMEM- for 24 hours followed by treatment with PGE_2 (10 µM), salbutamol (10 µM), forskolin (10 µM), PF 04418948 (10 µM), and L-161,982 (1 µM), respectively and with or without CSE for 24 hours. To examine the effect of CSE on the inhibitory effect of corticosteroids, cells were pre-treated with or without CSE (3.5%) for 24 hours followed by treatment with fluticasone (10^{-11}-10^{-6} M) for 1 hour before incubation with

CSE, TNFα (1ng/ml), or CSE+ TNFα for 24 hours. At the end of each experiment, the medium was collected and stored at -20 °C until required.

2.6 Cell lysate preparation

At the end of each experiment, the medium was removed from the plates and the cells were washed with PBS. Subsequently, 150 µl of radioimmunoprecipitation assay (RIPA) buffer with 2 Mm of Phenylmethylsulphonyl Fluoride (PMSF), 1 mM of Protease Inhibitor Cocktail (PIC), and 1 mM of Sodium Orthovanadate. (Appendix, section 7.4) were added to each well. A cell scraper was used to scrape the cells. Then, cell lysates were collected and centrifuged at 4°C for 5 minutes to remove any cellular debris. Thereafter, proteins were collected and transferred to fresh tubes (1.5 ml Eppendorf) and stored at -80 °C.

2.7 Bicinchoninic acid protein assay

The total protein concentration was measured with a bicinchoninic acid (BCA) assay kit (Appendix, section 7.5). The bovine serum albumin (BSA) standard solutions (0–2000 µg/ml) were prepared according to the manufacturer's guidelines (Appendix, section 7.4). The protein samples (5 µl) were duplicated into a 96-well plate, as well as the BSA standards. BCA reagents A and B were prepared (1:50) and mixed. From the BCA reagent mixture, 95 µl was added to each well. The plate was then covered and incubated at room temperature for 30 minutes. A FLUOstar Omega microplate reader (BMG labBtech) at 562 nm was used to measure the absorbance. Omega analysis

software was used to measure the protein concentrations, which were compared against the BSA standard and calculated as µg/ml.

2.8 Enzyme-Linked immunosorbent assay (ELISA)

IL-8 concentrations in the cell supernatants were measured by DuoSet ELISA kits (Appendix, section 7.5). The kit was used as per the guidelines of the manufacturer.

2.8.1 Method

For each ELISA, a 96-well plate (high binding) was prepared. The monoclonal antibody (mouse anti-human IL-8) was diluted in PBS, and 100 µl of the antibody solution was added to each well. The plate was then sealed and incubated at room temperature overnight. After incubation, the plate was washed three times with 400 µl of wash buffer (0.05% Tween 20 in PBS) to remove unbound antibodies, and then the plate was kept upside down on paper towels for drying. Next, 300 µl of block buffer (1% BSA in PBS) was added to each well, and the plate was sealed and incubated for 1 hour at room temperature. Standard was prepared to obtain seven points of the standard curve; the standard was diluted in DMEM- using 2-fold serial dilution with a concentration from 2000 to 31.3 pg/ml. After incubation, the plate was washed three times with 400 µl of wash buffer, and 100 µl of the diluted standards and samples were added to the wells, all of them in duplicate. The plate was incubated for 2 hours on a shaker (400 rpm). Thereafter, the plate was washed three times with 400 µl of wash buffer and 100 µl of a polyclonal antibody (biotinylated goat anti-human IL-8) specific

for IL-8 was added to each well and incubated for 2 hours at room temperature on the shaker (400 rpm). The plate was then washed three times with 400 µl of wash buffer and 100 µl of streptavidin horseradish peroxidase (HRP) was added to each well and incubated for 20 minutes. The plate was then washed three times with 400 µl of wash buffer and 100 µl of substrate solution (tetramethylbenzidine and hydrogen peroxide) was added to each well and incubated for 20 minutes. The last step was to add 50 µl of stop solution (sulphuric acid) to each well to stop the enzymatic reaction. The FLUOstar Omega microplate (BMG labBtech) at 450 nm was used to measure the optical density of each well, and Omega software was used to analyse the data. All data were normalised to total amount of protein, the average of the duplicated IL-8 concentrations (pg/ml) were divided by the total amount of protein (the protein concentration mg/ml) then multiplied by total volume of protein lysate (0.15 ml) and presented as pg/mg protein.

2.9 Bio-Plex Pro™ Human Cytokine Assays

The Bio-plex Pro™ Human Cytokine Assays kit (Appendix, section 7.5) was used to detect the concentration of a panel of cytokines including T2 and non-T2 inflammatory cytokines in the cell supernatants. The kit was used as per the guidelines of the manufacturer. A 50 µl of the capture antibodies were added to each well. Once the capture antibodies were added the Bio-Plex wash buffer was used immediately to wash the plate manually two times each time with 100 µl in each well. After the plate was emptied of the wash buffer, 50 µl of samples, standards with a dilution factor of

1:4, blank, and controls were transferred to their appropriate wells of the assay plate. Thereafter the plate was covered and incubated at room temperature on a shaker at 850 rpm for 30 minutes. After incubation, the plate was washed three times with 100 µl of the wash buffer, then 25 µl of the detection antibodies were added to each well and incubated for 30 minutes on a shaker with 850 rpm at room temperature. Then, the plate was washed as the previous wash step and 50 µl of streptavidin-phycoerythrin (SA-PE) was transferred to each well and the plate was covered and incubated for 10 minutes at room temperature on a shaker at 850 rpm. After incubation, the plate was washed as described before and 125 µl of assay buffer was added to each well to resuspend the beads for reading the plate and kept on a shaker at 850 rpm for 30 seconds at room temperature. Thereafter, the Bio-Plex^{TM} 200 System (Bio-Rad) was used to read the plate, and the Bio-Plex Manager software version 6.1 was used to calculate the concentration of different biomarkers. A photomultiplier tube (PMT) with a low setting was used to detect the lowest range of 7-17 pg/ml. All data were normalised as explained in 2.8.1 and then the concentration of the biomarker of interest was presented as pg/mg protein.

2.10 Eicosanoid release analysis

2.10.1 PGE_2 ELISA

2.10.1.1 Principle

PGE_2, a potent inflammatory mediator, is formed via the COX-2 pathway from arachidonic acid. The concentration of PGE_2 in the cell culture supernatant was measured by using the PGE_2 ELISA kit (Appendix, section 7.5). This is a competitive assay used to assess the concentration of PGE_2 in the sample, with a range of 7.8-1000 pg/ml and a sensitivity of 15 pg/ml. The cross-reactivity of the assay is listed in Table

Table 2.2. Cross-reactivity of the PGE_2 ELISA kit.

Cross-reactivity	**(%)**
Prostaglandin E_2	100%
Prostaglandin E_2 Ethanolamide	100%
Prostaglandin E_2-1-glyceryl ester	100%
Prostaglandin E_3	43.0%
Prostaglandin E_1	18.7%
8-*iso* Prostaglandin E_2	2.5%
Sulprostone	1.25%
6-keto Prostaglandin $F_1\alpha$	1.0%
8-*iso* Prostaglandin $F_2\alpha$	0.25%

Prostaglandin A_2	0.04%
13,14-dihydro-15-keto Prostaglandin E_2	0.02%
All others	<0.01%

This assay is based on competitive binding between the PGE_2 in the sample and a PGE_2-acetylcholinesterase (AChE) conjugate (PGE_2 tracer). Based on the variable amount of PGE_2 in the sample competes with the constant amount of PGE_2 tracer to bind to a limited amount of PGE_2 monoclonal antibody, the amount of PGE_2 in the sample is inversely proportional to the amount of PGE_2 tracer bound to the PGE_2 monoclonal antibody. This antibody- PGE_2 complex then binds to the pre-coated goat polyclonal anti-mouse IgG in the plate. After a series of washes to remove unbound reagents, an Ellman's reagent (substrate for AChE) is then transferred to the plate. The enzymatic reaction of Ellman's reagent caused a distinct yellow colour. The intensity of this yellow colour is proportional to the amount of PGE_2 tracer bound to the plate but is inversely proportional to the amount of PGE_2 in the sample.

2.10.1.2 Method

PGE_2 ELISA kit (Appendix, section 7.5) was used to assess the level of PGE_2 in the cell supernatants as per the guidelines of the manufacturer. PGE_2 ELISA standard was diluted in DMEM- using 2-fold serial dilutions to obtain an eight points standard curve ranging from 1000 pg/ml to 7.8 pg/ml. As the plate is already pre-coated with

goat polyclonal anti-mouse IgG and blocked, 50 µl of standards, blanks, samples, PGE_2 AChE tracer, and PGE_2 monoclonal antibody were transferred to the plate. The plate was then covered with plastic film and incubated for 18 hours at 4°C. Then, wash buffer was used to wash the plate five times, followed by 200 µl of Ellman's reagent for each well. After that, the plate was covered with a plastic film to enable the palate to develop in the dark and was incubated for 90 minutes at room temperature. A FLUOstar Omega microplate reader (BMG labBtech) was used to measure the optical density at 420 nm. PGE_2 concentration was normalised as explained in 2.8.1 and then the concentrations of PGE_2 was presented as pg/mg protein.

2.10.2 TXB_2 ELISA

2.10.2.1 Principle

Thromboxane A_2 (TXA_2) is produced through the conversion of arachidonic acid via the COX-2 pathway and rapidly converted to thromboxane B_2 (TXB_2) which is a stable metabolite. Therefore, the TXB_2 ELISA kit (Appendix, section 7.5) was used to measure TXB_2 concentration in the cell culture supernatants as an estimate of the release of TXA_2. This is a competitive assay used to assess the concentration of TXB_2 in the sample, with a range of 1.6-1000 pg/ml and a sensitivity of 5 pg/ml. The cross-reactivity of the assay is listed in Table 2.3.

Table 2.3. Cross-reactivity of the TXB_2 ELISA kit.

Cross-reactivity	**(%)**
Thromboxane B_2	100%
Thromboxane B_3	33.8%
2,3-dinor Thromboxane B_2	18.5%
11-dehydro Thromboxane B_2	5.4%
11-dehydro Thromboxane B_3	1.6%
Prostaglandin D_2	0.8%
Prostaglandin $F_{2\alpha}$	0.1%
All others	<0.01%

This assay is based on competitive binding between the TXB_2 in the sample and a TXB_2-acetylcholinesterase (AChE) conjugate (TXB_2 tracer). As the variable amount of TXB_2 in the sample competes with the constant amount of TXB_2 tracer to bind to a limited amount of TXB_2 specific rabbit antiserum, the amount of TXB_2 in the sample is inversely proportional to the amount of TXB_2 tracer bound to the TXB_2 specific rabbit antiserum. The TXB_2 specific rabbit antiserum then binds to the pre-coated mouse monoclonal anti-rabbit IgG in the plate. After a series of washes to remove unbound reagents, an Ellman's reagent (substrate for AChE) is then transferred to the plate. The enzymatic reaction of Ellman's reagent caused a distinct yellow colour. The

intensity of this yellow colour is proportional to the amount of TXB_2 tracer bound to the plate but inversely proportional to the amount of TXB_2 in the sample.

2.10.2.2 Method

TXB_2 ELISA kit (Appendix, section 7.5) was used to assess the level of TXB_2 in the cell supernatants as per the guidelines of the manufacturer. TXB_2 ELISA standard was diluted in DMEM- to obtain an eight points standard curve. The concentrations of these standards were 1000, 400, 160, 64, 25.6, 10.2, 4.1, and 1.6 pg/ml. As the plate is already pre-coated with mouse monoclonal anti-rabbit IgG and blocked, 50 µl of standards, blanks, samples, TXB_2 AChE tracer, and TXB_2 specific rabbit antiserum were transferred to the plate. The plate was then covered with plastic film and incubated for 18 hours at 4°C. Then, wash buffer was used to wash the plate five times, followed by 200 µl of Ellman's reagent for each well. After that, the plate was covered with a plastic film to enable the palate to develop in the dark and was incubated for 120 minutes at room temperature. A FLUOstar Omega microplate reader (BMG labBtech) was used to measure the optical density at 420 nm. TXB_2 concentration was normalised as explained in 2.8.1 and then the concentrations of TXB_2 was presented as pg/mg protein.

2.10.3 Cysteinyl leukotrienes ELISA

2.10.3.1 Principal

Cysteinyl leukotrienes (CysLT) including LTC_4, LTD_4, and LTE_4 are a group of metabolites that are produced upon the conversion of arachidonic acid through the activity of 5-lipoxygenase and are known as potent lipid mediators in inflammation which play a role in producing bronchoconstriction [182]. CysLT ELISA kit (Appendix, section 7.5) was used to measure the concentration of CysLT in the cell culture supernatants. This is a competitive assay used to assess the concentration of CysLT in the cell culture supernatant, with a range of 78.1-2500 pg/ml and a sensitivity of 26.6 pg/ml pg/ml. The cross-reactivity of the assay is listed in Table 2.4.

Table 2.4. Cross-reactivity of the CysLT ELISA kit.

Cross-reactivity	**(%)**
Leukotriene C_4	100%
Leukotriene D_4	115.12%
Leukotriene E_4	62.74%
Leukotriene B_4	1.16%
Arachidonic Acid	0.61%
PGE_2	0.48%
6-trans-LTB_4	0.45%
TXB_2	0.28%
All others	<0.2%

This assay is based on competitive binding between the CysLT in the sample and alkaline phosphatase-conjugated with LTC_4 (conjugate). As the variable amount of CysLT in the sample competes with the constant amount of CysLT conjugate to bind to a limited amount of CysLT mouse monoclonal antibody, the amount of CysLT in the sample is inversely proportional to the amount of CysLT conjugate bound to the CysLT mouse monoclonal antibody. The CysLT mouse monoclonal antibody then binds to the pre-coated goat anti-mouse IgG in the plate. After a series of washes to remove unbound reagents, a p-nitrophenyl phosphate (pNpp substrate) is then transferred to the plate. The enzymatic reaction of pNpp substrate reagent caused a distinct yellow colour. The intensity of this yellow colour is proportional to the amount of CysLT conjugate bound to the plate but inversely proportional to the amount of CysLT in the sample.

2.10.3.2 Method

CysLT ELISA kit (Appendix, section 7.5) was used to assess the level of CysLT in the cell supernatants as per the guidelines of the manufacturer. CysLT ELISA standard was diluted in DMEM- using 2-fold serial dilutions to obtain a six points standard curve ranging from 78.1 to 2500 pg/ml. As the plate is already pre-coated with goat anti-mouse IgG antibody and blocked, 50 µl of the prepared CysLT standards, samples, CysLT conjugate, and CysLT mouse monoclonal antibody were added in duplicate to the plate. The plate was then covered and incubated on the shaker at 500 rpm for two hours at room temperature. Then, a wash buffer was used to wash the plate three times, followed by 200 µl of pNpp substrate for each well. After that, the plate

was covered and incubated for two hours at 37°C. After the incubation, 50 µl of trisodium phosphate (stop solution) was added to each well to stop the enzymatic reaction. A FLUOstar Omega microplate reader (BMG labBtech) was used to measure the optical density at 405 nm. CysLT concentration was normalised as explained in 2.8.1 and then the concentrations of CysLT was presented as pg/mg protein.

2.10.4 LTB_4 ELISA

2.10.4.1 Principle

LTB_4, a potent neutrophilic chemotactic involved in airway inflammatory, is synthesised from arachidonic acid through the 5-lipoxygenase pathway [183]. LTB_4 ELISA kit (Appendix, section 7.5) was used to measure the concentration of LTB_4 in the cell culture supernatants. This is a competitive assay used to assess the concentration of LTB_4 in the cell culture supernatant, with a range of 11.7-3000 pg/ml and a sensitivity of 5.63 pg/ml pg/ml. The cross-reactivity of the assay is listed in Table 2.5.

Table 2.5. Cross-reactivity of the LTB_4 ELISA kit.

Cross-reactivity	**(%)**
Leukotriene B_4	100%
6-trans-12-epi-LTB_4	5.50%
6-trans-LTB_4	4.90%
12-epi-LTB_4	0.94%
All others	<0.2%

The LTB_4 ELISA is based on competitive binding between the LTB_4 in the sample and alkaline phosphatase-conjugated with LTB_4 (conjugate). As the variable amount of LTB_4 in the sample competes with the constant amount of LTB_4 conjugate to bind to a limited amount of LTB_4 rabbit polyclonal antibody, the amount of LTB_4 in the sample is inversely proportional to the amount of LTB_4 conjugate bound to the LTB_4 rabbit polyclonal antibody. The LTB_4 rabbit polyclonal antibody then binds to the pre-coated goat anti-rabbit IgG in the plate. After a series of washes to remove unbound reagents, a p-nitrophenyl phosphate (pNpp substrate) is then transferred to the plate. The enzymatic reaction of pNpp substrate reagent caused a distinct yellow colour. The intensity of this yellow colour is proportional to the amount of LTB_4 conjugate bound to the plate but inversely proportional to the amount of LTB_4 in the sample.

2.10.4.2 Method

LTB_4 ELISA kit (Appendix, section 7.5) was used to assess the level of LTB_4 in the cell supernatants as per the guidelines of the manufacturer. LTB_4 ELISA standard was diluted in DMEM- using 4-fold serial dilutions to obtain a five points standard curve ranging from 11.7 to 3000 pg/ml. As the plate is already pre-coated with goat anti-rabbit IgG antibody and blocked, 50 µl of the prepared LTB_4 standards, samples, LTB_4 conjugate, and LTB_4 rabbit polyclonal antibody were added in duplicate to the plate. The plate was then covered and incubated on the shaker at 500 rpm for two hours at room temperature. Then, a wash buffer was used to wash the plate three times, followed by 200 µl of pNpp substrate for each well. After that, the plate was covered and was incubated for two hours at 37°C. After the incubation, 50 µl of trisodium phosphate (stop solution) was added to each well to stop the enzymatic reaction. A FLUOstar Omega microplate reader (BMG labBtech) was used to measure the optical density at 405 nm. LTB_4 concentration was normalised as explained in 2.8.1 and then the concentrations of LTB_4 was presented as pg/mg protein.

2.11 Western blotting

2.11.1 Principle

Western blotting is an important technique to detect the protein of interest among a different complex mixture of proteins [184]. Each protein has a molecular weight therefore, proteins are separated based on their molecular weight by polyacrylamide gel electrophoresis (PAGE). The separated proteins are then transferred

to a nitrocellulose blotting membrane (Amersham Biosciences) through an electric current. Thereafter, the membrane is blocked and incubated with a primary antibody specific to the protein of interest and horseradish peroxidase (HRP) conjugated secondary antibody. Finally, enhanced chemiluminescence (ECL) substrate for detection of HRP enzyme activity is used to detect bands specific to the protein of interest.

2.11.2 Method

2.11.2.1 Gel electrophoresis

Proteins were collected and the concentrations of the protein were quantified using BCA assay as described in section 2.7. Protein samples were diluted in 4x Lamellae buffer (Appendix, section 7.4) with a dilution ratio of 1:4 and 5 µl of reducing agent (Appendix, section 7.4). Then, the samples were mixed and boiled for 10 minutes at 80°C to denature the structure of the protein. The Bolt Bis-Tris gel (Appendix, section 7.7), a pre-cast polyacrylamide gel with a concentration of 4-12%, was used to separate the protein of interest based on its molecular weight. Thereafter, 20 µg of the samples and 10 µl of the pre-stained protein marker (Appendix, section 7.4) were loaded into the wells of the gel. Then the electrophoresis tank was filled with running buffer (Appendix, section 7.4), connected to the electrical supply, and the electrophoresis was run for 60 minutes at 160V.

2.11.2.2 Transfer to nitrocellulose blotting membrane

Once the running step was completed, and to transfer the protein to the nitrocellulose blotting membrane (Appendix, section 7.7), the gel was removed from the electrophoresis tank and placed in 1x transfer buffer (Appendix, section 7.4). Thereafter, a sandwich of sponge, filter, paper, gel, A nitrocellulose blotting membrane, filter paper, and sponge was prepared in 1x transfer buffer and placed between positive and negative electrodes in the mini blot module and placed in the electrophoresis tank. Then, the electrophoresis tank was filled with 1x transfer buffer and run for 90 minutes at 10V.

2.11.2.3 Blocking and antibody incubation

Once the transfer step was completed, the membrane was placed in ponceau red stain (Appendix, section 7.4) to visualise the transferred protein in the membrane. Then, 1x tris buffered saline with tween 20 (TBS-T) buffer (Appendix, section 7.4) was used to wash the membrane three times. Thereafter, the membrane was incubated with block buffer (Appendix, section 7.4) at room temperature for 1 hour to decrease non-specific binding of antibodies to the membrane. Following the blocking step, the primary antibody was added to the membrane in the blocking buffer and incubated overnight at 4°C. The primary antibodies were used are mouse anti-human COX-2 monoclonal antibody (1:1000), rabbit anti-human thromboxane synthase monoclonal antibody (1:1000), rabbit anti-human 5-LO polyclonal antibody (1:1000), and rabbit anti-human mPGES-1 synthase monoclonal antibody (1:1000), mouse anti-human β-Actin

antibody (1:50000), and rabbit anti-human β-Actin antibody (1:50000) (Appendix, section 7.6). The next day, 1x TBS-T was used to wash the membrane three times each time for 10 minutes then the membrane was incubated with the secondary antibody in the blocking buffer for 1 hour at room temperate. The secondary antibodies were used are polyclonal goat anti-mouse immunoglobulins/HRP antibody (1:2500) and polyclonal goat anti-rabbit immunoglobins/HRP antibody (1:2500) (Appendix, section 7.6).

2.11.2.4 Detection step

After the incubation with the secondary antibody was completed, the membrane was washed with 1x TBS-T three times each time for 10 minutes. Finally, the membrane was incubated with a mixture of Enhanced chemiluminescence (ECL) solutions (1:1 ratio) (Appendix, section 7.4) for 5 minutes. ECL enable the visualization and the detection of the protein bands on the Licor C-DiGit scanner. Image studio software was used to quantify the protein band density on the membrane. The data was measured and normalised to the corresponding β-Actin and then to the control sample. The data were presented as a fold change over control.

2.12 Real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

2.12.1 Principle

RT-qPCR is one of the most common analyses used to assess gene expression by evaluating the level of the messenger RNA (mRNA) in the cells. The RT-qPCR analysis consists of three steps. These steps are; first is to isolate the total RNA from the samples, the second step is the reverse transcription (RT) which converts the mRNA into complementary DNA (cDNA), and the third step is qPCR. The qPCR step is based on three distinct steps: denaturation, annealing, and extension. The fluorescent signal requires several cycles to cross the threshold which is called cycle threshold (C_T). The increase in the level of cDNA in the sample is proportional to the increase in fluorescent signal.

2.12.2 Total RNA isolation

Before total RNA was extracted with the NucleoSpin II kit (Appendix, section 7.5), the cells were seeded in a 12-well plate until confluence then cells were serum-starved for 24 hours, followed by the required treatment. At the end of the experiment, the cell culture medium was collected and stored at -80 until needed, then cells were washed with PBS. Once the cells were washed with PBS, 350 µl of lysis buffer RA1 with β-mercaptoethanol 10 µl/ml was added to the cells. The lysed sample was filtered through a NucleoSpin filter into a 2 ml collection tube, centrifuged for one minute at 11,000 rpm. Then, the filter was removed and 350 µl of 70% ethanol was added to the

collection tube and mixed well with the sample to adjust the RNA binding conditions. Thereafter, the lysate was transferred to a new NucleoSpin RNA column and centrifuged for one minute at 11,000 rpm. After the RNA was bound to the silica membrane of the column, the column was placed in a new 2 ml collection tube and 350 µl of membrane desalting buffer (MDB) was added into the column and centrifuged for one minute at 11,000 rpm to eliminate any contaminating salt. Then, 95 µl of deoxyribonuclease (DNase) was loaded to each column and incubated for 15 minutes at room temperature to digest any contaminating DNA on the membrane. Then 200 µl of RAW2 buffer was added to wash the columns prior to centrifugation for one minute at 11,000 rpm. Thereafter, RA3 buffer was used to wash the silica membrane twice, the first time with 600 µl followed by centrifugation for 1 minute, then the second wash by 250 µl followed by centrifugation for 1 minute. Finally, to collect the RNA, 50 µl of RNase-free water was added, centrifuged for 1 minute at 11,000 rpm. Due to the instability of RNA, the isolated RNA was used immediately after isolation or stored at -80°C.

2.12.3 RNA quantification

The concentration of the RNA was measured by the NanoDrop spectrophotometer (Thermo Fisher Scientific). From each RNA sample, 2 µl was loaded onto the lower measurement pedestal of the NanoDrop. RNA concentrations were determined by two-wavelength spectrophotometry 260 and 280 nm.

2.12.4 Reverse transcription (RT)

To convert RNA into cDNA, a SuperScript™ IV Reverse Transcriptase kit (Appendix, section 7.5) was used. The volume of mixture components per reaction was prepared based on the guidelines of the supplier as follows: 0.5 µg of total RNA, 50 µM of Oligo $d(T)_{20}$ primer, 10 mM dNTPs mix (10 mM each), and nuclease-free water (Table 2.6). Thereafter, 13 µl of the mixture was loaded into each well of the reaction plate, centrifuged for 30 seconds, heated for 5 minutes at 65°C. Following heating, the volume mixture was made up to 20 µl with 5x SSIV Buffer, 100 mM DTT, RNase inhibitor, and SuperScript™ IV Reverse Transcriptase (200 U/µl) (Table 2.7). The reaction plate was then sealed and placed in a thermal cycler (BioRad). The thermal cycler was programmed according to the protocols of the supplier as follows: 55°C for 10 minutes and 80°C for 10 minutes. Resulting cDNA was stored at -20°C until needed.

Table 2.6. List of a SuperScript™ IV Reverse Transcriptase.

Component	**Volume**
50 µM of Oligo $d(T)_{20}$ primer	1 µl
10 mM dNTPs mix (10 mM each)	1 µl
Total RNA	Up to 11 µl
Nuclease-free water	Up to 13 µl

Table 2.7. List of RT reaction mixture.

Component	**Volume**
5x SSIV Buffer	4 µl
100 mM DTT	1 µl
RNase inhibitor	1 µl
SuperScript™ IV Reverse Transcriptase 200 U/µL	1 µl

2.12.5 Specificity of the primers

A serial dilution of cDNA from untreated cells (1:10, 1:100, 1:1000, and 1:10000) was used to test the specificities of all primers. Dissociation curves were generated after the RT-qPCR was conducted. When dissociation curves contained a single peak, primers were considered specific.

2.12.6 qPCR

The stored cDNA product was used for the qPCR process. A volume of 20 µl per reaction was prepared as a mixture of 1 µl of cDNA products, 0.4 µl of each forward (FW) and reverse (RV) of targeted gene primer, 10 µl of KAPA Taq DNA polymerase, and 8.2 µl nuclease-free water. The control (nuclease-free water) and the mixture volume were loaded in duplicate into each well of the qPCR plate. The qPCR reaction was performed in three stages: denaturation, annealing, and extension, which took place at different temperatures. To separate double-stranded DNA into single-stranded DNA, denaturation was performed for three minutes at 95°C. Then, the primers were bound to the template through the annealing step by reducing the temperature to 65°C for 15 seconds. Finally, the extension step was at a temperature of 65°C for 30 seconds, in this step DNA polymerase enzyme formed a new strand of DNA. Exponential amplification of the selected target sequence was made by repeating the steps of denaturation, annealing, and extension for 40 cycles by using Stratagene Mx3000P qPCR System (Agilent Technologies). After the C_T values were generated from both the treated samples and control, MxPro qPCR software (Agilent Technologies) was used to analyse the qPCR results. The expression of βeta-2-Microglobulin (β2M) was stable across samples, therefore, it was used as the reference (housekeeping gene) for all experiments. After obtaining average C_T values for the tested samples and β2M gene, the differences (ΔC_T = C_T tested samples - C_T β2M) were calculated, and then double delta C_T values ($\Delta\Delta C_T$ (ΔC_T tested samples -ΔC_T control) were obtained. Thereafter, the values of $2^{-\Delta\Delta CT}$ were calculated to obtain fold changes of the gene expression over control. Data were expressed as relative expression (fold increase).

Table 2.8. List of the target gene and their upstream and downstream primer sequences. All primers were designed using the national center for biotechnology information except VEGF primers were designed by Rachel Clifford [185]

Target gene	Primer sequences
IL-6	**FW:** 5'-CCAGAAAGGAAAGGAACTTC-3' **RV:** 5'-CTGCATTAGGAGGTCTTTG-3'
IL-8	**FW:** TCTCAGCCCTCTTCAAAAACTTCT **RV:** ATGACTTCCAAGCTGGCCGTG GCT
VEGF	**FW:** GAGTATGCCTGCCGTGTG **RV:** AATCCAAATGCGGCATCT
COX-2	**FW:** AAGCAGGCTAATACTGATAGG **RV:** TGTTGAAAAGTAGTTCTG GG
PTGES / mPGES-1	**FW:** CCTCCCAAGGTTTGAGTCCC **RV:** CACACATCTCAGGTCACGGG
TXAS	**FW:** CTACTGCAATTACACCACAG **RV:** AAGAGTAAAACCAGGATAGGTC
β2M	**FW:** AAGGACTGGTCTTTCTATCTC **RV:** GATCCCACTTAACTATCTTGG

2.13 Cell viability assay

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used to measure the cytotoxicity of the different treatments towards HASMCs. After the cells were confluent and treated with the designed treatment for each experiment, the medium was then removed, and 250 µl of 1 mg/ml MTT solution (dissolved in DMEM-) was added. The plates were incubated for 30 minutes in a humidified incubator. Then, the plates were dried at room temperature for 6 hours. Next, 250 µl of DMSO was added to each well and then transferred to a 96-well plate. The FLUOstar Omega microplate reader (BMG LABTECH) was used to measure the absorbance at 550 nm and to assess the metabolic activity of living cells. The viability data were presented relative to the control group (100%).

2.14 Statistical analysis

All data were analysed by GraphPad Prism software (version 9). Two-way analysis of variance (ANOVA) was used to compare the difference between two or more groups. One-way ANOVA followed by Student's *t-test* was used to determine any significant difference between the control and the treated cells. A *p*-value less than 0.05 was statistically significant. Holm-Sidak multiple comparisons test was used to compare the differences in the inhibitory effect of fluticasone at the same concentration between different groups. To calculate the half-maximal inhibitory concentration (IC_{50}), the drug concentrations were converted to log10 and plotted on the X-axis, and the normalised response of IL-8 production (as 100%) was plotted on the Y-axis. Nonlinear regression analysis was used to draw the curve and calculated the IC_{50} value.

Chapter 3. Effect of cigarette smoke extract on inflammatory cytokine and chemokine production and gene expression in HASMCs

3.1 Introduction

Asthma is a chronic lung disease characterised by the presence of one or more respiratory symptoms, such as wheezing, chest tightness, breathlessness and coughing, which are attributed to airflow obstruction, airway hyperresponsiveness and airway inflammation [1]. Airway inflammation in asthma is categorised into endotypes, namely, Type2 (T2) asthma and non-Type2 (non-T2) asthma. T2 asthma is characterised by the presence of T2 inflammatory cytokines, including Th2 cytokines (IL-4, IL-5 and IL-13) and eosinophil chemokines (RANTES, IP-10 and eotaxin), whereas non-T2 asthma is associated with an increase in non-T2 inflammatory cytokines, such as the neutrophil chemoattractant IL-8 and other cytokines (e.g. IL-6 and VEGF) in the airways [59, 82, 83]. HASMCs are crucial components of the airway structural wall owing to their ability to display an inflammatory response by secreting cytokines and chemokines, as well as to their ability to alter contractility of airways in response to inflammatory stimuli [3, 4].

CS is a known risk factor for the development of airway inflammation leading to asthma. It induces cytokine release, proliferation, migration and contraction in human bronchial smooth muscle cells (HBSMCs), all of which contribute to airway remodelling associated with asthma [186]. Studies have shown that CSE augments the release of various cytokines and chemokines from HASMCs which can contribute to the development of airway inflammation [187]. In particular, CSE induces the production of IL-8 in HASMCs, leading to neutrophilic influx into the airways, ultimately causing neutrophilic airway inflammation [188].

Although there are different endotypes of asthma and evidence suggests that CSE can promote neutrophilic airway inflammation, whether CSE can contribute to asthma endotype switching by affecting T2 inflammatory cytokines in HASMCs *in vitro* remains unclear.

3.2 Hypothesis and Aims

In this chapter we hypothesised that CSE affect the production of T2 and non-T2 inflammatory cytokines in HASMCs, contributing to the development of corticosteroid insensitive non-T2 asthma endotype

In this chapter, we aimed to:

- Determine the effect of CSE on the production of T2 and non-T2 inflammatory cytokines with and without TNFα stimulation in HASMCs.
- Assess the effect of CSE on the mRNA expression of non-T2 inflammatory cytokines in HASMCs.

3.3 Materials and methods

CSE was prepared from the smoke of two cigarettes (3R4F) bubbled into 20 ml of cell culture medium (Materials and Methods, section 2.3). Confluent and serum-starved HASMCs were pre-treated with and without CSE (3.5%) for 24 hours followed by incubation with CSE (3.5%), TNFα (1ng/ml) or CSE + TNFα for 24 hours. A Bio-plex ProTM Human Cytokine Assay kit was used to detect the concentration of T2 and

non-T2 inflammatory cytokines in the cell supernatants. All data were normalised to the amount of HASMC protein in the culture well and expressed as pg/mg protein. (Materials and Methods, section 2.9). RT-qPCR analysis was used to measure the mRNA expression of non-T2 inflammatory cytokines. For all RT-qPCR experiments, β2M was used as a reference gene (housekeeping gene) and data were expressed as relative expression compared to the control sample (Materials and Methods, section 2.12).

3.4 Results

3.4.1 Effect of CSE on the production of wide range of inflammatory cytokines and chemokines in HASMCs

In this chapter, we started to investigate the effect of CSE with and without TNFα on the production of a wide range of cytokines and chemokines that could play a role in airway inflammation in asthma. The results, as shown in Figure 3.1 revealed that HASMCs produced A) IL-2, B) IL-7, C) IL-9, D) IL-10, E) IL-12, F) IL-15, G) TNFα, H) IL-1ra, I) IL-1b, J) MIP-1a, K) MIP-1b, L) MCP-1, M) IFNϒ, N) FDF basic, O) PDGF-bb basally (383 ± 69, 946 ± 242, 1034 ± 23, 896 ± 166, 374 ± 5, 850 ± 55, 409 ± 19, 750 ± 132, 359 ± 57, 446 ± 31, 1064 ± 79, 231370 ± 46860, 3449 ± 560, 438 ± 56, 519 ± 39, pg/mg protein, respectively). CSE had no effect on all cytokines and chemokines that we tested but it inhibited and induced T2 and non-T2 inflammatory cytokines respectively (explained in sections 3.4.2 and 3.4.3, respectively). TNFα significantly increased the production of all the cytokines and chemokines that have been tested compared with the control.

Our results suggest that cigarette smoke could play a role in modifying inflammatory response in asthma by inhibiting T2 inflammatory cytokines and increasing non-T2 inflammatory cytokines in asthma.

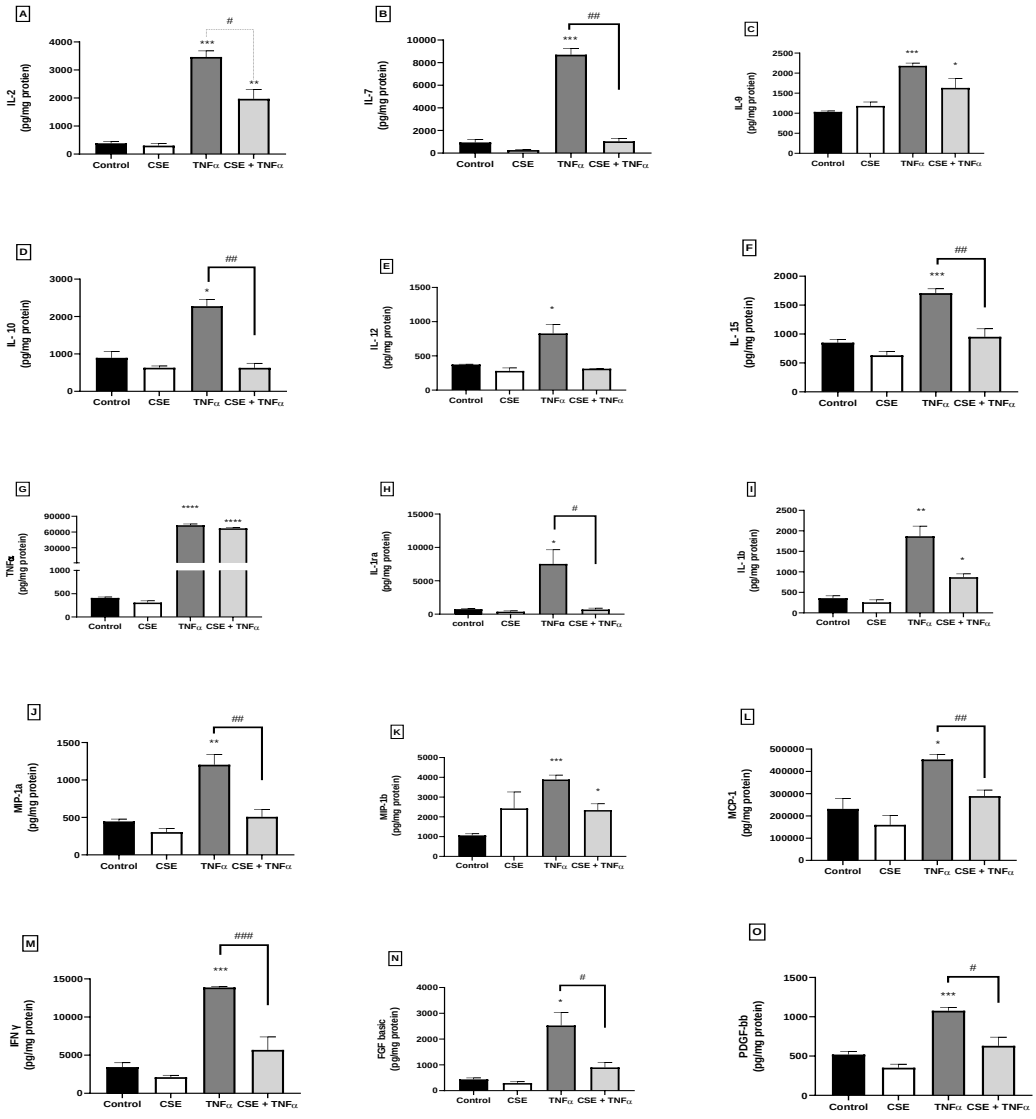

Figure 3.1. Effect of CSE on the production of different inflammatory mediators in HASMCs.

Confluent and serum-starved HASMCs were pre-treated with or without CSE 3.5% for 24 h, followed by incubation with CSE, TNFα (1ng/ml), or CSE+ TNFα for 24 h. The collected supernatants were used to measure the concentration of A) IL-2, B) IL-7, C) IL-9, D) IL-10, E) IL-12, F) IL-15, G) TNFα, H) IL-1ra, I) IL-1b, J) MIP-1a, K) MIP-1b, L) MCP-1, M) IFNY, N) FDF basic, O) PDGF-bb by bio-plex ELISA. Data were normalised to total protein. Each data point represents mean ± SEM of three experiments from three cell lines. *p<0.05,**p<0.01, ,***p<0.001, and ,****p<0.0001 compared with control and #p<0.05, ##p<0.01, ###p<0.001, and ####p<0.0001 compared with TNFα alone.

3.4.2 Effect of CSE on the production of Th2 cytokines

Th2 cytokines such as IL-4, IL-5 and IL-13 are associated with eosinophilic infiltration, mucus hypersecretion, immunoglobulin (Ig) E production, bronchial hyperresponsiveness and asthma exacerbations [58]. While the effect of CSE on neutrophilic airway inflammation is well documented, the effect of CSE on the release of Th2 cytokines IL-4, IL-5, and IL-13 in HASMCs *in vitro* has not previously been investigated. Thus, we examined the effect of CSE on the production of IL-4, IL-5, and IL-13 in HASMCs.

The results, as shown in Figure 3.2 A, B, and C revealed that HASMCs produced IL-4, IL-5 and IL-13 basally (883 ± 148, 683 ± 11 and 730 ± 89 pg/mg protein, respectively). CSE inhibited the production of IL-4 and IL-13 by 69% and 66% compared with control ($p<0.05$ for both, Figure 3.2 A and C respectively), but it had no effect on the production of IL-5 (Figure 3.2 B). TNFα increased the production of IL-4, IL-5 and IL-13 (4335 ± 391, $p<0.01$, 1993 ± 148, $p<0.001$ and 4335 ± 391 pg/mg protein, $p<0.01$, respectively) compared with control. CSE inhibited TNFα-induced IL-4 and IL-13 by 84% and 91% compared with the effect of TNFα alone ($p<0.01$ for both, Figure 3.2 A and C, respectively), but it had no effect on TNFα-induced IL-5 (Figure 3.2 B).

This inhibition of Th2 cytokines by CSE in HASMCs suggests that cigarette smoke may attenuate T2 airway inflammation in asthma.

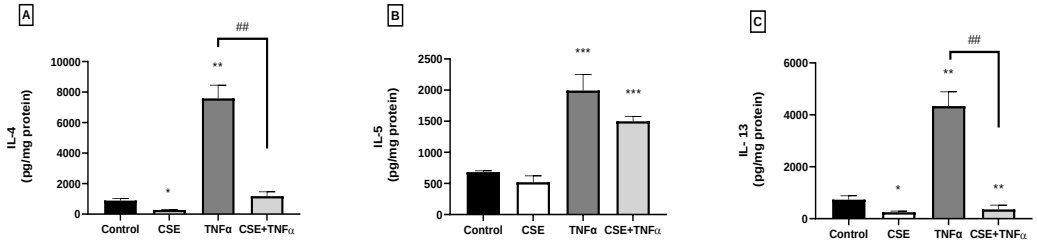

Figure 3.2. Effect of CSE and TNFα on the production of IL-4, IL-5, and IL-13 in HASMCs.

Confluent and serum-starved HASMCs were pre-treated with or without CSE 3.5% for 24 h, followed by incubation with CSE, TNFα (1ng/ml), or CSE+ TNFα for 24 h. The collected supernatants were used to measure the concentration of A) IL-4, B) IL-5, and C) IL-13 by bio-plex ELISA. Data were normalised to total protein. Each data point represents mean ± SEM of three experiments from three cell lines. *p<0.05, **p<0.01 and ***p<0.001 compared with control and ##p<0.01 compared with TNFα alone.

3.4.3 Effect of CSE on the production of eosinophil chemokines

The eosinophil chemokines eotaxin, IP-10 and RANTES are linked to eosinophil recruitment in the airway [189]. Eosinophils are a hallmark of T2 asthma, and they play an important role in airway inflammation [72]. In our study above, CSE significantly inhibited the Th2 cytokines, however, the effect of CSE on the production of eosinophil chemokines remains unknown. Thus, in this part of our study, we examined the effect of CSE on the production of eotaxin, IP-10 and RANTES. The results, as shown in Figure 3.3 A, B, and C revealed that HASMCs produced eotaxin, IP-10 and RANTES basally (62890 ± 16455, 1002 ± 140 and 1274 ± 134 pg/mg protein, respectively). CSE significantly inhibited the production of eotaxin, IP-10 and RANTES by 95%, 67%, and 71% compared with control ($p<0.05$, $p<0.01$, and $p<0.01$, Figure 3.3 A, B, and C, respectively). A significant increase in eotaxin, IP-10, and RANTES production was observed when HASMCs were stimulated with TNFα (263653 ± 38237, $p<0.01$, 173749 ± 45849, $p<0.05$, and 26652 ± 5680.7 pg/mg protein, $p<0.05$, respectively) compared with control. Interestingly, CSE inhibited TNFα-induced the production of eotaxin, IP-10 and RANTES by 91%, 98%, and 95% compared with the effect of TNFα alone ($p<0.01$, $p<0.05$, and $p<0.01$, Figure 3.3 A, B, and C, respectively). These findings strongly suggest that CSE may attenuate T2 airway response in HASMCs.

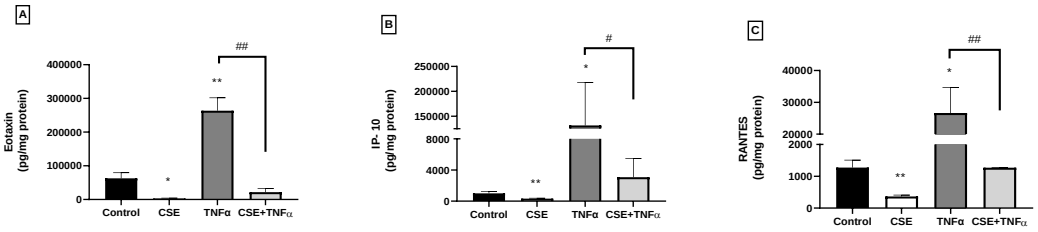

Figure 3.3. Effect of CSE and TNFα on the production of eotaxin, IP-10, and RANTES in HASMCs.

Confluent and serum-starved HASMCs were pre-treated with or without CSE 3.5% for 24 h, followed by incubation with CSE, TNFα (1ng/ml), or CSE+ TNFα for 24 h. The collected supernatants were used to measure the concentration of A) Eotaxin, B) IP-10, and C) RANTES by bio-plex ELISA. Data were normalised to total protein. Each data point represents mean ± SEM of three experiments from three cell lines. *p<0.05 and **p<0.01 compared with control and #p<0.05 and ##p<0.01 compared with TNFα alone.

3.4.4 Effect of CSE on the production of non-T2 inflammatory cytokines

Non-T2 inflammatory cytokines, including IL-6, IL-8 and VEGF, are associated with neutrophilic airway inflammation and airway remodelling [190, 191]. In this part of our study, we examined the effect of CSE on the production of IL-6, IL-8 and VEGF in HASMCs. Figure 3.4 A, B, and C revealed that HASMCs produced IL-6, IL-8 and VEGF basally (18210 ± 1125, 1188 ± 122 and 1373 ± 51 pg/mg protein, respectively). As shown in the figure, CSE significantly increased the production of IL-6, IL-8, and VEGF (42417 ± 6414, $p<0.05$, 20564 ± 4248, $p<0.05$ and 4214 ± 843 pg/mg protein, $p<0.05$, respectively) compared with control. TNFα induced the production of IL-6, IL-8 and VEGF (314692 ± 19793, $p<0.001$, 61156 ± 7585, $p<0.01$ and 3965 ± 318 pg/mg protein, $p<0.01$, respectively) compared with control. CSE significantly enhanced TNFα-induced IL-8 production by 2-fold ($p<0.01$) compared with the effect of TNFα alone (Figure 3.4 B), but it had no effect on TNFα-induced production of IL-6 and VEGF (Figure 3.4 A and C, respectively). These findings, along with the previous results presented in sections 3.4.1, 3.4.2, and 3.4.3 suggest that CS may attenuate T2 airway inflammation by inhibiting Th2 cytokines IL-4 and IL-13 and eosinophil chemokines but promotes non-T2 airway inflammation by increasing non-T2 inflammatory cytokines in asthmatic smokers.

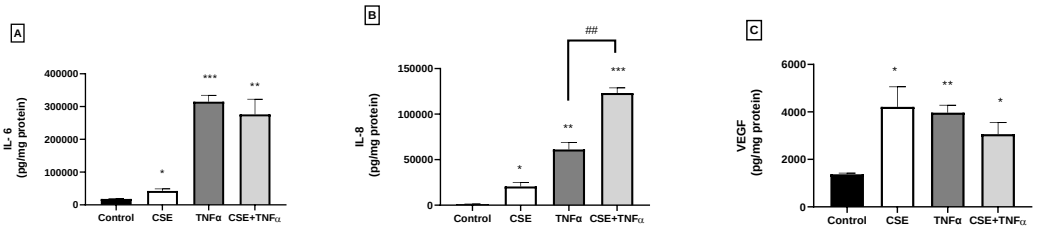

Figure 3.4. Effect of CSE and TNFα on the production of IL-6, IL-8, and VEGF in HASMCs.

Confluent and serum-starved HASMCs were pre-treated with or without CSE 3.5% for 24 h, followed by incubation with CSE, TNFα (1ng/ml), or CSE+ TNFα for 24 h. The collected supernatants were used to measure the concentration of A) IL-6, B) IL-8, and C) VEGF by bio-plex ELISA. Data were normalised to total protein. Each data point represents mean ± SEM of three experiments using three different cell lines. *p<0.05, **p<0.01, and ***p<0.001 compared with control and ##p<0.01 compared with TNFα alone.

3.4.5 Effect of CSE on the mRNA expression of non-T2 inflammatory cytokines

We presented in section 3.4.3 that CSE stimulated the production of IL-6, IL-8 and VEGF, which are associated with non-T2 airway inflammation [192] and most of the research in asthma has focused on the T2 inflammation, while the main phenotype in the asthmatic smokers is associated with neutrophilic airway inflammation [193] therefore, we were concentrated on non-T2 airway inflammation and explored the effect of CSE on the mRNA expression of non-T2 inflammatory cytokines in HASMCs.

The results showed that CSE increased IL-6 mRNA expression in a time-dependent manner with significant upregulation at 4, 16 and 24 hours ($p<0.05$, $p<0.01$ and $p<0.001$, respectively) compared with control (0 hours) (Figure 3.5 A). Additionally, CSE significantly upregulated IL-8 mRNA expression at all time points ($p<0.05$, $p<0.01$, $p<0.0001$, $p<0.01$, and $p<0.01$, respectively) compared with control (Figure 3.5 B), albeit in a biphasic manner with peaks in expression at 8 and 24 hours. Similarly, a significant upregulation of VEGF mRNA expression was noted following CSE stimulation at all time points ($p<0.05$, $p<0.01$, $p<0.05$, $p<0.01$, and $p<0.001$) compared with control (Figure 3.5 C). The above findings suggest that CSE-induced production of IL-6, IL-8 and VEGF in HASMCs could be via transcriptional regulation of the genes. This study supports our previous findings and strongly suggests that CS promotes non-type 2 airway inflammation in asthma.

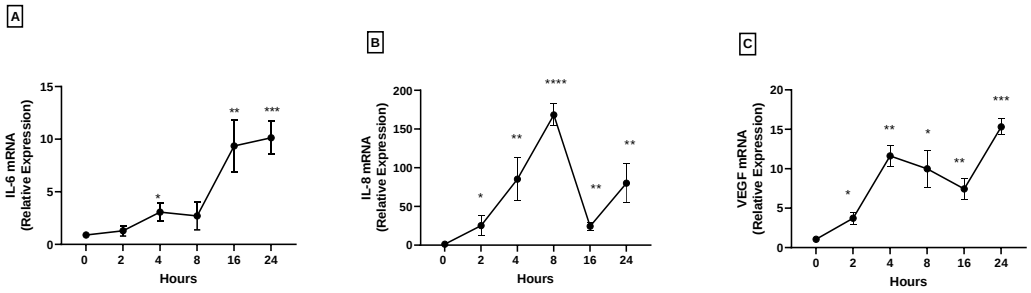

Figure 3.5. Effect of CSE on the mRNA expression of IL-6, IL-8, and VEGF in HASMCs.

Confluent and serum-starved HASMCs were treated with CSE (3.5%) for 2h, 4h, 8h, 16h and 24h before total RNA isolation. The samples were assayed for the mRNA expression of A) IL-6, B) IL-8, and C) VEGF by RT-qPCR. Each data point represents mean ± SEM of three experiments using three different cell lines. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 compared untreated cells.

3.5 Discussion

Asthma is a chronic inflammatory disease of the airways which occurs as a response to inhaled allergens, environmental factors and infections [191, 194]. HASMCs play a significant role in the pathogenesis of airway inflammation. They are responsible for bronchoconstriction due to their contractile property. Specifically in asthma, HASMCs release pro-inflammatory mediators in response to stimuli [195]. CSE can modulate airway inflammation, and significantly affects the proliferation, migration and contraction of HASMCs, all of which contribute to the airway remodelling process associated with asthma [186]. The main aim of this chapter was to examine whether CSE can influence the production of T2 inflammatory cytokines and non-T2 inflammatory cytokines in HASMCs and whether CSE could induce the gene expression of non-T2 inflammatory cytokines. In this chapter, we have examined the effect of CSE on the production of a wide range of different inflammatory cytokines and chemokines which are associated with airway inflammation in asthma. The key findings of this chapter are that CSE inhibited the production of Th2 cytokines IL-4 and IL-13 but had no effect on IL-5. Furthermore, CSE inhibited the production of the eosinophilic chemokines eotaxin, IP-10 and RANTES but induced the production and gene expression of non-T2 inflammatory cytokines IL-6, IL-8, and VEGF in HASMCs. Interestingly, CSE inhibited TNFα-induced production of Th2 cytokines IL-4 and IL-13 and eosinophilic chemokines but enhanced TNFα-induced IL-8 production. These results are summarised in Table 3.1.

The results presented in this chapter demonstrate for the first time that CSE inhibited the production of T2 inflammatory cytokines while simultaneously

stimulating the production of non-T2 inflammatory cytokines in HASMCs. The induction of non-T2 inflammatory cytokines in HASMCs may potentially contribute to neutrophilic airway inflammation and remodelling observed in asthmatic smokers. Our results suggest that CS may attenuate T2 airway inflammation but promote non-T2 airway inflammation. This is a novel observation that has not been previously reported.

Table 3.1. A summary of the results of chapter 3.

Type 2 inflammatory cytokines	Effect of CSE compared with control	Effect of TNFα compared with control	Effect of CSE + TNFα compared with TNFα
IL-4	↓	↑	↓
IL-5	↔	↑	↔
IL-13	↓	↑	↓
Eotaxin	↓	↑	↓
IP-10	↓	↑	↓
RANTES	↓	↑	↓
Non-Type 2 inflammatory cytokines			
IL-6	↑	↑	↔
IL-8	↑	↑	↑
VEGF	↑	↑	↔

A summary of the effect of CSE, TNFα, and CSE+ TNFα on the production of Type 2 inflammatory cytokines and non-Type 2 inflammatory cytokines in HASMCs. ↑ , Induced; ↓ , Inhibited; ↔, Unchanged.

3.5.1 CSE and T2 inflammatory cytokines

Asthmatics who smoke have airway inflammation (neutrophilic airway inflammation) that differs from that of non-smoking asthmatics (eosinophilic airway inflammation) [196], and clinical studies that have examined the effect of CS on airway inflammation have generally excluded smokers to avoid the effect of CS on the studies' outcomes [197]. Therefore, there is a lack of information about the effect of CS on airway inflammation in asthma patients.

Th2 cytokines IL-4, IL-5 and IL-13 play an important role in the pathogenesis of T2 inflammation in asthma. IL-4 promotes Ig isotype switching in B cells, leading to the generation of IgE and IgG [73]. IL-5 has been demonstrated to promote the maturation, proliferation, migration and survival of eosinophils, whereas IL-13 induces both airway hyperresponsiveness and mucus metaplasia [73]. Furthermore, there is a correlation between the level of eosinophils and the production of Th2 cytokines (IL-4, IL-5 and IL-13) and eosinophil chemokines (eotaxin, IP-10 and RANTES) in asthma [69, 70], and the cooperation between Th2 cytokines and eosinophil chemokines has been shown to play a significant role in eosinophil recruitment *in vivo* [71]. We demonstrated for the first time that CSE inhibits the production of both IL-4 and IL-13 but has no effect on IL-5. In addition, CSE inhibited TNFα-induced IL-4 and IL-13 but had no effect on TNFα-induced IL-5 in HASMCs. Our findings suggest that in HASMCs, IL-4, IL-5 and IL-3 respond differently to CSE, which may modulate airway inflammation in asthma. It has been reported that in *vivo* studies T cells were induced to express IL-5 in CS-treated mice [109]. In another *in vivo* study, mice exposed to CS showed increased IL-5 in the bronchial alveolar lavage fluid (BALF), but their IL-4 and

IL-13 levels did not change [111]. The function of IL-5 but not IL-4 or IL-13 in *in vivo* models is affected by CS; however, our study showed that IL-4 and IL-13 but not IL-5 is inhibited by CSE in HASMCs. The differences in experimental models are the main reason for the disparity between our findings and those of the reported studies [109, 111]. It also could be due that *In vivo,* exposure to CSE results in a large number of different cell types being exposed to CS making it difficult to discern whether the effect described is due to HASMs or another cell type. We have specifically studied the effect of CSE on HASM cells in isolating to understand what the effect is on a cell type that is crucial to asthma pathogenesis.

Our findings suggest that CS affects the functions of Th2 cytokines in different regulatory processes. We expected CSE to inhibit TNFα-induced IL-5 as demonstrated by my fellow PhD student, who showed that CSE inhibited IL-17-induced IL-5 production (unpublished data). However, we found that CSE did not inhibit TNFα-induced IL-5 production. This could be because he used IL-17 as opposed to TNFα, which was used in our study. IL-5 has been shown to stimulate the maturation of eosinophils in bone marrow (28). Since we found that CSE had no effect on IL-5, CSE may not have an effect on the maturation, proliferation and migration of eosinophils from bone marrow, however more detailed studies would be needed to confirm this. Eosinophil chemokines, such as eotaxin, IP-10 and RANTES, recruit eosinophils to the site of inflammation [78, 198]. In the present study, CSE inhibited eosinophil chemokines. This suggests that CSE does not affect IL-5 function (maturation of eosinophils from bone marrow) but may inhibits the recruitment of eosinophils from the blood to the inflammatory site in the airway.

The presence of eosinophil chemokines in the airway is associated with T2 inflammation in asthma [199]. It has been reported that the level of eosinophils in BALF increased in asthmatics compared with non-asthmatic [200]. An *in vivo* study showed that asthmatic mice exposed to CS had significantly reduced eosinophil levels in their BALF compared with controls (asthmatic mice not exposed to CS) [112], but the mechanism driving this effect was not explored. In the present study, we provide strong evidence that CSE can suppress T2 airway inflammation in asthma. This finding is supported by *Oltmanns et al.* [113] and *Eddleston et al.* [114]. The authors of these studies examined the effect of CSE on the production of eosinophil chemokines (RANTES and eotaxin) in HASMCs [113] and human bronchial epithelial cells (16-HBE and BEAS-2B) [114, 115] and showed that CSE can supress the production of RANTES and eotaxin, suggesting an association between CSE and eosinophil chemokines inhibition in airway structural cells in asthma. Our findings are further supported by the fact that the level of eosinophils in the blood is increased in ex-smokers compared with non-smokers [201], suggesting that CS can affect eosinophil production in asthma leading to the modulation of airway inflammation in asthmatic smokers.

Taken together, the findings of this part of our study demonstrated novel results: CSE inhibits the release of Th2 cytokines IL-4 and IL-13 and eosinophil chemokines in HASMCs. This suggests that cigarette smoke may attenuate type 2 airway inflammation in asthma patients.

3.5.2 CSE and non-T2 inflammatory cytokines

Our study showed that CSE stimulated the production of non-T2 inflammatory cytokines, IL-6, IL-8 and VEGF, in HASMCs and augmented TNFα-induced IL-8 production compared with the effect of TNFα alone. Furthermore, CSE induced the mRNA expression of IL-6, IL-8 and VEGF in HASMCs.

IL-6 is a potent pro-inflammatory cytokine that is considered a marker of inflammation and is implicated in the pathogenesis of asthma [73]. IL-8 is involved in the recruitment and activation of neutrophils and promotes neutrophilic accumulation within the airways. IL-8 levels are inversely proportional to pulmonary function and directly proportional to the severity of asthma [56]. VEGF is a potent angiogenic factor that plays an essential role in airway remodelling in asthma [92]. Our findings are in line with previous studies demonstrating that treatment of CSE induced the production of IL-6 in human airway epithelial cells (BEAS-2B) [202] and human tracheal smooth muscles (HTSMCs) [141], IL-8 in human-derived THP-1 cells [203], human tracheal epithelial cells (HTEpC) [117] and HASMCs [113, 118, 121, 188, 204-208] and VEGF in HASMCs [121] and fibroblast cells [120]. Based on our findings and other observations, CSE increased the expression of IL-6, IL-8 and VEGF in HASMCs, suggesting that airway smooth muscle is an important source of inflammatory cytokines and plays an important role in airway inflammation in asthmatic smokers.

In this chapter we also demonstrate that CSE enhanced TNFα-induced IL-8 production but not IL-6 or VEGF. HASM has been shown to be a rich source of cytokines and chemokines, including IL-8, which is released in large amounts in

response to TNFα [3, 164]. TNFα is a potent stimulator of IL-8, and the level of TNFα increases in asthmatic BALF and is associated with an increase in neutrophilia [209]. This suggests that the important role of IL-8 in recruiting neutrophils into the airways in asthma and the presence of TNFα in the lungs can explain the synergistic effect of CSE on TNFα-induced IL-8 release, which leads to neutrophilic airway inflammation in asthmatic smokers [113, 210]. In addition, neutrophilic airway inflammation appears to be the main endotype in asthmatic smokers that can contribute to glucocorticoid-insensitivity [107]; however, the underlying mechanism of CSE-induced neutrophilic airway inflammation is not clear. Therefore, we focused on non-T2 airway inflammation and investigated whether the induction of IL-6, IL-8 and VGEF could occur via a transcriptional mechanism in HASMCs.

We reported that CSE upregulated the mRNA expression of IL-6, IL-8 and VEGF in HASMCs. Studies have shown that CSE upregulates IL-8 mRNA levels, and this upregulation is associated with an increased production of IL-8 in human bronchial smooth muscle cells (HBSMCs) [206]. In an *in vivo* study, mice exposed to CS showed increased levels of IL-6 mRNA in their lung tissue compared with controls [211]. The same study also showed that CSE induced the expression of IL-6 mRNA in human bronchial epithelial cells (HBECs) [211]. In addition, it has been reported that CSE upregulated the expression and production of VEGF mRNA in HASMCs and normal human lung fibroblasts (NHLFs) [120]. Our findings are in line with these observations, suggesting that CSE is a potent stimulus for IL-6, IL-8 and VEGF in HASMCs. The increase in non-T2 inflammatory cytokines after CSE stimulation is associated with an increase in their mRNA expression, suggesting that CSE-induced production of IL-6,

IL-8 and VEGF in HASMCs could be via transcriptional regulation of the genes. Because studies suggest that IL-6, IL-8 and VEGF play a significant role in modulating lung function and are associated with the pathogenesis of asthma and because asthmatic smokers tend to demonstrate increased levels of non-T2 inflammatory cytokines [212], our results suggest that CS may contribute to non-T2 airway inflammation in asthmatic smokers through IL-6, IL-8 and VEGF.

In conclusion, this part of our study showed novel results demonstrating that CSE affects the release of cytokines and chemokines in HASMCs in different ways. CSE inhibited the release of T2 inflammatory cytokines while inducing the release of non-T2 inflammatory cytokines in HASMCs. These findings suggest that CS plays a role in regulating asthma endotypes; it may facilitate a shift from T2 inflammation to non-T2 inflammation by suppressing eosinophil airway inflammation and promoting neutrophil airway inflammation thereby contributing to glucocorticoid-insensitivity in asthma. However, the underlying signalling pathway of the effect of CSE on the production of inflammatory cytokines has not yet been fully investigated. Therefore, the next chapter will explore the singling pathway that may be involved in mediating the effect of CSE on the production of non-T2 inflammatory cytokines (IL-6, IL-8, and VEGF) in HASMCs.

Chapter 4. Mechanisms of CSE effect on the production of non-T2 inflammatory cytokines in HASMCs

4.1 Introduction

We demonstrated in Chapter 3 that CSE can induce and inhibit non-T2 and T2 inflammatory cytokines, respectively, in HASMCs. CSE is made up of over 4000 substances which makes CSE a potent stimulus. Nicotine is the major and addictive component in cigarettes which acts via nicotinic acetylcholine receptors (nAChRs) [213] It has been shown that nicotine upregulated the production of IL-8 in human periodontal ligament cells [214]. In addition, there is extensive evidence linking oxidative stress to CSE induced airway inflammation. In particular, *Oltmanns et al.* [128] showed that thirty minutes pre-treatment with an oxidative stress inhibitor glutathione (GSH), a potent antioxidant [129], showed inhibition of CSE-induced IL-8 production in HASMCs which suggest that oxidative stress could play a role in the CSE induced production of IL-8 [128]. Another study showed that CSE-induced production of IL-8 and VEGF in HASMCs was inhibited by glycogen synthase kinase-3 (GSK-3) inhibitor SB216763, and the effect of CSE was suggested to be through the activation of NF-κB pathway [121]. Pang and Knox showed another possible mechanism of COX pathway. They demonstrated that the inflammatory mediator bradykinin induced the production of IL-8 through both COX-1 and COX-2 in HASMCs [155].

Despite the studies described above in various other cell types, the exact mechanism behind the effect of CSE-induced IL-6, IL-8 and VEGF release in HASMCs is not known. Therefore, in this chapter, we focus on understanding the role of nicotine and the mechanism driving CSE-induced production of IL-6, IL-8 and VEGF in HASMCs.

4.2 Hypothesis and Aims

Our hypothesis in this chapter is that CSE affect non-T2 inflammatory response via COX-2 and PGE_2 pathway through oxidative stress.

In this chapter, we aim to understand the underlying mechanism of CSE effect on the production of non-T2 inflammatory cytokines in HASMCs. We will:

- Examine the role of nicotine in mediating the stimulatory effect of CSE on the production of non-T2 inflammatory cytokines by applying nicotine and the α7 nAChR antagonist MG624.
- Explore the role of oxidative stress in CSE-induced the production of non-T2 inflammatory cytokines by applying the oxidative stress inhibitor GSH.
- Investigate the role of COX-2 pathway in CSE-induced the production of non-T2 inflammatory cytokines by applying the COX-2 inhibitor NS-398 and determine the effect of CSE on the mRNA and protein expression of COX-2, mPGES-1 and PGE_2 release.
- Assess the effect of oxidative stress inhibitor GSH on CSE-induced the protein expression of COX-2 and PGE_2 release.
- Investigate the role of PGE_2 on CSE-induced the production of non-T2 inflammatory cytokines by applying exogenous PGE_2 and the EP_2 receptor antagonist PF 04418948 and EP_4 receptor antagonist L-161,982.
- Explore the effect of β_2-agonist salbutamol on the production of non-T2 inflammatory cytokines in HASMCs.

4.3 Materials and methods

CSE was prepared from the smoke of two cigarettes (3R4F) bubbled into 20 ml of cell culture medium (Materials and Methods, section 2.3). To explore the effect of nicotine, confluent HASMCs were serum-starved with DMEM- for 24 hours followed by treatment with nicotine (500 μM) and with or without CSE for 24 hours. To assess the effect of α7 neuronal nicotinic receptor antagonist, oxidative stress inhibitor, COX-2 inhibitors, EP_2 receptor antagonist, and EP_4 receptor antagonist, on the effect of CSE, confluent HASMCs were serum-starved with DMEM- for 24 hours followed by treatment with or without MG 624 (1 μM), GSH (100 μM), NS-398 (10 μM), PF 04418948 (10 μM), and L-161,982 (1 μM), respectively for 1 hour before incubation with CSE for 24 hours. To explore the effect of PGE_2, forskolin, and salbutamol, confluent HASMCs were serum-starved with DMEM- for 24 hours followed by treatment with PGE_2 (10 μM), forskolin (10 μM), and salbutamol (10 μM), respectively and with or without CSE for 24 hours. (Materials and Methods, section 2.4). A Bio-plex Pro™ Human Cytokine Assay kit was used to detect the concentration of non-T2 inflammatory cytokines and PGE_2 in the cell supernatants. All data were normalised to the amount of HASMC protein in the culture well and expressed as pg/mg protein. (Materials and Methods, section 2.9). Western blot was used to assess the protein expression of COX-2 and mPGES-1 (Materials and Methods, section 2.11). β-actin was used as a loading control to ensure equal loading of samples. RT-qPCR analysis was used to measure the mRNA expression of COX-2 and mPGES-1**.** For all RT-qPCR experiments, β2M was used as a reference gene (housekeeping gene) and data were

expressed as relative expression compared to the control sample (Materials and Methods, section 2.12).

4.4 Results

4.4.1 Role of nicotine on the production of non-T2 inflammatory cytokines in HASMCs

We have shown in chapter 3 that CSE drives IL-6, IL-8 and VEGF production in HASMC at translational and transcriptional levels. To determine whether the major active component of CSE, nicotine, was responsible for this induction we cultured ASMCs with CSE in the presence and absence of a potent inhibitor (MG624) of the major nicotine receptor a7 nAChR [215] of HASMC. We found that in the presence of the inhibitor there was no reduction in the production of IL-6, IL-8 and VEGF in response to CSE. The results as shown in Figure 4.1 revealed that HASMCs produced A) IL-6, B) IL-8 and C) VEGF basally. Nicotine had no effect on the production of IL-6, IL-8 and VEGF in HASMCs (Figures 4.1 A, B and C, respectively). In addition, MG624 had no effect on CSE-induced production of IL-6, IL-8 and VEGF in HASMCs (Figures 4.1 A, B and C, respectively). Collectively, these results suggest that the effect of CSE on the production of non-T2 inflammatory cytokines in HASMCs is not mediated by nicotine.

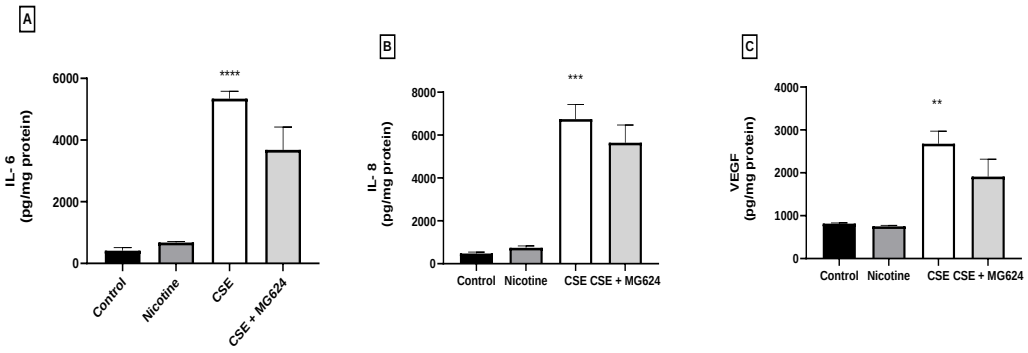

Figure 4.1. Role of nicotine on the production IL-6, IL-8, and VEGF in HASMCs.

Confluent and serum-starved HASMCs were pre-treated with MG624 (1 µM) for 1h, followed by treatment with or without nicotine (500 µM) or CSE (3.5%) for 24h. The collected supernatants were used to measure the concentration of A) IL-6, B) IL-8, and C) VEGF by bio-plex ELISA. Data were normalised to total protein. Each data point represents mean ± SEM of three experiments from three different donors carried out in duplicate $^{**}p<0.01$, $^{***}p<0.001$, and $^{****}p<0.0001$ compared with the control.

4.4.2 Effect of the oxidative stress inhibitor GSH on CSE-induced the production of non-T2 inflammatory cytokines in HASMCs.

Although the mechanism involved in the effect of CSE-induced non-T2 inflammatory cytokines is not fully understood, it is widely recognised that oxidative stress plays a crucial role in the development of lung damage in smokers. Moreover, reactive oxygen species (ROS) cause infiltration of neutrophils in the airways [126]. Previous studies have shown that pre-treatment with an oxidative stress inhibitor glutathione (GSH) inhibits CSE-induced IL-8 production in HASMCs [128]. Thus, in this study, we used the oxidative stress inhibitor GSH to explore whether oxidative stress could mediate CSE effect on the production of non-T2 inflammatory cytokines in HASMCs. GSH inhibited CSE-induced production of IL-6 by 38% ($p<0.001$, Figure 4.2 A) and IL-8 by 41.2% ($p<0.01$, Figure 4.2 B) compared with CSE alone, but had no effect on the production of VEGF (Figure 4.2 C). This inhibition of the production of IL-6 and IL-8 by GSH suggests that oxidative stress may play a role in mediating CSE effect on the production of non-T2 inflammatory cytokines in HASMCs.

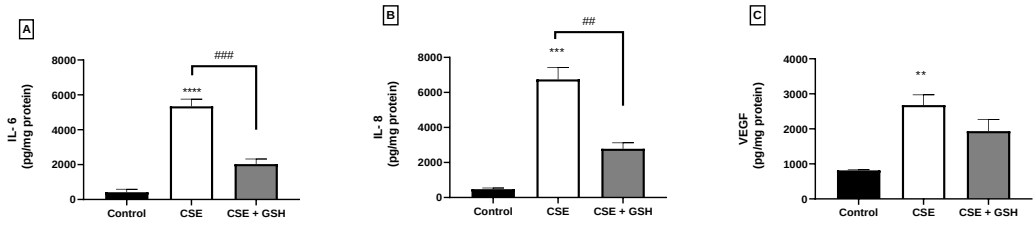

Figure 4.2. Effect of GSH on CSE-induced the production of IL-6, IL-8, and VEGF in HASMCs.

Confluent and serum-starved HASMCs were pre-treated with GSH (100 μM) for 1h, followed by treatment with CSE (3.5%) for 24h. The collected supernatants were used to measure the concentration of A) IL-6, B) IL-8, C) VEGF concentrations by bio-plex ELISA. Data were normalised to total protein. Each data point represents mean ± SEM of three experiments from three different donors carried out in duplicate. $^{**}p<0.01$, $^{***}p<0.001$ and $^{****}p<0.0001$ compared with control and $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ compared with CSE.

4.4.3 Effect of the COX-2 inhibitor NS-398 on CSE-induced the production of non-T2 inflammatory cytokines in HASMCs

Arachidonic acid is converted to prostaglandin H_2 via cyclooxygenase (COX) pathway, which can be further metabolised to prostaglandin E_2 [144] via microsomal prostaglandin E synthase (mPGES) [132]. It has been reported that CSE induces COX-2 expression in HASMCs [139]. However, whether the effect of CSE on the production of non-T2 inflammatory cytokines is mediated by COX-2 pathway is still not understood. Thus, in this study, we examined the effect of the COX-2 inhibitor NS-398 on CSE-induced the production of non-T2 inflammatory cytokines in HASMCs. CSE induced the production of IL-6, IL-8, and VEGF compared with control. Interestingly, NS-398 inhibited CSE-induced the production of IL-6, IL-8, and VEGF (45.5%, $p<0.01$, 66%, $p<0.05$ and 45.6%, $p<0.05$, respectively) compared with CSE alone (Figure 4.3 A, B, and C). These findings suggest that the effect of CSE on the production of non-T2 inflammatory cytokines is mediated by COX-2 in HASMCs.

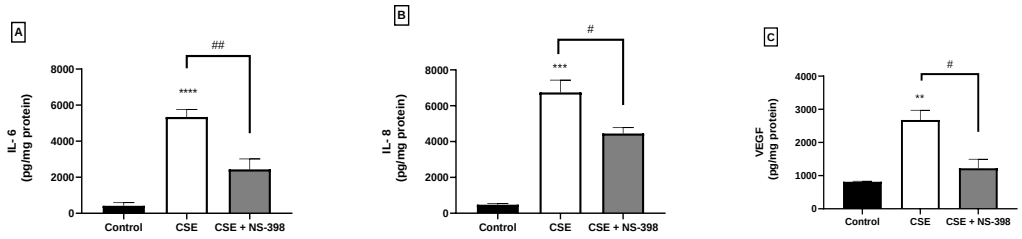

Figure 4.3. Effect of NS-398 on CSE-induced the production of IL-6, IL-8, and VEGF in HASMCs.

Confluent and serum-starved HASMCs were pre-treated with NS-398 (10 μM) for 1h, followed by treatment with CSE (3.5%) for 24h. The collected supernatants were used to measure the concentration of A) IL-6, B) IL-8, and C) VEGF by bio-plex ELISA. Data were normalised to total protein. Each data point represents mean ± SEM of three experiments from three different donors carried out in duplicate. $^{**}p<0.01$, $^{***}p<0.001$, and $^{****}p<0.0001$ compared with control and $^{\#}p<0.05$ and $^{\#\#}p<0.01$ compared with CSE.

4.4.4 Effect of CSE and nicotine on the protein and mRNA expression of COX-2, mPGES-1 and PGE_2 production in HASMCs

In section 4.4.3 we observed that CSE-induced non-T2 inflammatory cytokines were inhibited by the COX-2 inhibitor NS-398 which suggest that COX-2 activity and its downstream PGE_2 could play a role in mediating CSE effect on the production of non-T2 inflammatory cytokines in HASMCs. Thus, in this part of the study, we started by examining the effect of CSE and nicotine on the protein expression of COX-2 and mPGES-1. The results demonstrated that the protein expression of COX-2 and mPGES-1 were undetectable at basal levels in HASMCs (Figure 4.4 A and 4.5 A, respectively). Treatment with CSE induced the protein expression of COX-2 and mPGES-1 in HASMCs (Figure 4.4 A and 4.5 A, respectively). An optical densitometry analysis revealed that HASMCs following CSE stimulation significantly increased the protein expression of COX-2 (fold change 15 ± 1, $p<0.001$; Figure 4.4 B) and mPGES-1 (fold change 825 ± 140, $p<0.01$; Figure 4.4 B) over basal levels. In contrast, treatment with nicotine had no effect on the protein expression of COX-2 and mPGES-1 (Figure 4.4 A and 4.5 A, respectively).

These results suggest that the protein expression of COX-2 and mPGES-1 can be upregulated by CSE but not nicotine, which strongly support the previous observation in section 4.4.3 that CSE effect on non-T2 inflammatory cytokines is mediated by COX-2/mPGES-1.

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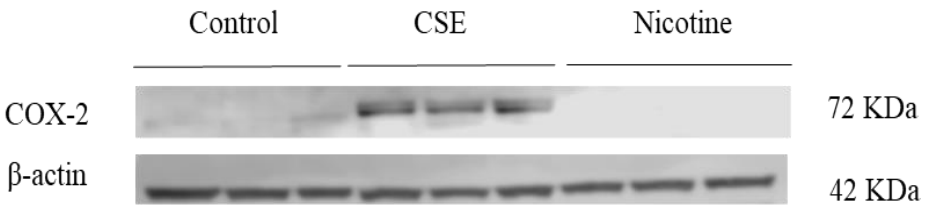

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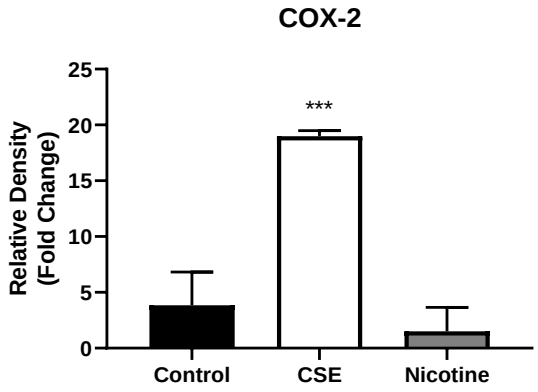

Figure 4.4. Effect of CSE and nicotine on the protein expression of COX-2 in HASMCs.

Confluent and serum-starved HASMCs were treated with CSE (3.5%) and nicotine (500 μM) for 24h. The collected cell lysates were used to detect the protein expression of COX-2 by Western blot analysis. A, a representative Western blot of three cell lines showing the effect of CSE and nicotine on the protein expression of COX-2. B, an optical densitometry analysis of Western blotting bands. Data were normalised with loading control β-actin and were expressed as fold change over control. Each data point represents mean ± SEM of three experiments using three different cell lines. ***$p<0.001$ compared with control.

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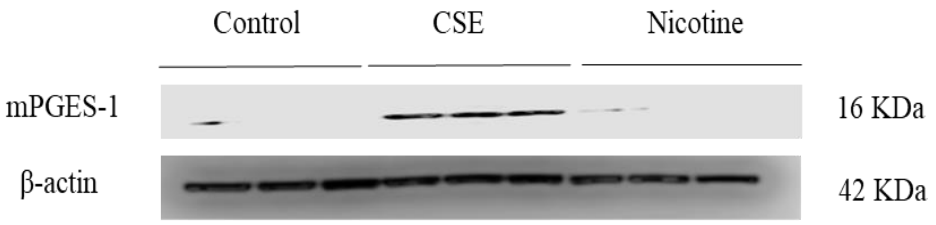

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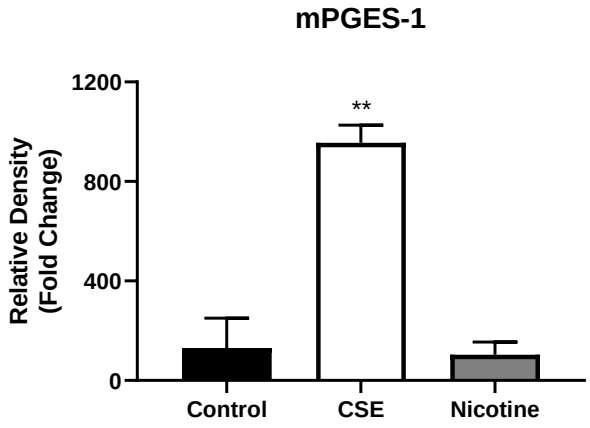

Figure 4.5. Effect of CSE and Nicotine on the protein expression of mPGES-1 in HASMCs.

Confluent and serum-starved HASMCs were treated with CSE (3.5%) and Nicotine (500 μM) for 24h. The collected cell lysates were used to detect the protein expression of mPGES-1 by Western blot analysis. A, a representative Western blot of three cell lines showing the effect of CSE and Nicotine on the protein expression of mPGES-1. B, an optical densitometry analysis of Western blotting bands. Data were normalised with loading control β-actin and were expressed as fold change over control. Each data point represents mean ± SEM of three experiments using three different cell lines. $^{**}p<0.01$ compared with control.

We next questioned whether the induction of COX-2 and mPGES-1 by CSE at the protein level could be via transcriptional regulation of the genes. Thus, we explored the effect of CSE on the mRNA expression of COX-2 and mPGES-1 in HASMCs. The results showed that the mRNA expression of COX-2 was significantly upregulated by CSE at 2 and 8 hours ($p<0.001$, and $p<0.01$, respectively) compared with control (0 hours) (Figure 4.6 A). After that, COX-2 expression returned to the basal level after 16 hours. Similarly, CSE significantly upregulated mPGES-1 mRNA expression at 2 hours ($p<0.01$), followed by a reduction in expression close to basal level at 8 hours. However, at 16 hours we observed a significant upregulation ($p<0.001$) compared with control (Figure 4.6 B). These findings suggest that CSE can upregulate the gene expression of COX-2 and mPGES-1 in HASMCs via a transcriptional mechanism.

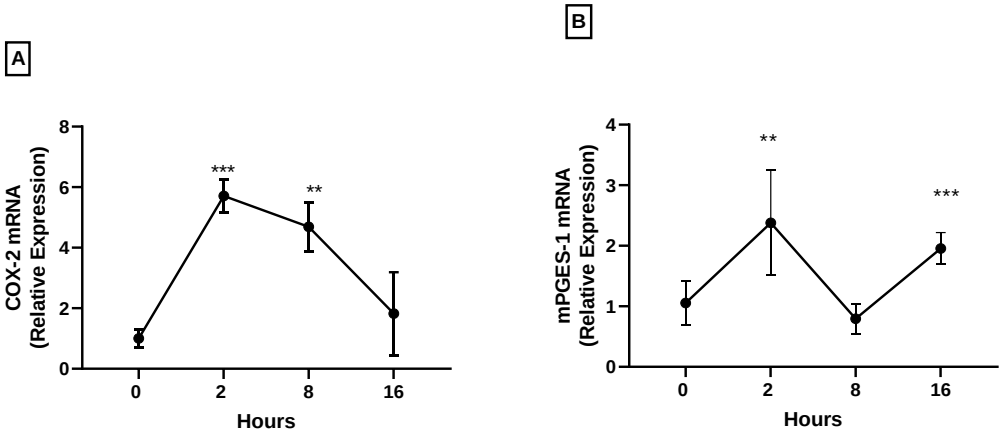

Figure 4.6. Effect of CSE on the mRNA expression COX-2 and mPGES-1 in HASMCs.

Confluent and serum-starved HASMCs were treated with CSE (3.5%) over three-time points (2h, 8h, and 16h) prior to total RNA isolation. The samples were assayed for the mRNA expression of A) COX-2 and B) mPGES-1 by RT-qPCR. Each data point represents mean ± SEM of three experiments using three different cell lines. $^{**}p<0.01$ and $^{***}p<0.001$ compared untreated cells.

PGE_2 is the downstream prostanoid product of COX-2 activity, and its production is mediated by mPGES-1 enzyme. The upregulation of COX-2 and mPGES-1 is expected to increase the production of PGE_2. Thus, we examined the effect of CSE on the production of PGE_2 in HASMCs. The results revealed that HASMCs produced PGE_2 basally (770 ± 64 pg/mg protein) (Figure 4.7). A significant increase in the production of PGE_2 was observed following CSE treatment (1780 ± 244 pg/mg protein, $p<0.05$) compared with control (Figure 4.7).

Collectively, our results provide strong evidence that the effect of CSE on non-T2 inflammatory cytokines is largely mediated by COX-2, mPGES-1 expression and PGE_2 release in HASMCs.

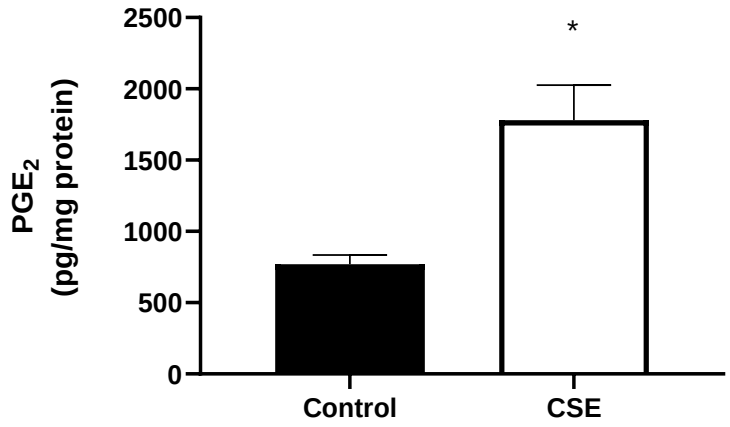

Figure 4.7. Effect of CSE on the production of PGE_2 in HASMCs.

Confluent and serum-starved HASMCs were treated with CSE (3.5%) for 24h. The collected supernatants were used to measure PGE_2 concentrations by ELISA. Data were normalised to total protein. Each data point represents mean ± SEM of three experiments from three different cell lines. *$p<0.05$ compared with control.

4.4.5 Effect of the oxidative stress inhibitor GSH on CSE-induced the protein expression of COX-2 and PGE_2 production in HASMCs.

Our previous findings showed that CSE-induced the production of non-T2 inflammatory cytokines, and this effect was inhibited by the oxidative stress inhibitor (GSH) in HASMCs, suggesting that oxidative stress plays a role in the effect of CSE on non-T2 inflammatory cytokines. We also demonstrated that the effect of CSE on non-T2 inflammatory cytokines is largely mediated by COX-2 expression. However, whether oxidative stress plays a role in mediating COX-2 expression and PGE_2 release in HASMCs has not been investigated. Thus, we started this part of the study by examining the effect of GSH on CSE-induced protein expression of COX-2 in HASMCs. The results revealed that, as we previously reported in section 4.4.4 (Figure 4.4 A and B), the protein expression of COX-2 was undetectable at basal levels in HASMCs (Figure 4.8 A and B) and treatment with CSE significantly induced the protein expression of COX-2 (fold change 383 ± 41, $p<0.001$) over the basal level. Interestingly, the protein expression of COX-2 was significantly reduced when HASMCs were pre-treated with GSH for 1 hour, followed by CSE treatment for 24 hours (Figure 4.8 A and B). Then, we assessed the effect of GSH on CSE-induced production of PGE_2 in HASMCs. As shown previously in section 4.4.4 (Figure 4.7), HASMCs produced the production of PGE_2 basally (Figure 4.9), and CSE significantly increased the production of PGE_2 compared with control (Figure 4.9). Interestingly, GSH inhibited CSE-induced PGE_2 production by 79% and 47% compared with CSE and control, respectively (Figure 4.9).

Collectively we established that GSH inhibited CSE-induced protein expression of COX-2 and PGE_2 release in HASMCs. These results suggest that the effect of CSE is mediated by COX-2/mPGES-1/PGE_2 via oxidative stress in HASMCs.

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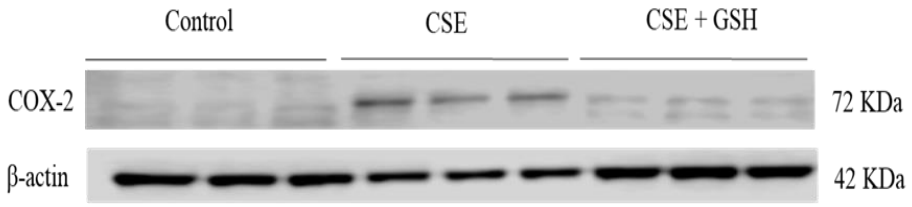

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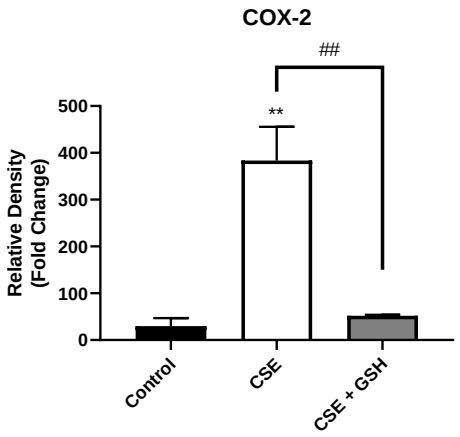


Figure 4.8. Effect of GSH on CSE-induced the protein expression of COX-2 in HASMCs.

Confluent and serum-starved HASMCs were pre-treated with GSH (100 μM) for 1h followed by CSE (3.5%) for 24h. The collected cell lysates were used to detect the protein expression of COX-2 by Western blot analysis. A, a representative Western blot of three cell lines showing the effect of CSE with and without GSH on the protein expression of COX-2. B, an optical densitometry analysis of Western blotting bands. Data were normalised with loading control β-actin and were expressed as fold change over control. Each data point represents mean ± SEM of three experiments using three different cell lines. ** $p<0.01$ compared with control and ## $p<0.01$ compared with CSE.

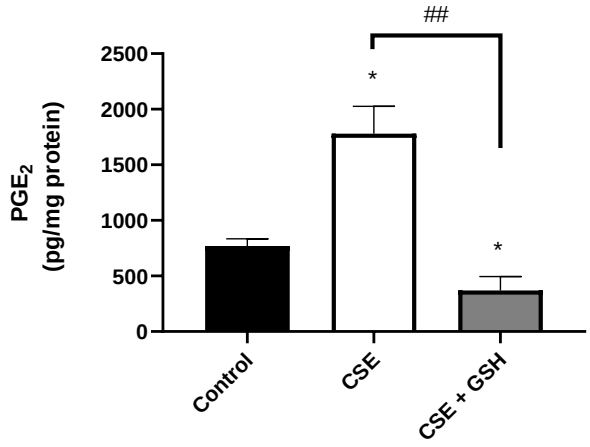


Figure 4.9. Effect of GSH on CSE-induced the production of PGE_2 in HASMCs.

Confluent and serum-starved HASMCs were pre-treated with GSH (100 µM) for 1h, followed by CSE (3.5%) for 24h. The collected supernatants were used to measure PGE_2 concentrations by ELISA. Data were normalised to total protein. Each data point represents mean ± SEM of three experiments from three different cell lines. *p<0.05 compared with untreated HASMCs and ##p<0.01 compared with CSE.

4.4.6 Effect of exogenous PGE_2 on the production of non-T2 inflammatory cytokines in HASMCs

Our previous findings suggest that the effect of CSE is largely mediated by COX-2/mPGES-1/PGE_2 via oxidative stress. PGE_2 is a pro-inflammatory meditator, playing an important role in airway inflammation. In previous studies, an increase in the production of PGE_2 has been reported in tracheal smooth muscle cells following CSE treatment [144]. Pang and Knox have previously demonstrated that PGE_2 induces IL-8 production in HASMCs [155]. We demonstrated that CSE induced the production of PGE_2 in HASMCs, and in order to explore the role of PGE_2 in mediating CSE effect on the production of non-T2 inflammatory cytokines, we used exogenous PGE_2 as a tool to support our previous studies that PGE_2 can mediate CSE effect on the production of non-T2 inflammatory cytokines. The results as shown in Figure 4.10 A, B, and C, revealed that HASMCs produced IL-6, IL-8, and VEGF basally, and CSE significantly induced these cytokines consistent with previous findings in sections 4.4.1, 4.4.2, and 4.4.3. Similarly, exogenous PGE_2 increased the production of IL-6, IL-8, and VEGF (190109 ± 3580, $p<0.0001$, 1406 ± 92, $p<0.01$, and 2589 ± 56 pg/mg protein, $p<0.0001$, respectively) compared with control. In addition, when we used both PGE_2 and CSE we observed an additive effect on the release of the production of IL-6, IL-8, and VEGF by 5-fold ($p<0.001$), 1-fold ($p<0.01$), and 5-fold ($p<0.0001$), respectively compared with CSE alone (Figure 4.10 A, B, and C). Collectively, these findings, along with the previous results, suggest the involvement of PGE_2 in the release of non-T2 inflammatory cytokines, confirming that PGE_2 largely mediates the effect of CSE on non-T2 inflammatory cytokines.

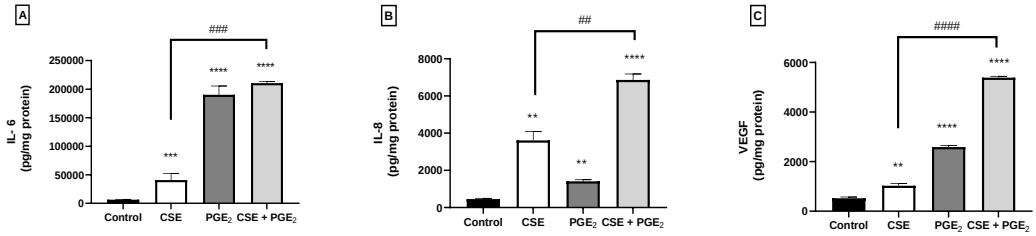


Figure 4.10. Effect of PGE_2 and CSE on IL-6, IL-8, and VEGF production in HASMCs.

Confluent and serum-starved HASMCs were treated with CSE (3.5%) and/or PGE_2 (10 µM) for 24h. The collected supernatants were used to measure the concentration of A) IL-6, B) IL-8, and C) VEGF by bio-plex ELISA. Data were normalised to total protein. Each data point represents mean ± SEM of three experiments from three cell lines. $^{**}p<0.01$, $^{***}p<0.001$ and $^{****}p<0.0001$ compared with control and $\#\#p<0.01$, $\#\#\#p<0.001$, and $\#\#\#\#p<0.0001$ compared with CSE.

4.4.7 Effect of the EP_2 and EP_4 receptor antagonists on CSE-induced the production of non-T2 inflammatory cytokines in HASMCs.

In the previous section 4.4.6, we have reported that exogenous PGE_2 stimulates non-T2 inflammatory cytokines in HASMCs. The effect of PGE_2 is mediated by four different E prostanoid (EP_1- EP_4) receptors. These EP receptors, including EP_2 and EP_4, are linked to the G protein-coupled receptor family [154]. EP_2 and EP_4 are associated with cyclic adenosine monophosphate (cAMP), and the activation of these receptors will lead to an increase of cAMP activity [161]. However, whether PGE_2 mediates the effect of CSE on non-T2 inflammatory cytokines via EP_2 and EP_4 receptors in HASMCs is unknown. Thus, we assessed the effect of EP_2 receptor antagonist PF 04418948 and EP_4 receptor antagonist L-161,982 on CSE-induced non-T2 inflammatory cytokines. CSE significantly induced the production of IL-6, IL-8, and VEGF compared with control (Figure 4.11 A, B, and C, respectively), consistent with previous findings. PF 04418948 significantly inhibited CSE-induced the production of IL-6 by 61% ($p<0.001$), IL-8 by 71% ($p<0.01$), and VEGF by 55% ($p<0.01$) compared with CSE alone. Similarly, L-161,982 was also observed to significantly inhibit CSE-induced the production of IL-6 by 75% ($p<0.01$), IL-8 by 42% ($p<0.001$), and VEGF by 67% ($p<0.05$) compared with CSE, as shown in Figure 4.11 A, B, and C, respectively.

Collectively, EP_2 and EP_4 antagonists inhibited CSE-induced the production of non-T2 inflammatory cytokines in HASMCs. These results strongly suggest that the effect of CSE is mediated by PGE_2 via its receptors (EP_2 and EP_4). In addition, our observation also suggests that downstream cAMP may play a role in the effect of CSE.

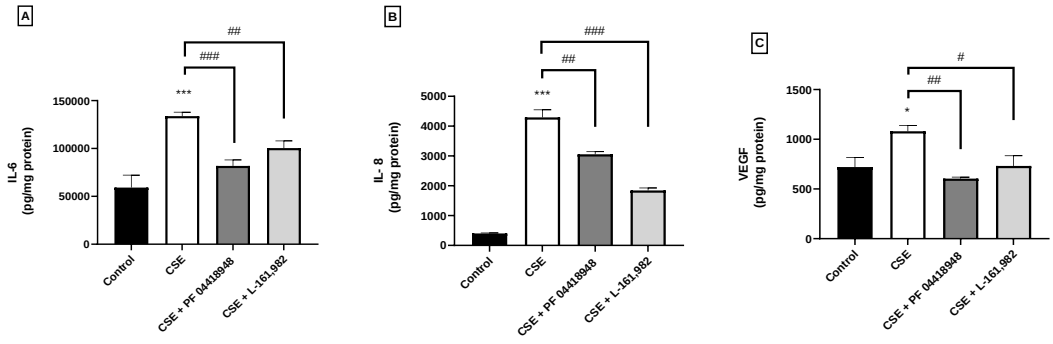


Figure 4.11. Effect of EP_2 and EP_4 on CSE-induced the production of IL-6, IL-8, and VEGF in HASMCs.

Confluent and serum-starved HASMCs were pre-treated with or without EP_2 receptor antagonist PF 04418948 (10 µM) or EP_4 receptor antagonist L-161,982 (1 µM) for 1h, followed by CSE (3.5%) for 24h. The collected supernatants were used to measure the concentration of A) IL-6, B) IL-8, and C) VEGF by using bio-plex ELISA. Data were normalised to total protein. Each data point represents mean ± SEM of three experiments from three cell lines. *p<0.05 and ***p<0.001 compared with untreated HASMCs and #p<0.05, ##p<0.01 and ###p<0.001 compared with CSE.

4.4.8 Effect of forskolin on the production of non-T2 inflammatory cytokines in HASMCs.

We demonstrated in 4.4.7 that CSE increased through EP_2 and EP_4 possibly via local induction of PGE_2; therefore, we assessed whether cAMP is involved in mediating the effect of CSE. Thus, we examined the effect of forskolin (a potent and rapid activator of adenylyl cyclase (AC)) on the production of IL-6, IL-8, and VEGF in HASMCs. As shown previously in sections 4.4.1, 4.4.2, and 4.4.3, CSE significantly induced the production of IL-6, IL-8, and VEGF compared with control. Similarly, forskolin induced the production of IL-6, IL-8, and VEGF (7869 ± 894, $p<0.01$, 1763 ± 183, $p<0.001$, and 7992 ± 583 pg/mg protein, $p<0.001$, respectively) compared with control. Interestingly, forskolin and CSE caused an additive effect on the release of the production of IL-6 by 33-fold ($p<0.001$, Figure 4.12 A) and VEGF by 3-fold ($p<0.001$, Figure 4.12 C) but not IL-8 compared with CSE alone (Figure 4.12 B).

Collectively, our observations suggest that the effect of CSE on non-T2 inflammatory cytokines is mediated through COX-2/mPGES-1/PGE_2/EP_2/EP_4/cAMP pathway via oxidative stress.

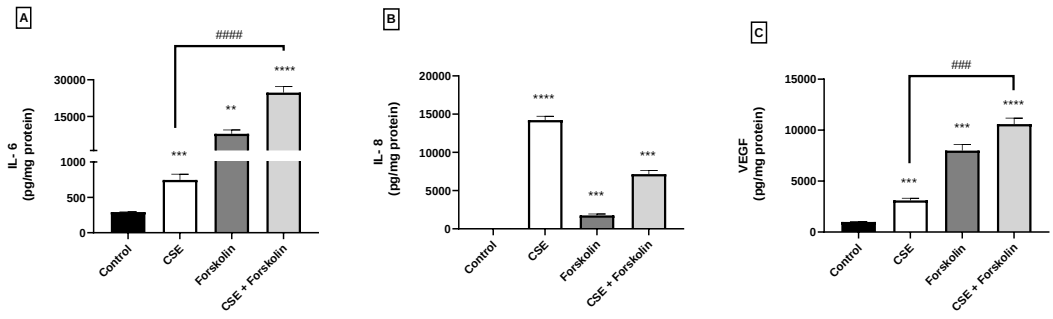


Figure 4.12. Effect of forskolin and CSE on IL-6, IL-8, and VEGF production in HASMCs.

Confluent and serum-starved HASMCs were treated with CSE (3.5%) and/or forskolin (10 µM) for 24h. The collected supernatants were used to measure the concentration of A) IL-6, B) IL-8, and C) VEGF by bio-plex ELISA. Data were normalised to total protein. Each data point represents mean ± SEM of three experiments from three cell lines. **$p<0.01$, ***$p<0.001$ and ****$p<0.0001$ compared with control and ####$p<0.0001$ compared with CSE.

4.4.9 Effect of β_2-agonist salbutamol and CSE on the production of non-T2 inflammatory cytokines in HASMCs.

Salbutamol is one of the effective treatments that has been used with asthmatic patients [216]. It has been reported that salbutamol increased the production of IL-8 in HASMCs [164]. We previously demonstrated that the production of IL-6, IL-8, and VEGF are increased by exogenous PGE_2, which is coupled to AC and elevations in cAMP. Since salbutamol can affect cAMP, we investigated whether salbutamol would have the same effect as PGE_2 in promoting non-T2 inflammatory cytokines production in HASMCs. The results, as shown in Figure 4.14 A, B, and C, revealed that CSE significantly induced the production of IL-6, IL-8, and VEGF compared with control. Similarly, salbutamol induced the production of IL-6, IL-8, and VEGF (100650 ± 4835, $p<0.0001$, 976 ± 128, $p<0.05$, and 895 ± 53 pg/mg protein, $p<0.01$, respectively) compared with control (Figure 4.13 A, B, and C). Interestingly, when we used both salbutamol and CSE, we observed an additive effect on the release of the production of IL-6, IL-8, and VEGF by 4-fold, $p<0.001$, 2-fold, $p<0.01$, and 3-fold, $p<0.001$, respectively, compared with CSE alone (Figure 4.13 A, B, and C).

These observations demonstrated that salbutamol had a similar effect as PGE_2 and CSE on the production of non-T2 inflammatory cytokines in HASMCs, suggesting that salbutamol could contribute to non-T2 airway inflammation by promoting these cytokines. The results also provide further evidence to support the concept that the effect of CSE on the production of non-T2 inflammatory cytokines in HASMCs is mediated through cAMP pathway.

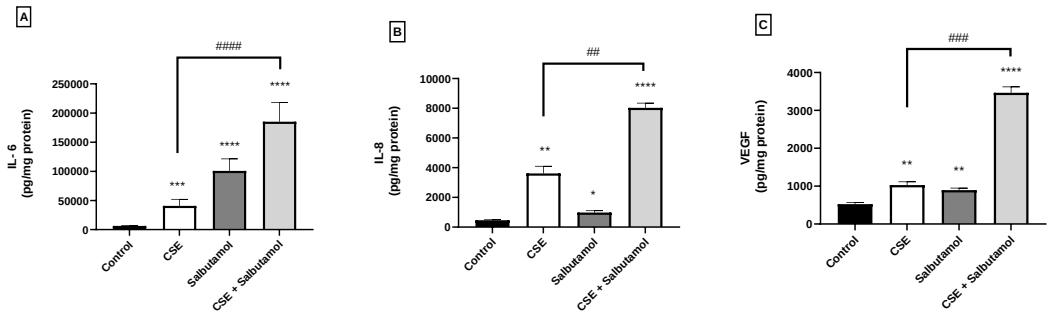


Figure 4.13. Effect of salbutamol and CSE on IL-6, IL-8, and VEGF production in HASMCs.

Confluent and serum-starved HASMCs were treated with CSE (3.5%) and/or salbutamol (10 µM) for 24h. The collected supernatants were used to measure the concentration of A) IL-6, B) IL-8, and C) VEGF by bio-plex ELISA. Data were normalised to total protein. Each data point represents mean ± SEM of three experiments from three cell lines. *p<0.05, **p<0.01, and ****p<0.0001 compared with control and ##p<0.01, ###p<0.001 and ####p<0.0001 compared with CSE.

4.5 Discussion

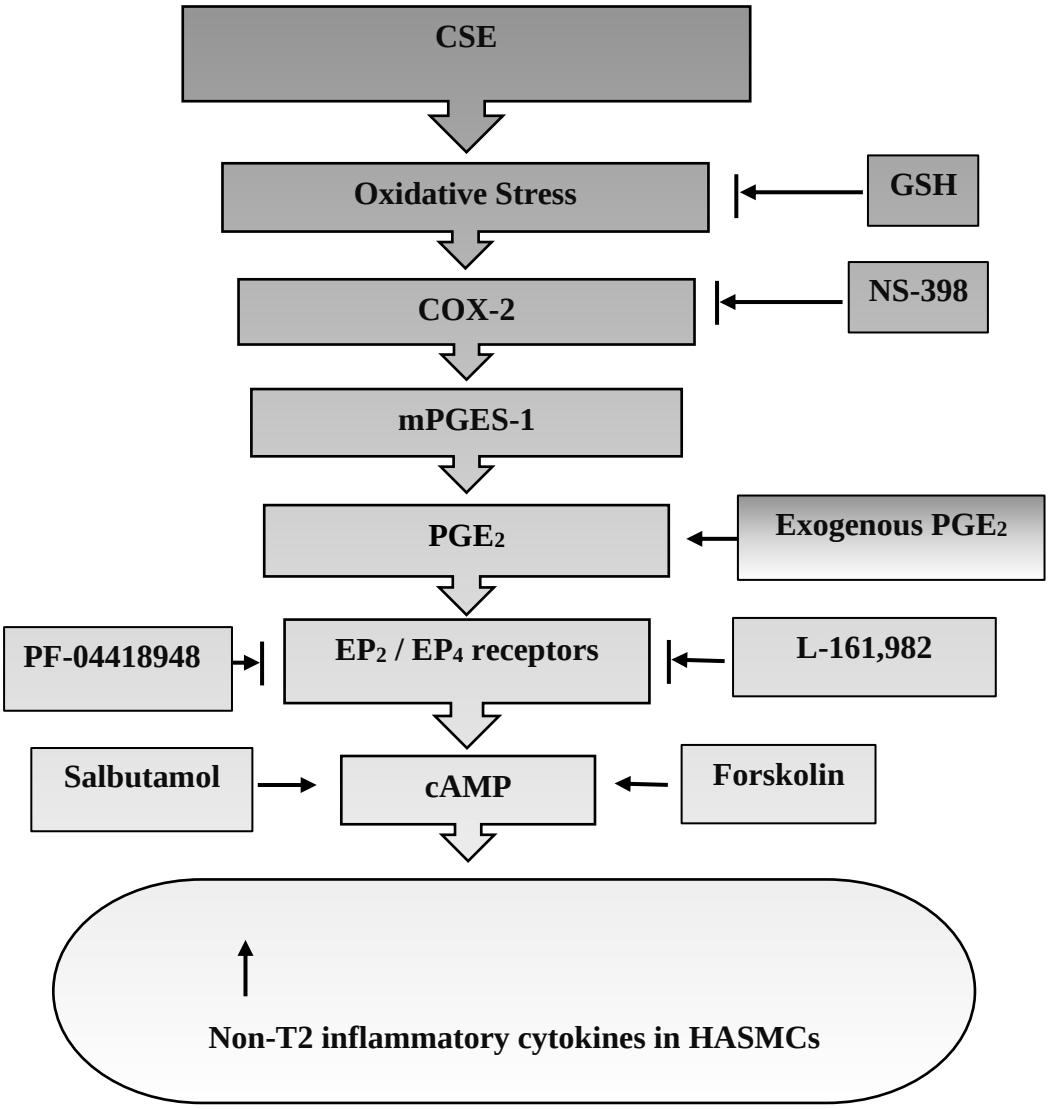
We showed in chapter 3 that CSE can modify the inflammatory responses in human airway smooth muscle cells, favouring a shift from T2 airway inflammation to non-T2 airway inflammation by promoting non-T2 inflammatory response and suppressing T2 inflammatory response. The main aim of this chapter was to determine the mechanism behind CSE effect on the production of non-T2 inflammatory cytokines in HASMCs. This study provides evidence for the first time that the effect of CSE on the production of non-T2 inflammatory cytokines is largely mediated by COX-2/mPGES-1/PGE_2/EP_2 and EP_4/cAMP pathway via oxidative stress, which eventually contributes to non-T2 airway inflammation in asthma. This is a novel observation that has not been previously reported. A summary of the result is described in Table 4.1 and Diagram 4.1.

Table 4.1. A summary of the results of chapter 4.

	Effect on non-T2 inflammatory cytokines			Effect on CSE-induced non-T2 inflammatory cytokines		
Drugs \ Cytokines	**IL-6**	**IL-8**	**VEGF**	**IL-6**	**IL-8**	**VEGF**
Nicotine	↔	↔	↔	X	X	X
MG264	X	X	X	↔	↔	↔
GSH	X	X	X	↓	↓	↔
NS-398	X	X	X	↓	↓	↓
Exogenous PGE_2	↑	↑	↑	↑	↑	↑
PF 04418948	X	X	X	↓	↓	↓
L-161,982	X	X	X	↓	↓	↓
Forskolin	↑	↑	↑	↑	↔	↑
Salbutamol	↑	↑	↑	↑	↑	↑

A summary of the effects of the drugs that have been used in this chapter. ↑ , increased; ↓ , Inhibited; ↔, Unchanged and X; not applied.

Diagram 4.1. A summary of the results of chapter 4.



Schematic diagram illustrating the proposed signalling pathway involved in CSE-induced the production of IL-6, IL-8, and VEGF in HASMCs. ↑, increased and ⊣, inhibited or blocked.

4.5.1 Role of nicotine and oxidative stress in CSE effect on non-T2 inflammatory cytokines

Nicotine is an important element in cigarette smoke extract, which binds to nicotine acetylcholine receptors (nAChRs) [217]. Studies showed that α7nAChR is the most highly nAChR expressed in HASMCs, which is further increased in smokers and asthmatic patients [215]. It has been reported that α7nAChR is increased by pro-inflammatory cytokines like TNFα compared to other nAChR like α4nAChR, suggesting that α7nAChR could be more involved in smokers and asthmatic patients [215].

Our results provide evidence that nicotine did not have any effect on the production of non-T2 inflammatory cytokines in HASMCs by using nicotine and the α7nAChR antagonist MG624, suggesting that the effect of CSE on non-T2 airway inflammation is not mediated by nicotine. Our data is supported by a recent study in which nicotine had no effect on the production of IL-6 and IL-8 in epithelial cells [218]. Our findings are consistent with these observations suggesting that non-T2 inflammatory cytokines released upon exposure to cigarette smoke is not mediated by nicotine. In contrast, 2.5 and 3 mM of nicotine have been reported to induce neutrophils (from citrated venous blood of healthy non-smoker subjects) to produce the production of IL-8 in a time and dose-dependent manner [219]. Another study showed that 1 mM of nicotine induced the production of IL-6 in human tracheal smooth muscles (HTSMCs) [141].

The differences in experimental models and the use of different concentrations of nicotine and passages of HTSMCs (4 to 7) could be the main reasons for the disparity between our findings and those of the reported studies [141, 219]. In the present study, we conducted all of our experiments by using HASMCs with passage 6 and applying 500 µM of nicotine, while Lin and colleagues used different cell passages.

Although the underlying mechanisms involved in the effect of CSE-induced non-T2 airway inflammation are not fully investigated, it is commonly believed that oxidative stress plays a role in lung damage seen in smokers. For example, CSE increased the intracellular levels of reactive oxygen species (ROS) and resulted in an increased production of IL-6 and IL-8 levels in bronchial epithelial cells [127]. Our findings suggest that oxidative stress is crucial in mediating CSE-induced non-T2 airway inflammation. In line with our findings, *Oltmanns et al.* [128] showed that 30 minutes pre-treatment with GSH revealed inhibition of CSE-induced IL-8 production in HASMCs, which suggest that oxidative stress could play a role in the CSE induced production of IL-8 [128].

We showed that IL-6 and IL-8 but not VEGF are inhibited by GSH in HASMCs. We expected to see an inhibition by GSH on CSE-induced VEGF. Cell responses could be the most likely reason, as well as sample size because we have seen a trend in reduction in VEGF production which was not significant. An increase in sample size would provide greater statistical power to detect a reductio. According to our other results, VEGF is like other non-T2 inflammatory cytokines (IL-6 and IL-8), COX-2

mediates its production. If COX-2 is mediated by oxidative stress, then GHS should have an inhibitory effect on -induced VEGF production.

Collectively, the production of non-T2 inflammatory cytokines induced by CSE was inhibited by the oxidative stress inhibitor (GSH) in HASMCs, suggesting that the effect of CSE on non-T2 airway inflammation is mediated by oxidative stress.

4.5.2 PGE_2 and CSE effect on non-T2 inflammatory cytokines

We report in this study for the first time that in HASMCs CSE-induced production of IL-6, IL-8, and VEGF was mediated by COX-2 pathway, associated with PGE_2 synthesis. We also provide evidence that CSE upregulated the protein and mRNA expression of COX-2 and mPGES-1 in HASMCs, suggesting that CSE upregulates COX-2 and mPGES-1 expression via transcriptional regulation in HASMCs.

It is widely accepted that many cells, including HASMCs, constitutively express COX-1 while COX-2 is an inducible gene [141], and its expression and activity is increased in response to inflammatory stimuli like CSE [220]. In line with our study, *Yang et al*. report that CSE increased the protein expression of COX-2 and PGE_2 production in HASMCs. The authors also showed that CSE-induced PGE_2 was inhibited by the COX-2 inhibitor NS-398 [139]. Our findings and these observations suggest that CSE is a potent stimulant that can induce PGE_2 production through COX-2 pathway, which may contribute to airway inflammation in asthmatic smokers. These observations were also supported by a study that has been conducted by our group, which demonstrated that CSE induced the protein expression of COX-2, mPGES-1 and

PGE_2 production in pulmonary artery epithelium cells (PAECs) [143]. Increased COX-2 expression has been reported to be associated with increased release of cytokines like IL-6 in HTSMCs [141]. Our findings are the first to report the role played by COX-2 pathway as the underlying mechanism involved in the induction of IL-6, IL-8, and VEGF cytokines from HASMCs post-exposure to CSE through increased production of PGE_2.

Studies have previously shown that increased VEGF and IL-8 cytokines levels due to increased PGE_2 production in fibroblast cells is through COX-2 pathway [221]. In agreement with our findings, exogenous PGE_2 1.0 μM was observed to increase the production of IL-8 [155] and VEGF [156], while a higher concentration (10 μM) of PGE_2 increased IL-6 [141] in HASMCs. Our findings suggest that the effect of CSE-induced non-T2 inflammatory cytokines is largely mediated through COX-2/mPGES-1/PGE_2 in HASMCs.

It was interesting to investigate the involvement of oxidative stress in the expression of COX-2 and PGE_2 release. Our findings report for the first time that pre-treatment of GSH attenuated COX-2 expression and consequently PGE_2 production after CSE treatment in HASMCs, suggesting that oxidative stress plays a crucial role in mediating COX-2 expression and PGE_2 production, which in turn mediated the effect of CSE on the production of non-T2 inflammatory cytokines in HASMCs. Our results are supported by a study showing that pre-treatment of HTSMCs with nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (one of the main sources of ROS) inhibitor (Diphenyleneiodonium) reduced COX-2 expression [141]. Our findings

suggest that COX-2/mPGES-1/PGE_2 mediates CSE-induced non-T2 airway inflammation via oxidative stress.

We then investigated whether the effect of PGE_2 is through its receptors (EP_2 and EP_4). We found that pre-treatment with the EP_2 and EP_4 receptor antagonists inhibited CSE-induced the production of IL-6, IL-8, and VEGF in HASMCs, suggesting that the effect of CSE-induced these cytokines is mediated by PGE_2 through its receptors EP_2 and EP_4. Our findings are supported by a recent study showing that the EP_2 and EP_4 antagonists (AH6809 and AH23848, respectively) inhibited PGE_2-induced IL-6 and IL-8 production in nasal polyp-derived fibroblasts (NPDFs) [154]. Our data and these observations suggest that the effect of CSE-induced non-T2 airway inflammation is mediated through COX-2/mPGES-1/PGE_2 via EP_2 and EP_4 receptors through oxidative stress.

4.5.3 Role of cAMP in CSE effect on non-T2 inflammatory cytokines

It is known that EP_2 and EP_4 receptors are linked to cAMP, and the activation of these receptors will cause an increase in cAMP activity [161]. It has been reported that PGE_2 stimulation resulted in increased the mRNA and protein expression of IL-6 through cAMP signalling pathway. It further showed that forskolin, which increases cAMP activity, induced IL-6 production in human chondrocytes [222]. In the present study, we showed that both forskolin and CSE caused an additive effect on the release of IL-6 and VEGF production but not IL-8. These findings suggest that although the increase in cAMP activity significantly stimulated IL-6, VEGF, and IL-8 on its own, it

had no further effect on the accumulation of IL-8 induced by CSE which also suggest that cAMP is a potent inducer of IL-6 and VEGF, but relatively weak stimulant of IL-8 release in HASMCs. In line with our results, Pang and Knox showed that forskolin induced the production of IL-8 in HASMCs and had no further effect on TNFα-induced IL-8 production. Another study also showed that forskolin induced the production of IL-6 [165] and VEGF in HASMCs [166]. Our findings suggest that increased intracellular cAMP plays a role in mediating CSE effect on non-T2 inflammatory cytokines.

As we previously demonstrated that PGE_2, which is coupled to AC, stimulated the production of non-T2 inflammatory cytokines in HASMCs, we postulated that the β_2 agonist salbutamol, which is coupled to AC, may have a similar effect on the production of non-T2 inflammatory cytokines in HASMCs. We found that salbutamol induced IL-6, IL-8, and VEGF. In addition, salbutamol and CSE caused an additive effect in the release of the production of non-T2 inflammatory cytokines in HASMCs. This is the first study to report an increase in non-T2 inflammatory cytokines in response to salbutamol and CSE. However, a similar effect of salbutamol alone on IL-8 (15) and IL-6 (31) in HASMCs has been previously reported. Several studies highlighted the association between the use of β_2 agonist and the increase in mortality rate in asthmatic patients [223]. This could indicate that β_2 agonist not only act as a bronchodilator but could also play a pro-inflammatory role. When given as a monotherapy, β_2 agonists can cause an increase in bronchial responsiveness after therapy is discontinued [224, 225]. Recently, β_2 agonist is seldom used as monotherapy in asthma [226], and the recommendations suggest using a combination of β_2 agonist

and corticosteroid therapy [227-230]. Our findings and other observations suggest that salbutamol could promote airway inflammation and remodelling through the release of non-T2 inflammatory cytokines and provide further evidence that cAMP plays a crucial role in CSE effect on IL-6, IL-8, and VEGF in HASMC.

In conclusion, our findings demonstrate novel results that CSE promote non-T2 inflammatory cytokines, largely through COX-2/mPGES-1/PGE_2/EP_2/EP_4/cAMP pathway via oxidative stress, thereby contributing to non-T2 airway inflammation in asthma.

Chapter 5. Impact of CSE on the inhibitory effect of fluticasone on TNFα-induced IL-8 production in HASMCs

5.1 Introduction:

Non-T2 asthma is associated with an increase in the production of the neutrophilic chemoattractant IL-8, which recruits neutrophils in the airway [56, 91]. It is widely believed that CS positively correlates with neutrophilic airway inflammation in asthmatic smokers.

We demonstrated in the previous chapters (3 and 4) that CSE suppressed T2 inflammatory cytokines and promote non-T2 inflammatory cytokines, largely through COX-2/mPGES-1/PGE_2/EP_2/EP_4/cAMP pathway via oxidative stress, thereby contributing to non-T2 airway inflammation in asthma.

Anti-inflammatory therapy, such as Inhaled corticosteroids (ICS), reduces asthma exacerbation, emergency visits or hospitalisations and airway hyper-responsiveness (AHR) incidents [38]. ICS has been used in asthmatic patients to maintain lung function and control asthma exacerbation. Several clinical studies have shown that asthmatic patients who smoke are less sensitive to corticosteroids and have higher neutrophils in their airways [176, 210, 231, 232]. It has been reported that pre-treatment with CSE for 6 hours reduces the inhibitory effect of corticosteroid dexamethasone on TNFα-induced the production of IL-8 in transformed human bronchial epithelial cells (BEAS-2B) [179]. However, whether CSE can induce corticosteroid insensitivity *in vitro* in HASMCs remains unclear. This study explores whether CSE can influence the anti-inflammatory effect of corticosteroids on TNFα-induced IL-8 production in HASMCs.

5.2 Hypothesis and Aims

In this chapter we hypothesised that CSE influence the anti-inflammatory effect of corticosteroids on TNFα-induced IL-8 production in HASMCs.

Our aim was to explore:

1. The influence of CSE on the anti-inflammatory effect of corticosteroids on TNFα-induced IL-8 production in HASMCs.

5.3 Material and Methods

The smoke of two cigarettes was bubbled into 20 ml of cell culture medium to make the CSE. HASMCs were pre-treated with and without CSE (3.5%) for 24h followed by treatment with fluticasone (10^{-11}-10^{-6} M) for 1h prior to incubation with CSE, TNFα (1 ng/ml), or CSE + TNFα for 24h. Enzyme-linked immunosorbent assays (ELISAs) were used to assess the production of IL-8 in the collected medium. GraphPad Prism was used to calculate the half-maximal inhibitory concentration (IC_{50}). The drug concentrations were converted to $\log_{10}$ and plotted on the X-axis, and the normalised response of IL-8 production (as 100%) was plotted on the Y-axis. Nonlinear regression analysis was used to draw the curve and calculate the IC_{50} value.

5.4 Results

5.4.1 Effect of CSE and TNFα on IL-8 production in HASMCs.

As shown previously in Chapter 3, HASMCs produced IL-8 basally (1608.073±534.163 pg/mg protein), CSE significantly increased the production of IL-8 in HASMCs (51189.186±12460.877 pg/mg protein (p<0.05)) compared with control, and TNFα stimulated the production of IL-8 in HASMCs (164158.86±23855.332 pg/mg protein (p<0.01)) compared with control, as shown in Figure 5.1. Furthermore, CSE and TNFα caused an additive effect on the release of the production of IL-8 by 3.6-fold (p<0.0001, Figure 5.1) compared with the effect of TNFα alone.

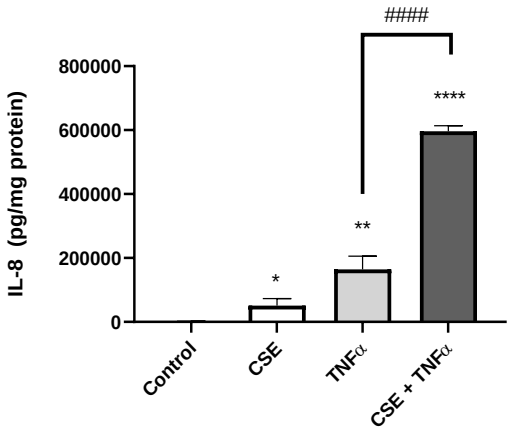


Figure 5.1. Effect of CSE and TNFα on IL-8 production in HASMCs.

Confluent and serum-starved HASMCs were pre-treated with or without CSE (3.5%) for 24 hr prior to treatment with TNFα (1 ng/ml) or CSE (3.5%) for 24 hr. Then, medium was collected to measure IL-8 concentration by ELISA. Data were normalised to total protein, and each data point represents mean ± SEM of three experiments from three different donors carried out in triplicate. *p<0.05, **p<0.01, and ****p<0.0001 compared control and ####p<0.0001 compared with TNFα alone.

5.4.2 Effect of fluticasone on CSE- and TNFα-induced IL-8 production in HASMCs

The results showed that, as we reported previously in section 5.4.1, HASMCs produced IL-8 basally. CSE and TNFα significantly increased the production of IL-8 in HASMCs (51189.186±12460.877 pg/mg protein ($p<0.05$) (Figure 5.2) and 164158.86±23855.332 pg/mg protein ($p<0.01$) (Figure 5.3), respectively) compared with control. Fluticasone inhibited CSE-induced the production of IL-8 in a concentration-dependent manner with a significant inhibition start at a concentration of 10^{-10} M (by 87.9% ($p<0.05$)) compared with CSE alone (Figure 5.2). Moreover, Fluticasone inhibited TNFα-induced the production of IL-8 in a concentration-dependent manner with a significant inhibition start at a concentration of 10^{-8} M (by 50.3% ($p<0.05$)) compared with TNFα alone (Figure 5.3). Our findings suggest that CSE- and TNFα-induced IL-8 production can be inhibited by fluticasone in HASMCs.

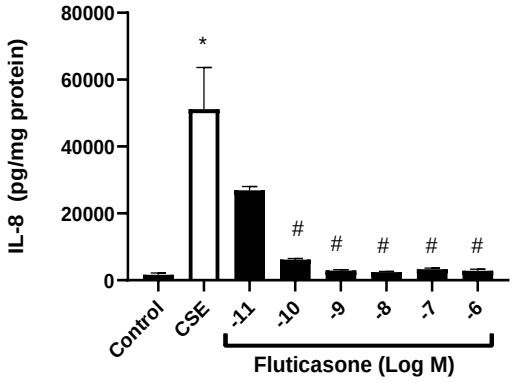


Figure 5.2. Effect of fluticasone on CSE- induced IL-8 production in HASMCs.

Confluent and serum-starved HASMCs were pre-treated with CSE (3.5%) for 24h prior to treatment with fluticasone (10^{-11}-10^{-6} M) for 1h followed by incubation with CSE for 24h. The collected supernatants were used to measure IL-8 concentration by ELISA. Data were normalised to total protein. Each data point represents mean ± SEM of three experiments from three different donors carried out in triplicate. *p<0.05 compared with control and #p<0.05 with CSE.

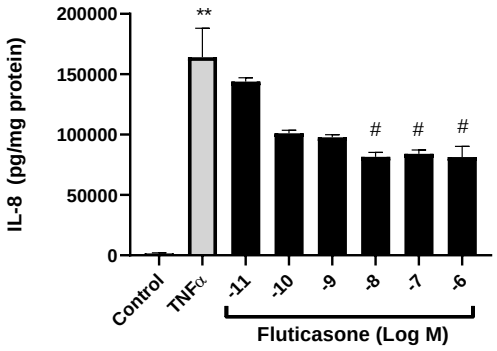


Figure 5.3. Effect of fluticasone on TNFα-induced IL-8 production in HASMCs.

Confluent and serum-starved HASMCs were pre-treated with fluticasone (10^{-11}-10^{-6} M) for 1h prior to incubation with TNFα (1 ng/ml) for 24h. The collected supernatants were used to measure IL-8 concentration by ELISA. Data were normalised to total protein. Each data point represents mean ± SEM of three experiments from three different donors carried out in triplicate. **p<0.01 compared with control and #p<0.05 compared with TNFα.

5.4.3 Effect of fluticasone on CSE and TNFα (CSE+TNFα)-induced IL-8 production in HASMCs.

The results showed that, as we reported previously in section 5.4.1, HASMCs produced IL-8 basally, and CSE with TNFα together induced IL-8 in HASMCs (613569.16± 9138.889 pg/mg protein (p<0.0001)) compared with control. Fluticasone inhibited (CSE+TNFα)-induced the production of IL-8 in a concentration-dependent manner with a significant inhibition by 27.5% (p<0.01) observed at a concentration of 10^{-8} M compared with CSE and TNFα in HASMCs (Figure 5.4). Our findings suggest that CSE and TNFα-induced IL-8 production can be inhibited by fluticasone in HASMCs.

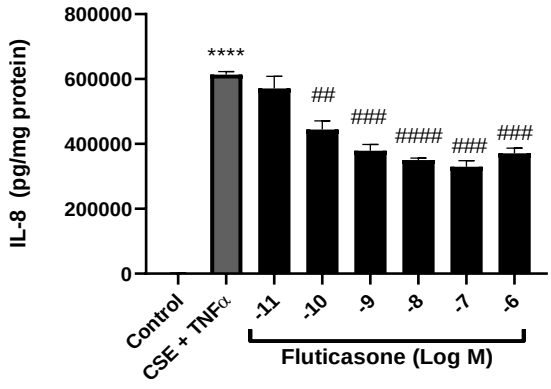


Figure 5.4. Effect of fluticasone on (CSE+TNFα)-induced IL-8 production in HASMCs.

Confluent and serum-starved HASMCs were pre-treated with CSE (3.5%) for 24h followed by treatment with fluticasone (10^{-11}-10^{-6} M) for 1h prior to incubation with CSE (3.5%) + TNFα (1 ng/ml) for 24h. The collected supernatants were used to measure IL-8 concentration by ELISA. Data were normalised to total protein. Each data point represents mean ± SEM of three experiments from three different donors carried out in triplicate. ****p<0.0001 compared with control and ##p<0.01, ###p<0.001, and ####p<0.0001 compared with CSE+TNFα.

5.4.4 Impact of CSE on the inhibitory effect of fluticasone on TNFα-induced IL-8 production in HASMCs

We showed that CSE induced the production of IL-8 in HASMCs; thereby contributing to non-T2 airway inflammation, which could cause glucocorticoid insensitivity. However, whether CSE affects the inhibitory effect of glucocorticoid *in vitro* remains unclear. Thus, we assessed the impact of CSE on the inhibition by fluticasone on TNFα-induced IL-8 production in HASMCs. The inhibitory effect of fluticasone on (CSE+TNFα)-induced IL-8 (Figure 5.4) and on TNFα-induced IL-8 (Figure 5.3) were plotted together as two curves for comparison.

The results in Figure 5.5 showed that (CSE+TNFα)- and TNFα-induced IL-8 production were inhibited by fluticasone in a concentration-dependent manner with IC_{50}= 0.07653 nM and IC_{50}= 0.0347 nM, respectively. Two-way ANOVA analysis showed no significant difference in the inhibitory effect of fluticasone between the two groups.

Our findings suggest that (CSE+TNFα)- and TNFα-induced IL-8 production inhibited by fluticasone similarly in HASMCs; CSE had no effect on the inhibitory effect of fluticasone on TNFα-induced IL-8 production in HASMCs.

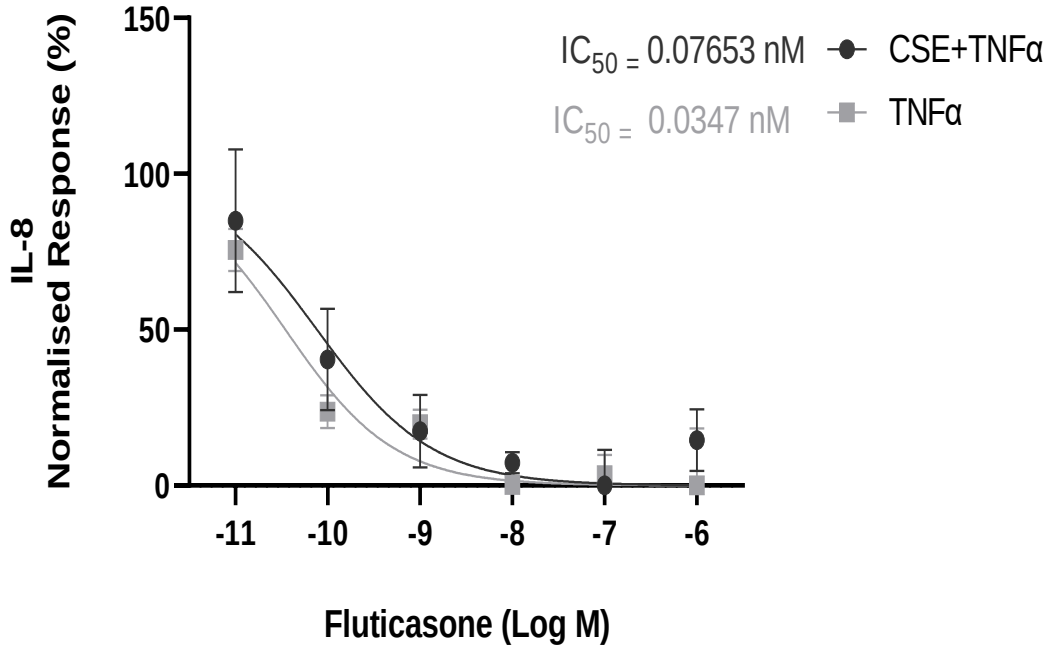


Figure 5.5. Impact of CSE on the inhibitory effect of fluticasone on TNFα-induced IL-8 production in HASMCs.

Confluent and serum-starved normal HASMCs were pre-treated with or without CSE (3.5%) for 24h, and then the cells were pre-treated with fluticasone (10^{-11}-10^{-6} M) for 1h prior to incubation with CSE (3.5%) and TNFα (1ng/ml) for 24h. The collected supernatants were used to measure IL-8 concentration by ELISA. Data were normalised to total protein and then expressed as a percentage against either CSE+TNFα or TNFα alone (100%). Each data point represents mean ± SEM of three experiments from three different donors carried out in triplicate

5.5 Discussion

In the previous chapters (3 and 4) we demonstrated that CSE can modulate the inflammatory responses in HASMCs, favouring a switch from T2 airway inflammation to non-T2 airway inflammation in asthmatic smokers by promoting non-T2 inflammatory response and suppressing T2 inflammatory response, largely through COX-2/mPGES-1/PGE_2/EP_2/EP_4/cAMP pathway via oxidative stress.

The main aim of this chapter was to explore whether CSE affects the inhibitory effect of fluticasone on TNFα-induced the neutrophilic chemoattractant IL-8 production in HASMCs.

Our findings showed that CSE and TNFα, either individually or in combination, increased the production of IL-8, which was inhibited by fluticasone in a concertation dependent-manner. In agreement with our findings several studies showed that CSE induced the production of IL-8 in HASMCs [233, 234]. TNFα is a potent pro inflammatory mediators, and an increase in its production has been associated with asthma [235]. Evidence also showed that in HASMCs, TNFα stimulated the production of IL-8 [3, 181, 236, 237]. It is widely known that TNFα-induced-IL-8 production in asthma can be inhibited by corticosteroids [3, 181, 236, 237]. Our findings along with these observations suggest that CSE and TNFα are potent stimulants for IL-8 production thereby contributing to non T2 airway inflammation in asthma.

Several clinical studies have been conducted to assess the effect of corticosteroids therapy in asthmatic patient. One study conducted on 114 subjects (55 never smoke, 30 current smokers, and 29 ex-smokers) by using fluticasone 500 µg/day for 1 year and they found that among the three groups there were no differences in the

lung functions in response to ICS treatment. [238]. Another study showed that budesonide 400 µg/day for 3 years had similar effect in 492 asthmatic smokers and 2,432 non-smokers [30]. In the present study we showed that CSE had no effect on the inhibitory effect of fluticasone on TNFα-induced the production of IL-8 in HASMCs suggesting that corticosteroid therapy is effective in reducing IL-8 production in asthmatic smokers. In contrast, an *in vitro* study showed that the effectiveness of the inhibitory effect of fluticasone on (CSE +TNFα)-induced IL-8 production was reduced compared with CSE alone [181]. The differences between our study and their study could be due to the comparison between groups. In their study, they aimed to compare the effects of a high level of TNFα in COPD smokers; therefore, they compared the effects of (CSE+TNFα)-induced IL-8 production with CSE alone. In our study, we aimed to assess the effect of CSE on the inhibitory effect of fluticasone on TNFα-induced IL-8 production in asthmatic smokers. That is why we compared the effect of (CSE+TNFα)-induced IL-8 production with TNFα alone.

In conclusion, our findings suggest that CSE did not influence the anti-inflammatory effects of fluticasone on TNFα-induced production of IL-8 in HASMCs. However, CSE contribute to non-T2 airway inflammation by promoting non-T2 inflammatory response.

Chapter 6. General discussion, limitations, future studies and conclusions

6.1 General discussion

The main aims of my thesis were to explore the effect of CSE on the production of T2 and non-T2 inflammatory cytokines and to investigate the underlying mechanism of CSE effect on the production of non-T2 inflammatory cytokines in HASMCs. In addition, we were also interested in evaluating the effect of CSE on the inhibitory effect of corticosteroids in HASMCs.

The key findings of this project were that CSE inhibited the production of T2 inflammatory cytokines IL-4, IL-13, eotaxin, IP-10, and RANTES, but had no effect on IL-5 while it induced the production and mRNA expression of non-T2 inflammatory cytokines IL-6, IL-8, and VEGF in HASMCs. The effect of CSE on the production of non-T2 inflammatory cytokines was largely mediated by COX-2/mPGES-1/PGE_2/EP_2/EP_4/cAMP pathway via oxidative stress. In addition, CSE-induced production of neutrophilic chemoattractant IL-8 was inhibited by corticosteroids fluticasone in a concentration-dependent manner. We observed that CSE had no effect on the inhibitory effect of fluticasone on TNFα-induced IL-8 production in HASMCs.

Our novel findings in this thesis suggest that CSE plays a role in modifying asthma endotypes; it may facilitate a shift from T2 airway inflammation to non-T2 airway inflammation by suppressing T2 inflammatory response and promoting non-T2 inflammatory response. This indicates that asthmatic smokers develop neutrophilic airway inflammation, which is insensitive to corticosteroid therapy compared with eosinophilic airway inflammation. Therefore, our study suggests that smoking cessation could be a very important therapeutic intervention to control the symptoms in asthmatic smokers. Clinical studies showed that asthmatic smokers who started

smoking cessation improved their asthma control and reduced neutrophil counts but with no change in eosinophil counts [239]. It has been observed that ex-smokers display an increased number of eosinophils in their blood count [201, 240] and there are several studies that have reported an approved biological therapy which targets the specific receptors in T2 asthma. For example, anti-IL-5 (mepolizumab) [241], anti-IL-4/IL-13 (dupilumab) [242] and anti-IgE (omalizumab) [243] showed a potent effect in reducing asthma exacerbation and reduced hospitalisation admissions in T2 asthma. Therefore, smoking cessation may help suppress neutrophilic airway inflammation and switch to eosinophilic airway inflammation, shown to be controlled by asthma treatment.

The data presented in this thesis highlighted the signalling pathways that may have an essential role in mediating CSE effect on the production of non-T2 inflammatory cytokines in HASMCs. Some studies link oxidative stress to airway inflammation (7), and our novel result shows that CSE induced the production of non-T2 inflammatory cytokines in HASMCs through COX-2/PGE_2 pathway via the oxidative stress. In addition, we are the first to emphasise the importance of COX-2 and PGE_2 pathway in mediating the CSE effect on non-T2 inflammatory cytokines. Previous studies assessed the effect of CSE on COX-2 expression and PGE_2 production in HASMCs. For example, *Yang et al*. reported that CSE increased the protein expression of COX-2 and PGE_2 production in HASMC [139]. This indicates that these signalling pathways play a crucial role in mediating CSE effect on non-T2 asthma and that the COX-2 inhibitor, EP_2, and EP_4 antagonists examined in this study may prevent non-T2 airway inflammation in asthmatic smokers. An inhibition in COX pathway and PGE_2 have been reported to play an important role in shifting inflammation from COX

pathway to 5-lipoxygenase (5-LO) pathway, another arachidonic acid enzyme, resulting in mediating aspirin-exacerbated respiratory diseases (AERD). Non-steroidal anti-inflammatory drugs (NSAIDs) like COX inhibitors are associated with increased bronchoconstriction because an inhibition in COX pathway reduces PGE_2 synthesis, leading to increased cysLT levels [148]. However, further studies need to be conducted to evaluate the dual COX-2/5-lipoxygenase inhibitors such as (BW 755C and SF&F 86002) with asthmatic smokers.

We reported an increase in non-T2 inflammatory cytokines in response to salbutamol and CSE. Several studies have shed light on the association between the usage of β_2 agonist and an increased risk of mortality in asthmatic patients [223]. Recently, β_2 agonist have been rarely used as monotherapy in asthma [226], and recommendations advocate combining them with corticosteroids [227-230]. This indicates that salbutamol could promote airway inflammation and remodelling through the release of non-T2 inflammatory cytokines and provide further evidence that cAMP plays a crucial role in CSE-induced neutrophilic airway inflammation.

Clinically, asthmatic patients with neutrophilic airway inflammation have shown a poor response to corticosteroid therapy [244]. In addition, it has been reported that CSE induced corticosteroid insensitivity in human lung epithelial cells [179]. Our study showed that corticosteroids fluticasone inhibited CSE- and TNFα-induced IL-8 production and CSE did not influence the anti-inflammatory effect of fluticasone. This indicates that CSE is a potent stimulant which promotes neutrophilic airway inflammation by increasing non-T2 inflammatory cytokines, including the neutrophilic chemoattractant IL-8, which recruits neutrophils in the airway, suggesting that CSE

may induce corticosteroid insensitivity by suppressing the production of T2 inflammatory cytokines and promoting the production of non-T2 inflammatory cytokines contributing to non-T2 asthma, which is insensitive to corticosteroids.

6.2 Conclusion

Airway inflammation in asthma is categorised into endotypes, namely, T2 and non-T2 asthma. The findings presented in this thesis provide for the first-time evidence that CSE modifies the inflammatory responses in HASMCs, favouring a shift from corticosteroid-sensitive T2 to corticosteroid-insensitive non-T2 airway inflammation by suppressing T2 inflammatory response and promoting non-T2 inflammatory response, thereby contributing to corticosteroid insensitivity. Our findings also suggest that CSE promotes non-T2 airway inflammation largely through the COX-2/mPGES-1/PGE_2/EP_2/EP_4/cAMP pathway via oxidative stress. Therefore, targeting this pathway (e.g. COX-2 and PGE_2) may provide potentially novel therapeutics for controlling non-T2 airway inflammation in asthmatic smokers.

6.3 Limitations and Future Studies

- We investigated the effect of CSE on T2 and non-T2 inflammatory cytokines in HASMCs from healthy donors. However, whether CS has a similar effect in diseased HASMCs was not explored. Therefore, it is necessary to use HASMCs from asthmatic smokers and non-smokers to validate our findings further.

- Our study only used HASMCs from three different donors, which is why we observed variation in the cellular response to GSH, particularly in VEGF production. Therefore, increasing sample numbers would provide statistical power to minimise variations among cellular responses.
- We used only one cell type of airway structural cell, HASMCs. However, other cell types may or may not respond similarly to CSE. Therefore, further studies are needed to assess the effect of CSE in human bronchial epithelial cells (HBECs) as it is the first layer of the airway structural wall. In addition, it would be interesting to examine the effect of CSE on epithelial-smooth muscle cell interaction in co-culture models.
- In our thesis, we assessed the effect of CSE on the production of inflammatory cytokines in HASMCs in *vitro.* However, the effect of CSE may be different from CS. Therefore, it is necessary to assess the effect of CS in animal model studies to validate our findings.
- We treated HASMCs by CSE for a maximum of 48 hours *in vitro* which is a very short exposure compared with asthmatic smokers who smoke for a long time. Whether the effect in our study can be observed in asthmatic smokers is unknown. Therefore, further studies are needed by using patient samples such as BALF from asthmatic smokers and non-smokers for analysing inflammatory cell profiles and T2 and non-T2 cytokine levels, which will further validate our findings.

Chapter 7. Appendix

7.1 Materials for cells culture and MTT assay

Dulbecco's Modified Eagle's Medium (DMEM).	Sigma Aldrich
Amphotericin-B 250 mg/ml in deionised water, sterile-filtered.	Sigma Aldrich
L-glutamine solution, 200 mM solution, sterile-filtered.	Sigma Aldrich
Penicillin/streptomycin 5000 units of penicillin and 5 mg of streptomycin/ml, sterile filtered.	Sigma Aldrich
Foetal bovine serum (FBS)	Thermo Fisher Scientific
DMEM+ Supplemented with 10% of FBS, 0.02% antibiotics (penicillin/streptomycin), 0.02%L-glutamine, and 0.01% amphotericin-B.	
DMEM- Supplemented with 0.5% FBS, 0.02% antibiotics (penicillin/streptomycin), 0.02% L-glutamine, and 0.01% amphotericin B.	

7.2 CSE Materials

3R4F research-grade cigarettes, one cigarette contains 9.4 mg of tar, 0.7 mg of nicotine, 12 mg of carbon monoxide cigarette pump.	Kentucky Tobacco Research and Development

	Centre, University of Kentucky, USA
DA7C Pump.	Charles Austen Pumps.

7.3 Table of Drugs

Fluticasone propionate. Mass:5 mg. Molecular weight: 500.57. To make original stock of 0.5 M, dissolve in 19.98 µl of dimethyl sulfoxide (DMSO).	Sigma Aldrich
Recombinant human tumour necrosis factor (TNFα). Mass: 5 mg. Molecular weight: 17.5. Original stock solution: 20 µg diluted in 1 mL of PBS (0.1% BSA) for a final concentration of (20,000 ng/mL).	R&D Systems
NS-398. Mass: 5 mg. Molecular weight: 314.4. To make original stock of 0.05 M, dissolve in 318 µl of DMSO.	Cayman Chemical
MG 624.	Sigma Aldrich

Mass: 5 mg. Molecular weight: 451.39. To make original stock of 0.05 M, dissolve in 251.5 µl of DMSO.	
L-Glutathione (GSH). Mass: 10 mg. Molecular weight: 307.32. To make original stock of 0.05 M, dissolve in 650.6 µl of water.	Sigma Aldrich
(-)-Nicotine. Mass: 1.010 g/ml. Molecular weight: 6.23. To make original stock of 100 mM, 80.3 µl of (-)-nicotine was dissolved in 5 ml of ethanol.	Sigma Aldrich
Forskolin. Mass: 5 mg. Molecular weight: 410.5. To make original stock of 0.05 M, dissolve in 243.6 µl of DMSO.	R&D Systems
Salbutamol. Mass: 25 mg. Molecular weight: 239.31.	Sigma-Aldrich

To make original stock of 0.05 M, dissolve in 2,088 µl of methanol (MeOH).	
Prostaglandin E_2 (PGE_2). Mass: 10 mg. Molecular weight: 352.47. To make original stock of 0.05 M, dissolve in 647.4 µl of DMSO	Cayman Chemical
PF 04418948. Mass: 10 mg. Molecular weight: 409.41. To make original stock of 0.05 M, dissolve in 488.4 µl of DMSO.	Tocris bioscience
L-161,982. Mass: 10 mg. Molecular weight: 654.72. To make original stock of 0.05 M, dissolve in 304 µl of DMSO.	Tocris bioscience

7.4 Buffers and reagents

Radioimmunoprecipitation assay (RIPA) buffer.50 mM Tris-HCL, PH 8.0, 0.5% Sodium Deoxycholate, 150 mM Sodium Chloride, 0.1% Sodium Dodecyl Sulphate (SDS). After RIPA buffer preparation, then the following contents were added to do protein collection: 2 Mm of Phenylmethylsulphonyl Fluoride (PMSF), 1 mM of Protease Inhibitor Cocktail (PIC), and 1 mM of Sodium Orthovanadate.	
Tween 20	Sigma Aldrich
Tris-buffered saline (TBS). 20 mM Trizma hydrochloride (Tris-HCI) and 150 mM NaCl.	Sigma Aldrich
Bovine serum albumin (BSA)	Thermo Fisher Scientific.
Block buffer for CXCL-8 DuoSet ELISA: 1% BSA in 100 ml of PBS Filtered by 0.02-µm pore syringe filter (PH 7.2–7.4).	
Wash buffer for CXCL-8 DuoSet ELISA: 0.05% (0.5 ml) Tween and 5 tablets of PBS in each 1000 ml of distilled water (PH 7.2-7.4).	
Reagent diluent for CXCL-8 DuoSet ELISA:	

0.1% BSA, 24.2 g of TBS, 87.6 g of NaCL, and 0.05% Tween 20 (PH 7.2 – 7.4). Filtered by 0.2 μm pore size syringe filter.	
Substrate solution: 1:1 mixture of Colour Reagent A (H2O2) and B (Tetramethylbenzidine)	Thermo Fisher Scientific
Phosphate-buffered saline (PBS).	Thermo Fisher Scientific
10x TBS-T buffer: 1000 ml of dH2O, 87.6 g NaCL, 0.2 M tris base, 10 Tween 20 (pH 7.4-7.6). To make 1x TBS-T: 900 ml of dH2O + 100 ml of 10x TBS-T buffer.	
Blocking buffer for Western Blotting. 5% of non-fat dry milk in TBS-T (Tris-Buffered Saline with 0.1% Tween 20)	
20x Bolt MOPS SDS running buffer	Thermo Fisher Scientific
Bolt Transfer Buffer (20x)	Thermo Fisher Scientific
Bolt Antioxidant	Thermo Fisher Scientific

4x Bolt LDS Sample Buffer	Thermo Fisher Scientific
MagicMark XP Western Protein Standard	Thermo Fisher Scientific
Spectra Multicolor Broad Range Protein Ladder	Thermo Fisher Scientific
10x Bolt Sample Reducing Agent	Thermo Fisher Scientific
Running buffer: 50 ml of 20x MOPS SDS running buffer + 950 ml of dH2O.	
Transfer buffer: 50 ml of 20x Transfer buffer with 100 ml methanol, 1 ml of Antioxidant + 849 ml of dH2O.	
Clarity and clarity Max ECL Western Blotting Substrates	Bio-Rad Laboratories
Bovine serum albumin	Thermo Fisher Scientific
Deoxynucleotide triphosphates	Promega, USA
Dimethyl sulfoxide (DMSO).	Sigma Aldrich
Ethanol	Sigma Aldrich
Methanol	Sigma Aldrich

β-mercaptoethanol	Sigma Aldrich
Non-fat dry milk	Santa Cruz Biotechnology, USA
Phosphate buffered saline tablets	Sigma Aldrich
Phenylmethanesulfonyl fluoride	Sigma Aldrich
Protease inhibitor cocktails	Sigma Aldrich
Sodium chloride	Sigma Aldrich
Sodium dodecyl sulfate	Sigma Aldrich
KAPA Taq DNA polymerase	Sigma Aldrich
Trypsin–Ethylenediamine Tetraacetic Acid (EDTA)	Sigma Aldrich
Thiazolyl Blue Tetrazolium Bromide	Sigma Aldrich
Ponceau S Staining Solution	Thermo Fisher Scientific
Trypan Blue Solution	Sigma Aldrich

7.5 Kits

Human CXCL-8 DuoSet ELISA Kit	R&D Systems
Human VEFG DuoSet ELISA Kit	R&D Systems
Human IL-6 DuoSet ELISA Kit	R&D Systems
Bio-Plex Pro Human Cytokine 27-plex Assay Kit	Bio-Rad Laboratories
LTB_4 ELISA Kit	Enzo Life Sciences

Cysteinyl leukotriene ELISA Kit	Enzo Life Sciences
Prostaglandin E_2 ELISA-Monoclonal Kit	Cayman Chemical
Thromboxane B2 ELISA Kit	Cayman Chemical
BCA Protein Assay Kit	Thermo Fisher Scientific
NucleoSpin RNA Extraction Kit	Macherey-Nagel
SuperScript™ IV Reverse Transcriptase kit	Thermo Fisher Scientific

7.6 Antibodies

Mouse anti-human COX-2 monoclonal antibody	Cayman Chemical
Recombinant anti-Thromboxane synthase antibody	Abcam
Anti-5Lipoxygenase antibody	Abcam
Recombinant Anti-Prostaglandin E Synthase/MPGES-1 Antibody	Abcam
Goat Anti-Mouse Immunoglobulins/HRP	Dako
Goat Anti-Rabbit Immunoglobulins/HRP	Dako

7.7 Materials

Nitrocellulose Blotting Membrane	Amersham Biosciences
Blot 4 to 12%, Bis-Tris, 1.0 mm Mini Protein Gel	Thermo Fisher Scientific
Mr. Frosty Freezing Container	Thermo Fisher Scientific
Nunc Biobanking and cell culture cryogenic tubes	Thermo Fisher Scientific

7.8 Effect of CSE or TNFα with fluticasone on cell viability

To evaluate the cytotoxicity of CSE (3.5%) or TNFα (1 ng/ml) with and without fluticasone (10^{-11}-10^{-6} M) on HASMCs, MTT assay was used. Confluent and serum-starved HASMCs were pre-treated with CSE for 24h followed by treatment with fluticasone (10^{-11}-10^{-6} M) for 1 hr prior to incubation with TNFα (1 ng/ml) or CSE for 24 hr (Figure 3.1). Results show no decrease on the cell viability when cells were treated with CSE (3.5%) or TNFα (1 ng/ml) with and without fluticasone (10^{-11}-10^{-6} M) when compared with control. Therefore, CSE (3.5%) or TNFα (1 ng/ml) with fluticasone are not toxic to the cells and were used in further experiments.

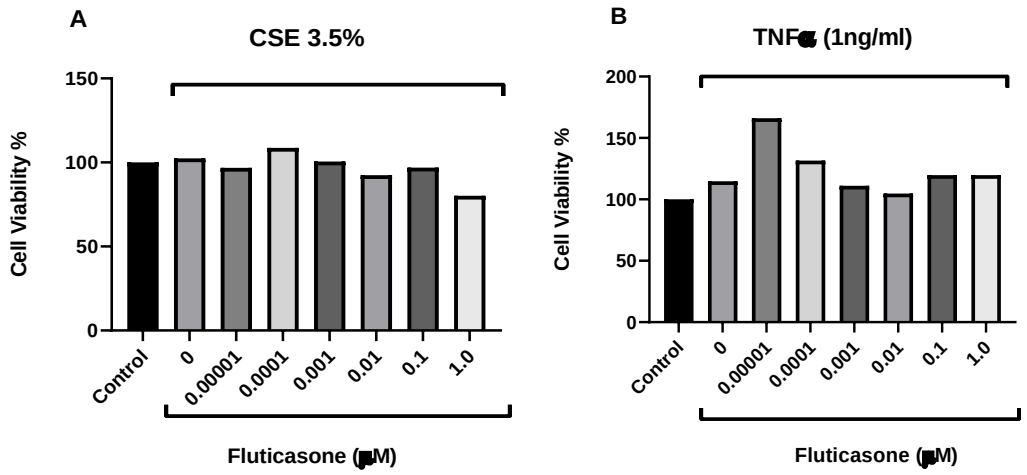


Figure 7.8.1. Effect of CSE or TNFα with fluticasone on cell viability.

Confluent and serum-starved HASMCs were pre-treated with fluticasone (10^{-11}-10^{-6} M) for 1 hr prior to incubation with (A) CSE (3.5%) or (B) TNFα (1 ng/ml) for 24 hours. MTT was used to assess the cell viability. Data is presented as average of duplicates wells versus control of one experiment.

7.9 Effect of CSE on the mRNA of housekeeping gene β2M in HASMCs

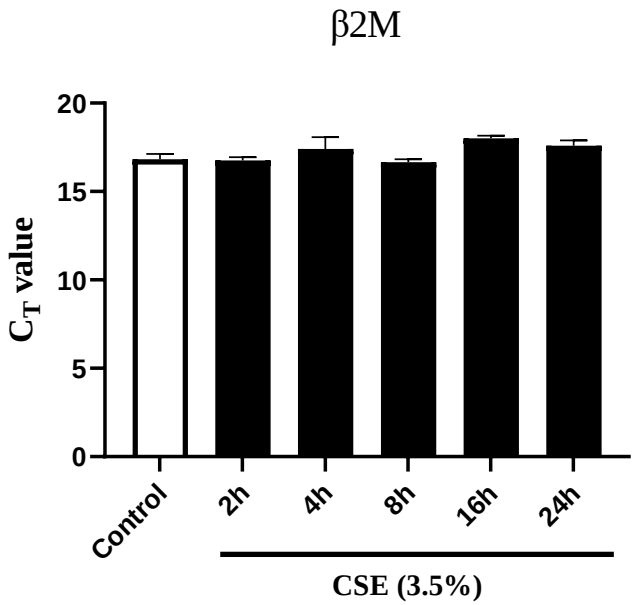


Figure 7.9.1. Effect of CSE on the mRNA of the housekeeping gene β2M in HASMCs.

Confluent HASMCs were treated with concentrations of CSE (3.5%) for 2, 4, 8, 16 and 24h before total RNA isolation. The samples were assessed by RT-qPCR. Data were expressed as fold change over the control. Each data point represents mean ± SEM from three independent experiments using three cell lines.

7.10 Effect of CSE with and without NS-398 on the production of LTB_4 and TXB_2 in HASMCs.

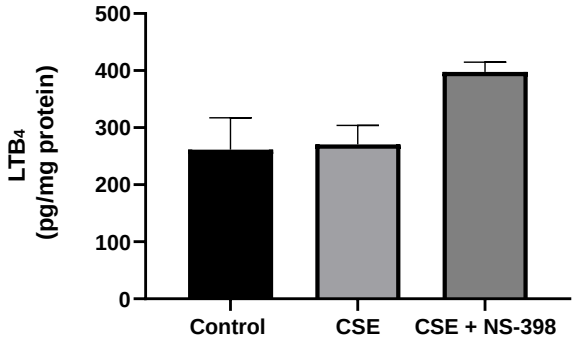


Figure 7.10.1. Effect of CSE with and without NS-398 on the production of LTB_4 in HASMCs.

Confluent and serum-starved HASMCs were pre-treated with NS-398 (10 μM) for 1h, followed by CSE (3.5%) for 24h. The collected supernatants were used to measure LTB_4 concentrations by ELISA. Data were normalised to total protein. Each data point represents mean ± SEM of three experiments from three different cell lines.

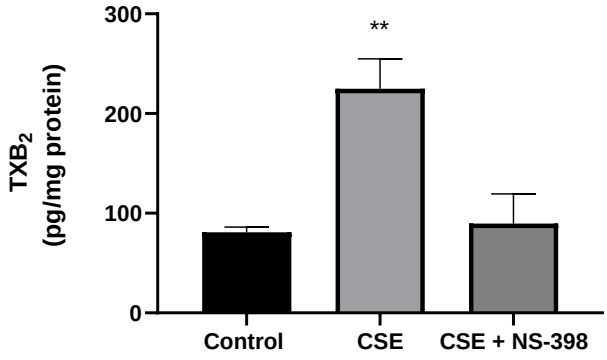


Figure 7.10.2. Effect of CSE with and without NS-398 on the production of TXB_2 in HASMCs.

Confluent and serum-starved HASMCs were pre-treated with NS-398 (10 μM) for 1h, followed by CSE (3.5%) for 24h. The collected supernatants were used to measure TXB_2 concentrations by ELISA. Data were normalised to total protein. Each data point represents mean ± SEM of three experiments from three different cell lines.

Chapter 8. References

8.1 References

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