Effect of cigarette smoke extract and IL-17A on T2 and non-T2 inflammatory responses in human airway smooth muscle cells

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Abbreviations

A

AA	Arachidonic acid
AC	Adenylyl cyclase
AHR	Airway hyperresponsiveness

B

BALs	Bronchoalveolar lavages
BTC	British Thoracic Society
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
CS	Cigarette smoke
CSE	Cigarette smoke extract
CT	Cycle threshold
cDNA	Complementary DNA

D

DALYs	Disability-adjusted life years lost
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

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

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSH	Glutathione
GR-beta	Glucocorticoid receptor-beta (GR-beta)

H

HASMCs	Human airway smooth muscle cells
HRP	Horseradish peroxidase

I

IC_{50}	Half-maximal inhibitory concentrationIL-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)

L

LABA	Long-acting β2-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

N

nAChR Nicotinic acetylcholine receptor

NSAID Nonsteroidal anti-inflammatory drug

NADPH Nicotinamide adenine dinucleotide phosphate

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 β2-adrenoreceptor agonists

SEM Standard error of the mean

T

TNFα Tumour necrosis factor alpha

TSLP Thymic stromal lymphopoietin

TBS-T Tris-buffered saline plus tween 20

V

VEGF Vascular endothelial growth factor

W

WHO World Health Organization

WST-1 Water-soluble tetrazolium-1

Abstract

Background: Asthma is a chronic inflammatory disease of the airways. Airway inflammation in asthma can be broadly classified as Type 2 (T2 or Th2-high), which responds well to glucocorticoids, or non-Type 2 (non-T2 or Th2-low), known to be insensitive to glucocorticoids, based on their cellular profile. Human airway smooth muscle cells (HASMCs) play a role in the inflammatory process in asthma by expressing a wide range of inflammatory cytokines and chemokines. Asthmatic smokers tend to have non-T2 airway inflammation and do not respond to glucocorticoids as effectively as non-smokers. Cigarette smoke (CS) and the Th17 cytokine IL-17A contribute to the non-T2 inflammatory response, but it is unknown how CS and IL-17A affect the T2 inflammatory response in HASMCs. Cyclooxygenase 2 (COX-2) and its downstream product prostaglandin E2 (PGE2), as well as oxidative stress, are involved in inflammatory responses. There is evidence that CSE induces COX-2 expression and PGE2 production in different cell types, including HASMCs. We hypothesise that CSE and IL-17A can affect T2 and non-T2 inflammatory responses through the COX-2/PGE2 pathway via oxidative stress in HASMCs and may contribute to glucocorticoid insensitivity in asthma.

Methods: CSE was prepared from the smoke of two research cigarettes bubbled into 20 ml of cell culture medium. Western blotting and real-time RT-PCR were used to assess the protein and mRNA expression of genes, respectively. ELISA and Bio-Plex assays were used to assess the production

of the T2 and non-T2 inflammatory cytokines. Statistical analysis was conducted using GraphPad Prism 9.

Results: CSE induced production of the non-T2 inflammatory cytokine IL-8 but inhibited production of T2 inflammatory cytokines, including Th2 cytokines IL-4 and IL-13 and eosinophil chemokines Eotaxin, IP-10 and RANTES in HASMCs**.** IL-17A stimulated the production of IL-8, Th2 cytokines and eosinophil chemokines. CSE inhibited IL-17A-induced production of Th2 cytokines and eosinophilic chemokines but significantly enhanced IL-17A-induced production of IL-8. The oxidative stress inhibitor glutathione (GSH) reversed the inhibition of IL-13 production by CSE and had no effect on the inhibition of IL-4 production. GSH reversed the inhibitory effect of CSE on IL-17A-induced production of IL-4 and IL-5 but not IL-13. GSH reversed the inhibition of Eotaxin, IP-10 and RANTES production by CSE. The COX-2 inhibitor NS-398 reversed the inhibition of the production of T2 inflammatory cytokines by CSE. CSE, but not IL-17A, induced the protein and mRNA expression of COX-2 and PGE_2 release. GSH inhibited CSE-induced COX-2 expression and PGE2 release, suggesting that CSE-induced COX-2 expression is mediated by oxidative stress. Our findings showed that exogenous PGE_2 alone had no effect on the production of T2 inflammatory cytokines, except Eotaxin, but inhibited IL-17A-induced production of T2 inflammatory cytokines. The prostaglandin EP_2 receptor antagonist PF 04418948 and EP_4 receptor antagonist L-161,982 reversed the inhibition of IL-4 and IL-13 production by CSE. PF 04418948 blocked the inhibitory effect of CSE on IL-17A-induced production of IL-4 and IL-5. L-161,982 blocked the inhibitory effect of CSE on IL-17A-induced production

of IL-4, IL-5 and IL-13. PF 04418948 and L-161,982 reversed the inhibition of Eotaxin, IP-10 and RANTES production by CSE on its own and together with IL-17A stimulation. The cAMP analogue 8-Bromo-cAMP reduced the production of T2 inflammatory cytokines after stimulation with IL-17A but had no effect on its own on the production of T2 inflammatory cytokines except Eotaxin. CSE induced production of IL-8 was inhibited by fluticasone. CSE reduced the inhibitory effect of fluticasone on IL-17A-induced IL-8 production.

Conclusion: Results from this thesis showed for the first time that CSE can modify the inflammatory responses in HASMCs, promoting a shift from glucocorticoid-sensitive T2 to glucocorticoid-insensitive non-T2 inflammatory response by suppressing T2 inflammatory cytokine production and promoting the non-T2 inflammatory cytokine IL-8 production. Interestingly, Th17 cytokine IL-17A can enhance T2 and non-T2 inflammatory responses by stimulating their cytokine production in HASMCs. Our results also demonstrated that CSE suppresses T2 inflammatory response largely through a COX-2/PGE2/EP2/EP4/cAMP pathway via oxidative stress. Our findings suggest that targeting this pathway may be of great potential for the development of novel treatments for asthmatic smokers.

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Chapter 1. Introduction

1.1 Asthma Definition, Diagnosis and Treatment

1.1.1. Definition

Asthma is a common chronic disorder of the airways affecting around 1-18% of the population in different countries, and around 339 million people worldwide are suffering from this disease (1, 2). Asthma disease is characterised by variable and recurring symptoms, airflow obstruction, bronchial hyperresponsiveness, and an underlying inflammation (1, 3). Asthma is considered an umbrella diagnosis for a collection of varying endotypes and phenotypes, all of which manifest with wheezing, shortness of breath, chest tightness, and cough along with variable airflow obstruction. However, the diverseness of asthma results in different responses to treatment (4).

1.1.2. Asthma Prevalence, Mortality and Morbidity

It is estimated that the number of asthmatic patients will increase by 100 million in 2025 (5, 6). 5.4 million people were diagnosed with asthma in the United Kingdom (UK) in 2018, of which 4.3 million were adults and 1.1 million were children (7). Asthma affects approximately 1 in every 11 people in the UK (7).

In 2015, 383,000 people died because of asthma worldwide, which increased to 420,000 in the following year (2, 6). In 2019, asthma caused around 455,000 deaths around the world (8). In the UK, 1410 people died because of asthma in 2016 (7). It is estimated that 3 people die every day due to asthma attacks in the UK (7). The mortality rate has increased around the world, and the UK has one of the worst asthma death rates in Europe,

with the rate of people died from an asthma attack increased by more than 20% in five years (2011 to 2015) (7).

Asthma imposes a huge burden on the health care systems and patients across the world. The estimated disability-adjusted life years lost (DALYs) due to asthma was 23.7 million worldwide and across all ages in 2016 (2). Further, asthma is one of the top-ranked causes of years lived with a disability and a major leading cause of the disease burden (2). In 2018, there was at least one admission to the hospital because of an asthma attack every 8 minutes in the UK, and the estimated annual cost to treat and manage asthma in National Health Service is more than £1.1 billion (7).

1.1.3. Diagnosis

In clinical practice, spirometry is the gold standard diagnostic tool for asthma patients (1). Spirometry is used to evaluate lung function by measuring forced expiratory volume per second (FEV_1) to the forced vital capacity (FVC). Asthmatic patients have a lower FEV_1 (<80% predicted) along with a decrease in FEV1/FVC ratio (<75% predicted), confirming the airflow limitation (1, 9). The reversibility of airflow obstruction is a strong indicator of asthma with the use of bronchodilators or glucocorticoids, so an improvement by more than 400 ml in FEV_1 indicates underlying asthma (10) After confirming the diagnosis, the treatment plan should be initiated in order to control the disease symptoms and prevent exacerbation.

1.1.4. Treatment

The treatment plan for asthmatic patients includes short-acting β2-adrenoreceptor agonists (SABA) such as salbutamol (11) and low-dose of inhaled corticosteroids (ICS). The SABA are used as bronchodilators to relieve acute asthma symptoms. Asthmatic patients are also recommended

to use low-dose of inhaled corticosteroids (ICS) like fluticasone as preventer treatment for obtaining overall therapy goals (12) by reducing the incidence of asthma exacerbations and improving asthma symptom control . In some asthmatics, the low-dose ICS may not be enough to keep certain asthma under control; therefore, inhaled long-acting β2-agonist (LABA) such as salmeterol is the first approach as an add-on treatment to ICS in adults, and it should be considered prior to increase the dose of ICS (12). Adding the LABA to ICS improves symptoms and lung function of asthmatics while decreasing asthma attacks (12). If asthma for some patients remains uncontrolled after adding a LABA, then increase the dose of ICS from low to medium or consider alternative therapies like the leukotriene receptor antagonist (LTRA) (12) (Figure 1.1).

There are some other specialist therapies to control asthma such as leukotriene receptor antagonists or theophylline, also known as 1,3-dimethylxanthine, which is a phosphodiesterase inhibiting drug used in the treatment of lung disease including asthma (11, 13). The leukotriene receptor antagonists such as zafirlukast and montelukast are used as maintenance therapy to treat chronic asthma (14). However, they are less effective than inhaled glucocorticoids, particularly for exacerbation reduction, but they may be appropriate for some patients who are unable or unwilling to use inhaled glucocorticoids or for patients who experience intolerable side effects (13). Theophylline has been used to treat asthma as a bronchodilator (15). Theophylline is a potential alternative to SABA for relieving asthma symptoms (13). However, theophylline has a slower onset of action and a higher risk of side effects than short-acting β2-agonists (13).

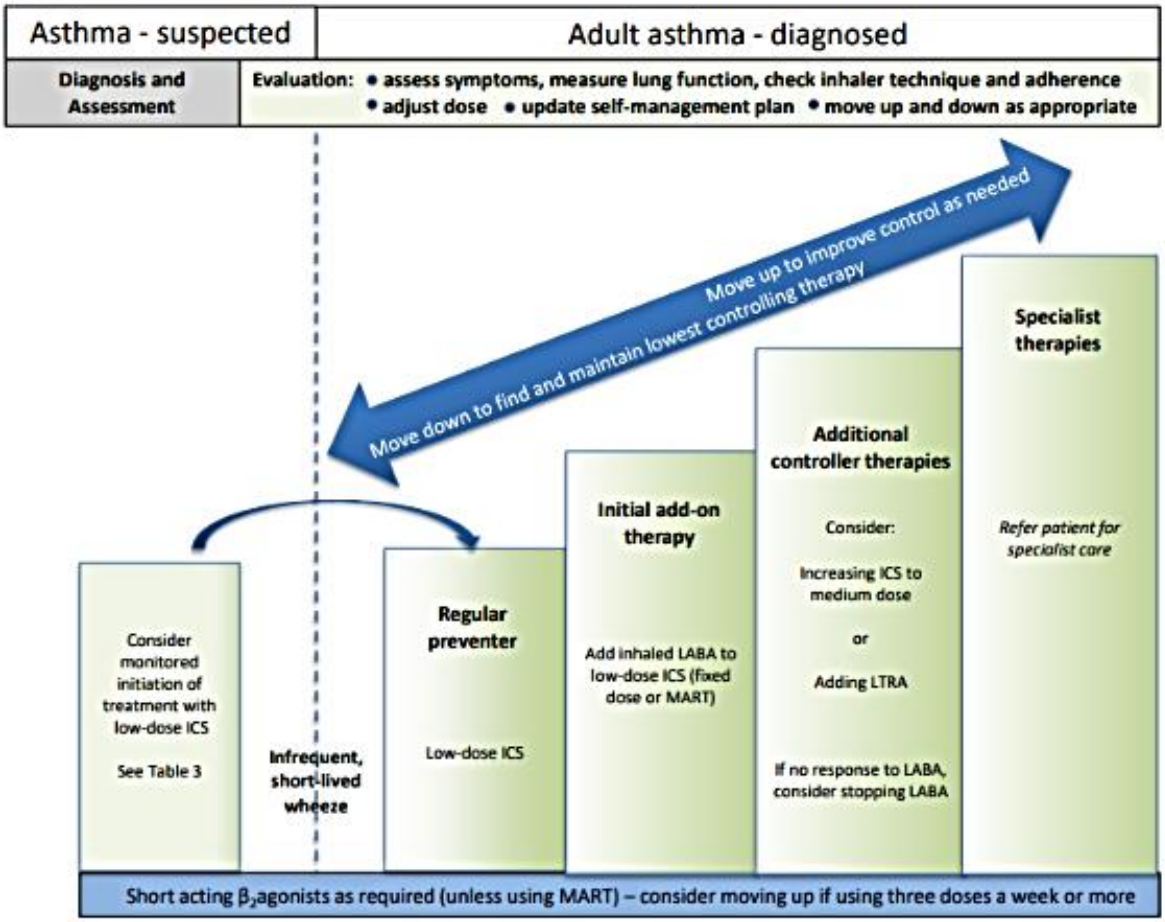


Figure 1.1 Management steps for patients with asthma

This diagram illustrates the management steps for asthmatics as per the national clinical guideline provided by the British Thoracic Society (BTS). Inhaled corticosteroids (ICS) inhaled long-acting β2 agonists (LABA) and short-acting β2 agonists (SABA) (12).

1.2. Airway Inflammation

Asthma has different endotypes, pathologies, responses to treatment and triggers. Airway inflammation is a hallmark pathological feature of asthma, described by airway inflammatory infiltrate including eosinophils, neutrophils, T lymphocytes and mast cells (16-18). An important understanding of the development of airway inflammation in asthmatics came when CD4+ T cells were discovered and classified into two distinct types based on T helper cells (Type 1 and Type 2) which could be distinguished based on their cytokine secretion profile [30]. Th1 cells are mainly responsible for secreting interleukin-2 (IL-2), interferon-γ and lymphotoxin-α. Th2 cells secrete cytokines such as IL-4, IL-5, IL-9, and IL-13, which play an important role in the inflammatory process and pathophysiology of asthma.

Airway inflammation can be divided into two types: type 2 (T2), also known as Th2 high, and non-T2, also known as Th2 low (Table 1.1) [19]. Around 50% of patients with asthma have a T2 airway inflammation, whereas the remaining patients have a non-T2 airway inflammation or mixed eosinophil and neutrophilic inflammation, which is commonly found in severe asthma (19). T2 airway inflammation is defined by increased eosinophilic counts in the sputum, airway and blood, and the best way to determine this type of inflammation is by sputum cellular profiles (20). Eosinophil levels higher than 2-3% in sputum can be defined as T2 asthma (20). There is no cut-off for eosinophil count in the airway, but a clinical study demonstrated that asthmatic patients with T2 airway inflammation showed an average of 20 eosinophils/mm2 of tissue (interquartile range, 16-31 eosinophils/mm2)

(21). T2 inflammation is characterised by the upregulation of Th2 cytokines IL-4 and IL-13, and IL-5 in asthma (22). Eosinophilic chemokines such as Eotaxin, IP-10 and RANTES are involved in the recruitment of eosinophils at the allergic inflammatory site (23). Activation of eosinophils and Th2 cells amplified the T2 inflammatory response characteristic for allergy and asthma (24).

Asthmatic patients with a non-T2 type of inflammation are differentiated based on the counts of sputum neutrophils ranging from as low as 40% to as high as 70% (20). We know from the available literature that the increase of non-T2 inflammation in asthmatic patients positively correlates with the increase in IL-8 levels [1]. IL-8 has also been detected in the serum and bronchial tissue of an asthmatic patient with severe asthma.

Table 1.1 The main endotypes of airway inflammation in asthma

	T2 airway inflammation	**Non-T2 airway inflammation**
Description	Allergic/ Eosinophilic/ Th2-high	Th2-low / Neutrophilic / Smoking related
Cytokine and chemokines	Th2 cytokines (IL-4, IL-5 and IL-13) Eosinophil chemokines (Eotaxin, IP-10 and RANTES)	Neutrophile chemoattractant (IL-8, IL-6 and GRO-α) Th17 cytokines (e.g. IL-17A)
Biomarkers	Eosinophils	Neutrophils
Treatment	Glucocorticoids, anti-IgE or anti-IL-4, IL-5, or IL-13	High dose of glucocorticoids, antibiotics, Smoking cessation

This table illustrates the main endotypes of airway inflammation of asthma (T2 and non-T2), the key cytokines and chemokines for each type, the biomarkers for each type (e.g., eosinophils and neutrophils) and the most common treatments for each.

1.2.1 T2 inflammation in asthma

Th2 cells play an integral role in the T2 airway inflammation process and pathophysiology of asthma by secreting inflammatory cytokines such as IL-4, IL-5 and IL-13. These cytokines are responsible for eosinophilia, overproduction of IgE and airway hyperresponsiveness, all observed in the airways of asthmatic patients (25). IL-5 has a crucial pathogenic role in eosinophil differentiation, recruitment and survival; therefore plays a pivotal role in the pathophysiology of T2 inflammation in asthma (26). IgE has an important role in allergic asthma as it mediates allergen-initiated bronchoconstriction and plays a part in the development of airway inflammation (25, 27). Cytokines produced by Th2 cells (e.g., IL-4 and IL-13) enhance the production of IgE antibodies, which then attach to cells via high-affinity IgE receptors (FcεR1) (28, 29). FcεR1 is expressed in different inflammatory cells such as mast cells and basophils, which are key mediators in T2 inflammation (30). Human airway smooth muscle cells (HASMCs) possess high-affinity IgE receptors (31), suggesting HASMCs may participate in T2 inflammation in asthma.

Th2 cytokines drive the recruitment of mast cells, basophils and eosinophils and induce isotype switching of B cells to produce IgE after exposure to antigens. Eosinophilic inflammation and IgE synthesis are therefore encompassed in T2 inflammation in asthma. Airway structural cells such as HASMCs can produce Th2 cytokines (32). It has also been shown that Th2 cytokines can be produced by other cell types, such as NK

cells, type 2 innate lymphoid cells (ILC2s), and even Th1 cells under certain conditions (33) (Figure 1.2).

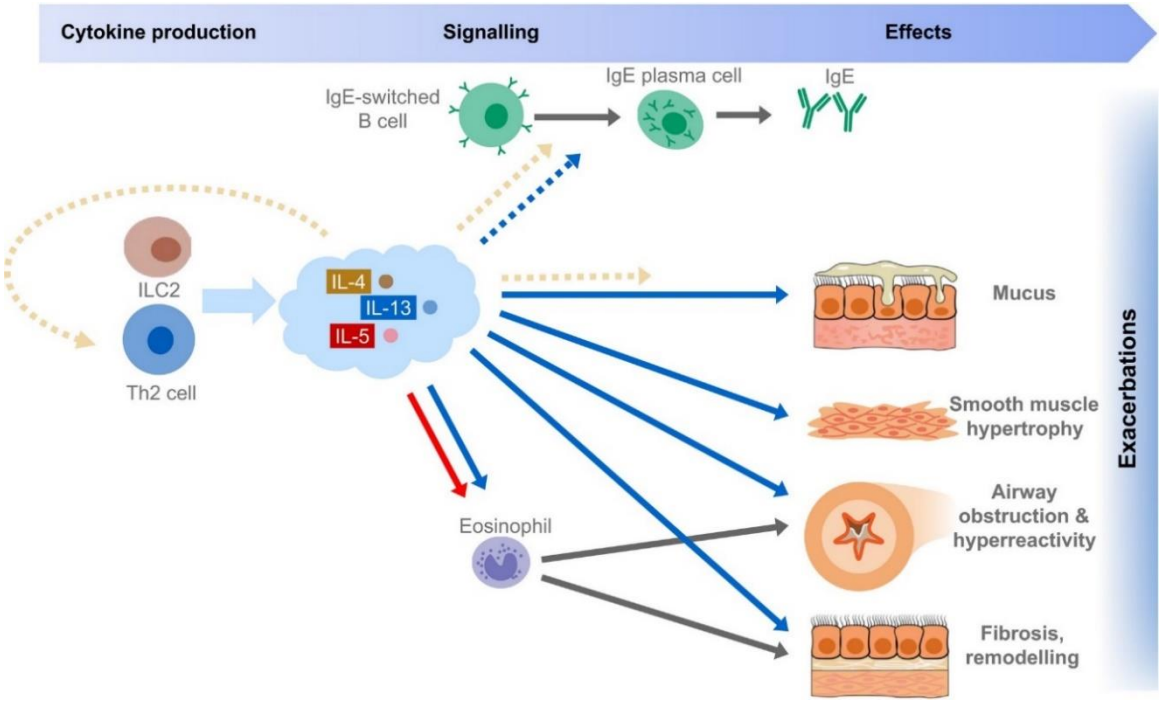


Figure 1.2 Effects of Th2 cytokines in asthma.

Th2 cytokines drive the recruitment of eosinophils and are also central to many of the hallmarks of asthma, including mucus production, smooth muscle hypertrophy, subepithelial fibrosis, bronchial remodelling, and airway obstruction and hyperresponsiveness (34).

Eosinophils are a hallmark of T2 asthma and play an important role in airway inflammation (35), and some asthmatics have shown elevated eosinophil counts in their airways (25, 27), sputum (36) and blood (37). Chemokines are selective in their recruitment of inflammatory cells; for instance, Eotaxin, IP-10 and RANTES mostly recruit eosinophils (38). Eosinophils are produced in the bone marrow from pluripotent hematopoietic stem cells (39). IL-5 was identified as a factor that promotes differentiation and proliferation of eosinophils in the bone marrow (40). Cooperation between eosinophil chemokines and IL-5 stimulates the fast build-up of

eosinophils in tissues exposed to an allergen (41). It is suggested that eosinophils can contribute to airways bronchoconstriction, mucus production, and tissue damage (42)

1.2.2 Th2 cytokines

Th2 cytokines play an integral role in the process of inflammation in asthma by recruiting inflammatory cells in the airways. There is a range of cytokines produced by Th2 cells, such as IL-4, IL-5, and IL-13. HASMCs are shown to produce Th2 cytokines such as IL-5 and IL-13 (32) and eosinophil chemokines like Eotaxin (43), IP-10 (44) and RANTES (45, 46), which contribute to T2 airway inflammation in asthma. Alarmins such as IL-25, IL-33 and Thymic stromal lymphopoietin (TSLP) which are released from tissue cells are playing a role in T2 inflammatory response in asthma (47).

IL-4 induces important pro-inflammatory functions in asthma, which include induction of the IgE isotype switch, promotion of eosinophil transmigration in the endothelium, mucus production, and differentiation of T2 lymphocytes leading to cytokine release (48). A study showed that IL-4 levels in bronchoalveolar lavage (BAL) fluid of mild T2 asthmatics were increased after the 18h allergen challenge (49). Stimulation with IL-25 increased IL-4, IL-5, and IL-13 production in mice resulting in increased blood eosinophils, IgE production, mucus production, and AHR (50). These observations suggest that IL-4 plays an important role in the T2 inflammatory response in asthma.

T cells from lung tissue samples of asthmatic patients have been shown to express a high level of IL-5 mRNA, which is associated with an increase in disease severity as well as eosinophil count (51-53). The use of

therapies targeting IL-5 signalling (anti-IL-5) showed a reduction in blood eosinophils and improved lung function (54). Therapies targeting IL-5 such as mepolizumab, reslizumab, and benralizumab, minimised asthma exacerbations and improved lung function in patients with severe T2 airway inflammation and poor control (55). Intravenous IL-5 enhanced the accumulation of eosinophils induced by intradermal injection of eotaxin in guinea pigs, suggesting cooperation between eosinophil chemokine and IL-5 simulated fast build-up of eosinophils in tissues exposed to an allergen (41). These studies illustrate that IL-5 is a key mediator in eosinophil activation, which may contribute to the T2 inflammatory response in asthma.

IL-13 is a pleiotropic cytokine produced by Th2, mast cells and basophils and is thought to be involved in many of the features of asthma, such as airway hyperresponsiveness (56, 57). IL-13 is implicated with T2 airway inflammation and has been recognised as a potential therapeutic target in the treatment of asthma. IL-13 level was increased in sputum, peripheral blood, bronchial sub-mucosa, and airway smooth muscle bundle of patients with asthma (58). Persistent elevation of IL-13 may contribute to refractory asthma, and therefore anti-IL-4/IL-13 therapies could be effective in those patients (59).

1.2.3 Eosinophils chemokines

Eotaxin is a small protein which is a potent chemoattractant for eosinophils and can be produced by HASMCs (60). Eotaxin induces eosinophil migration from pulmonary small blood vessels by acting on the CCR3 receptor, which found on the surface of leukocyte cell (61). The

production of Eotaxin by HASMCs is increased when stimulated with pro-inflammatory cytokines like Th2 cytokines, such as IL-13 (43). Eotaxin production has been shown to be increased in samples collected from bronchial mucosa of asthmatic patients (62, 63). Moreover, Eotaxin levels are also markedly increased in the sputum and plasma of patients with asthma compared to healthy subjects (64) and showed HASMCs have the ability to produce Eotaxin and could thereby contribute to the recruitment of eosinophils in the airways of asthmatic patients (60).

IP-10 is a small 10.8kD protein that is produced by different cell types. A study showed that the overexpression of IP-10 in the lung contributed to the development of AHR and a T2 inflammatory response, while a reduction of IP-10 showed a reduction of T2 airway inflammation (65). A study showed that IP-10 was expressed by asthmatic ASM in bronchial biopsies and ex vivo cells compared with healthy subjects (44). IP-10 levels were higher in BALF from asthmatic patients compared with non-smoker subjects (66). The production of IP-10 in HASMCs stimulated by a mixture of cytokines like IL-1β, TNF-α, and IFN-γ is greater in cells obtained from asthmatic patients than in cells from healthy subjects (67).

Regulated upon Activation, Normal T cell Expressed, and Secreted (RANTES) is an 8-kDa polypeptide of the C-C chemokine subfamily. RANTES play a role in T2 airway inflammation as it is a potent chemoattractant for eosinophils (38, 68). TNFα and IL-1β stimulated the production of RANTES in HASMCs (45, 46). RANTES levels in plasma are significantly increased during acute asthma attacks compared to that in

healthy subjects (69). In addition, RANTES levels in plasma are higher during asthma attacks than in asymptomatic asthma patients (69).

1.2.4 Biological therapies targeting T2 inflammation

In terms of the development of relevant biologic therapies, T2 is the most well-defined asthma endotype. The biologic therapies that have been authorised and the majority of those in development aim to target T2 airway inflammation. IL5 is the key cytokine responsible for activating eosinophils, which promote airway inflammation and are a hallmark of asthma. Studies demonstrate that monoclonal antibodies targeting IL5 or its receptor IL5R, such as mepolizumab, reslizumab, and benralizumab, minimised asthma exacerbations and improved lung function in patients with severe T2 airway inflammation and poor control (55). Different clinical trials worldwide have shown that difficult-to-treat asthmatic patients can benefit greatly from using mepolizumab (70). The anti-IgE drug, omalizumab, reduced the allergen response in asthmatic patients, which may reduce T2 inflammation (71). In addition, the anti-IgE treatment improved asthma control and decreased the severity of asthma exacerbations in patients with severe T2 asthma (72). Dupilumab is a monoclonal antibody that inhibits both IL-4 and IL-13 pathways by binding to the IL-4 receptor alpha-subunit (73). To date, the types of biologics licensed for use with severe asthma patients are omalizumab, mepolizumab, reslizumab, benralizumab and dupilumab (73).

1.2.5 Non-T2 inflammation in asthma

There is no clear definition of non-T2 inflammation in asthma. However, the non-T2 airway inflammation in asthma is characterised by neutrophilic inflammation, which tends to be less sensitive to glucocorticoids and involves various asthma phenotypes such as smoking or occupational

exposures (74). Research on healthy controls suggested that the average range of neutrophil percentages in the sputum is between 30 and 50% (75, 76). A study showed that most asthma exacerbations are non-T2, and patients with non-T2 exacerbations are mainly neutrophilic (68% neutrophils and 0.3% eosinophils) (77). IL-8 may promote the recruitment of neutrophils to the site of inflammation, which contributes to the non-T2 inflammatory response in asthma (78).

Increased neutrophils and T helper 17 cells (Th17) are a subset of T helper cells which defined by their production of interleukin 17 (IL-17) and have been linked to moderate and severe asthma phenotypes (79). The non-T2 inflammation in asthma is more strongly associated with the presence of Th17 cells (80). In fact, IL-17A and IL-17F are overexpressed in asthmatics' lung tissue, and their levels correlate with the severity of asthma, especially in patients with non-T2 inflammation and glucocorticoid resistance (81). Several studies demonstrated that some severe asthmatic patients had been linked to non-T2 inflammation and showed an increase in the production of the Th17 cytokines IL-17A and IL-17F (82). IL-17A and IL-17F can stimulate human airway epithelial cells to produce chemokines like GRO-α and IL-8, which may trigger neutrophil infiltration (82). IL-17A can contribute to non-T2 airway inflammation by attracting and activating neutrophils (83). These studies demonstrated that Th17 cells, by their ability to recruit the neutrophils, may play a role in the non-T2 inflammatory response.

1.2.6 Non-T2 cytokines

IL-8 is a neutrophilic chemoattractant that plays an integral role in the initiation of the non-T2 inflammatory response in the airways (84, 85). An

increase in non-T2 inflammation in asthmatic patients positively correlates with the increase in IL-8 levels (85). HASMCs have been shown to produce IL-8 in response to inflammatory mediators, such as TNFα (98). IL-8 protein and mRNA expression were higher in isolated HASMCs taken from patients with asthma than in non-asthmatic individuals (86). IL-8 is upregulated in isolated bronchial epithelium from symptomatic asthmatic patients (87). Patients with severe acute asthma have 19 times higher levels of IL-8 in samples from tracheal aspirates, which positively correlated with the number of neutrophils than those obtained from healthy subjects (88). IL-8 has also been detected in the serum and bronchial tissue of asthmatic patients with a severe form of asthma, but it was undetectable in normal subjects and patients with mild asthma (89). These studies suggest that IL-8 is involved in non-T2 airway inflammation, and the level of IL-8 could be an indicator of asthma severity.

IL-6 is a pleiotropic cytokine which acts as a pro-inflammatory mediator (90). IL-6 is primarily produced by the innate immune systems cells, but it has been reported that structural cells such as HASMCs can produce IL-6 (45, 91). There are many studies showing a high level of IL-6 in the serum and BAL fluid of patients with asthma, especially in an activated state of the disease (18, 91). IL-6 is involved in the differentiation of human Th17 cells (92). IL-17A enhances neutrophil migration by inducing the production of IL-6 in bronchial epithelial cells (93). These studies showed that IL-6 might contribute to non-T2 airway inflammation in asthma.

GRO-α/CXCL-1 is a chemokine which is structurally related to IL-8, but it is a more potent neutrophil chemoattractant than IL-8 (94). IL-17A

induced the production of the neutrophil chemoattractant GRO-α from airway epithelial cells and HASMCs, which may play a role in the non-T2 inflammation of severe forms of asthma (95, 96). Lipopolysaccharide (LPS) and TNFα induced GRO-A production and mRNA expression in bronchial epithelial cells (97). Furthermore, anti-inflammatory treatment (Fluticasone and Dexamethasone) minimally inhibited GRO-α α production in BEAS-2B cells (97). These results suggest that GRO-α may contribute to non-T2 airway inflammation, and it is insensitive to treatment with glucocorticoids.

Th17 cells secrete cytokines such as IL-17A, IL-17F and IL- 22 from humans (79). Th17 cytokines are considered an important regulator of non-T2 inflammation. IL-17A, IL-17F, and IL-22 enhanced non-T2 inflammatory response, mucous cell metaplasia, and smooth muscle proliferation and migration (Figure 1.3) (79). IL-17A and the closely related IL-17F are linked to non-T2 inflammation by stimulating the production of the neutrophil chemoattractant GRO-α and IL-8 from airway epithelial cells and ASMCs and thereby may play a role in the non-T2 inflammatory response (95, 96). IL-17A is the major cytokine secreted by Th17 cells, which contributes to the development of airway neutrophilia and induces the production of many pro-inflammatory cytokines and chemokines from different cell types (17). For instance, IL-17A induced the production of IL-1β and IL-6 from airway epithelial cells and endothelial cells, which leads to an increase in neutrophil infiltration (79). Overexpression of IL-17F in the airway of mice is associated with airway neutrophilia, the production of several cytokines, an increase in airway hyperreactivity, and mucus hypersecretion in asthma (98). Moreover, levels of IL-17A, IL-17F and IL22 are increased in bronchial biopsies and

bronchoalveolar lavage from severe and moderate asthmatic patients (79). The increase in Th17 cytokines level is positively correlated with asthma severity (79). Previous studies have demonstrated that IL-17 induced airway inflammation (99). IL-17 induced IL-8 from human bronchial smooth muscle cells and bronchial epithelial cells (100, 101). These studies demonstrate that Th17 cytokines contribute to non-T2 airway inflammation, which may lead to a severe form of asthma. The effect of IL-17A on the T2 inflammatory response in HASMCs is largely unknown.

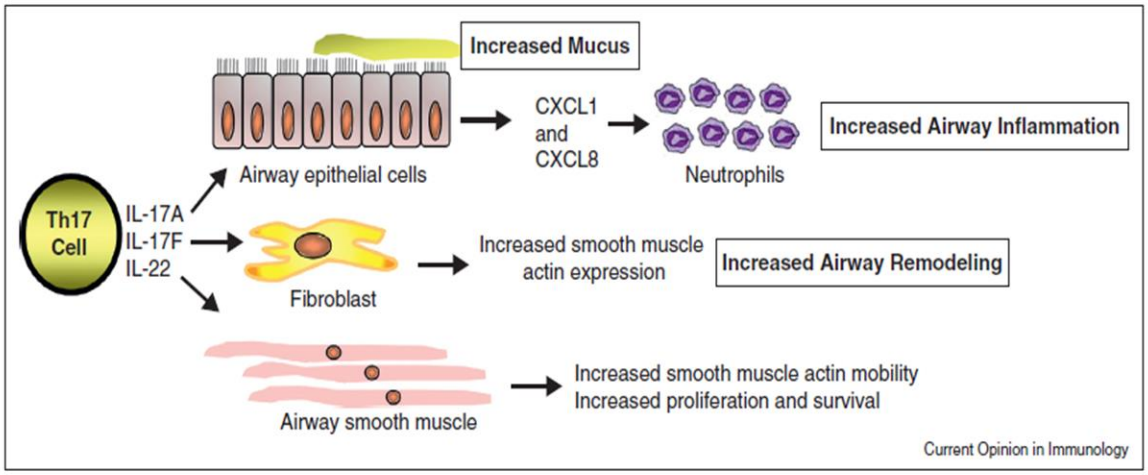


Figure 1.3. Effects of Th17 cytokines on the airways

Figure 1.3. Th17 cytokines such as IL-17A, IL17F and IL-22 contribute to non-T2 airway inflammation and remodelling in asthma by increasing airway neutrophil infiltration, mucous cell metaplasia, and smooth muscle proliferation and migration (79).

1.3. Human airway smooth muscle cells and airway inflammation

1.3.1 Synthetic function of HASMCs

The pro-inflammatory action of HASMCs results from their synthetic functions and produces a number of inflammatory mediators, such as cytokines, chemokines, and growth factors (38, 102). HASMCs have been shown to produce some pro-inflammatory cytokines such as IL-6 and IL-1β as well as some growth factors, such as VEGF, and matrix metalloproteinases (MMPs) which contribute to airway remodelling (38). HASMCs also produce lipid mediators, like leukotrienes, which enhance bronchoconstriction, and prostanoids and isoprostanes, which can have a several effects on HASMC's tone (38). A summary for inflammatory mediators produced by HASMCs is shown in Table 1.2.

Table.2. Inflammatory mediators produced by HASMCs

Chemokines	CCL5/RANTES CCL11/Eotaxin CCL2/MCP-1 CCL7 CCL8 CCL17/TARC CXCL10/IP-10 CXCL8/IL-8 CXCL1 CXCL2 CXCL3 CX3CL1	
Lipid mediators	Prostanoids	PGD_2 $PGF_{2\alpha}$ Thromboxane A_2 PGE_2 PGI_2
	Leukotrienes	LTD_4
Growth and Remodelling Factors	Matrix metalloproteinases (MMPs)	MMP-2 MMP-9 MMP-12
	Extracellular matrix components	Fibronectin Laminin

		Perlecan Chondroitin
	Other	TGF-β CTGF VEGF PDGF IGF bFGF
Other	Cytokines	IL-6 IL-5 GM-CSF Stem cell factor IL-1β IFN-γ

1.3.2 Role of HASMCs in airway inflammation

HASMCs produce a wide range of pro-inflammatory cytokines and chemokines, which orchestrates the role of HASMCs in the inflammatory

process in asthma by recruiting and activating the mast cells, leukocytes, neutrophils, and eosinophils (103). HASMCs contribute through their structural and contractile properties to the stabilisation and homeostasis of the airways system (104). The HASMCs also play an integral role in the pathology of asthma disease. For instance, they are involved in airway hyperresponsiveness and excessive bronchoconstriction by their contractile properties (105).

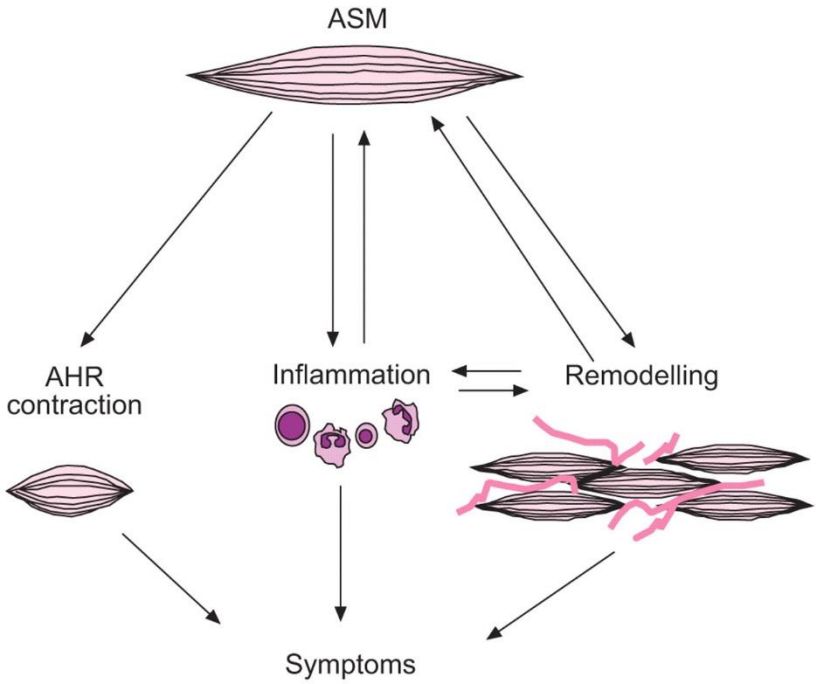


Figure 1.4. Schematic diagram of overview of the role of HASMCs in asthma
HASMCs are involved in airway hyperresponsiveness by an exaggerated response to bronchoconstrictor stimuli; remodelling due to an increase in HASMCa mass and altered deposition of extracellular matrix; and inflammation by producing cytokines, chemokines and growth factors. HASMCs by these three features contribute to asthma symptoms. (106).

1.3.3 HASMCs and T2 airway inflammation

HASMCs are shown to produce Th2 cytokines such as IL-5 and IL-13 (32), eosinophil chemokines like Eotaxin (43), IP-10 (44) and RANTES (45, 46), which contribute to T2 inflammatory response in asthma. The release

of Th2 cytokines IL-5 and IL-13 contributes to changes in HASMCs responsiveness, by increased cell proliferation or by hypertrophy of airway smooth muscle cell depending on the nature of the inflammatory stimulation in asthma, in asthma (32). IL-13 and IL-4 enhanced histamine-induced hyperresponsiveness of airway smooth muscle *in vitro* (107). IL-13 receptors are expressed in HASMCs and airway epithelial cells and studied demonstrated that IL-13 enhanced cholinergic-induced contractions of HASMCs *in vitro* (32, 108). IL-4 and IL-13 can contribute to airway inflammation by inducing cytokine release, such as the eosinophil chemoattractant Eotaxin in HASMCs (109). A study showed that IL-17A, involved mainly in inducing non-T2 inflammatory responses, may play a role in T2 inflammatory response by inducing Eotaxin production in HASMCs (110). However, the effect of IL-17A on the production of Th2 cytokines and other eosinophil chemokines in HASMCs is largely unknown. Taken together, HASMCs can produce some of the Th2 cytokines and eosinophil chemokines (T2 inflammatory cytokines), which may contribute to the T2 inflammatory response in asthma.

1.3.4 HASMCs and non-T2 airway inflammation

HASMCs have been shown to produce IL-8, which contributes to non-T2 inflammation, in response to inflammatory mediators, such as TNFα (111) and CSE (112). Studies demonstrated that isolated HASMCs from asthmatic patients produce a higher level of the neutrophilic chemoattractant IL-8 than non-asthmatic (84, 88). HASMCs also have been shown to produce IL-6 and IL-8 in response to bradykinin, an asthmatic mediator (113, 114).

Th17 cytokines are considered as an important regulator of neutrophilic inflammation.IL-17A and the closely related IL-17F are linked to non-T2 inflammation by induction of the production of the neutrophil chemoattractant GRO-α and IL-8 from HASMCs and thereby may play a role in the non-T2 inflammation in asthma (95, 96). IL-17A amplifies the synthetic function of HASMCs by enhancing TNFα-induced production of IL-6 from HASMCs. Taken together, Th17 cytokines may contribute to non-T2 inflammation and airway remodelling in asthma.

1.4. Cigarette smoke and airway inflammation in asthma

Cigarette smoke (CS) is an important cause of airway inflammation in asthmatic smokers (115). Studies have shown that CS and CS extract (CSE) induced the production of inflammatory cytokines and chemokines in various experimental designs.

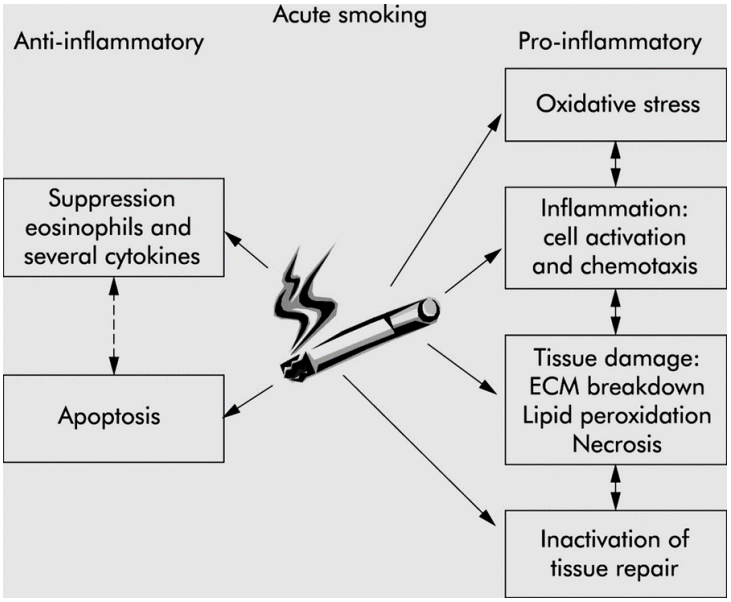


Figure 1.5 The effects of cigarette smoking on the airway

Cigarette smoke causes increased oxidative stress due to different mechanisms, including direct damage by radical species and the enhanced inflammatory response. Cigarette smoke can cause tissue damage, as there is evidenced by increased products of lipid peroxidation and degradation products of extracellular matrix proteins. Cigarette smoke also has an anti-inflammatory effect on the number of eosinophils and some inflammatory cytokines.

1.4.1 Cigarette smoke and T2 airway inflammation

The effect of CS on the T2 inflammatory response is unclear, and studies showed a different response to CS in the *in vivo* experimental models. It has been shown that CS can induce the production of the Th2 cytokine IL-4 in BALF from rats and, therefore, there possibility that may contribute to the T2 inflammatory response (116). IL-13 level was significantly increased in BAL of mice after CS exposure (117). IL-5 level was also increased in BAL after 4 days of CS exposure (118, 119). On the other hand, *in vivo* research found that CS inhibited the development of T2 airway inflammation by inhibiting the antigen-stimulated release of IL-4 in mice (120). Further, an *in vivo* study showed that CS attenuated house dust mite-mediated eosinophil response by reducing eosinophils in bronchoalveolar lavages (BALs) (121). An *in vivo* study showed that CS significantly reduced the number of eosinophils in BAL fluid of OVA-challenged mice, while in the same study, CS increased the number of neutrophils in nonallergic animals (122).

The effect of CS on the T2 inflammatory response in the *in vitro* studies showed a different response in the inflammatory cells and structural cells. An *in vitro* study demonstrated that cigarette smoke condensate (CSC) induced the production of IL-5 but not IL-13 in macrophage cells (123). A study showed that CSE (3% and 10%) increased the production of IL-4 and IL-5 in dendritic cells (124). CSE inhibited Eotaxin and RANTES production in HASMCs after stimulation with TNFα (125). Another study showed that CSE reduced LPS-induced production of IP-10 in an immortalised normal bronchial epithelial cell line (16-HBE) (126). These findings indicate that CSE may have a suppressive effect on the T2 inflammatory response in airway

structural cells. Considering HASMCs are an important source of inflammatory cytokines and chemokines and play a role in the inflammatory process in asthma; therefore, it is crucial to explore the effect of CSE on the production of T2 inflammatory cytokines in HASMCs.

1.4.2 Cigarette smoke and non-T2 airway inflammation

The majority of asthmatic smokers have non-T2 airway inflammation in the airways (127). An *in vivo* study showed that CS increased the number of neutrophils in nonallergic animals (122). Non-T2 inflammation involves the upregulation of IL-8 via the NF-κB pathway (78). IL-8 is crucial for the recruitment of neutrophils to the site of inflammation in the airways (128). CSE increased the production of IL-8 and GRO-α from HASMCs (129). IL-17A led to a slight increase in IL-8 production in human bronchial epithelial, but the co-treatment with CSE enhanced the IL-17A-induced production of IL-8 in human bronchial epithelial cells (BEAS-2B) (130), which may potentially further enhanced the non-T2 inflammatory response. CSE induced the production of IL-6 in human bronchial epithelial cells (131).

Th17 cytokines are generally regarded as an important regulator of non-T2 inflammation, and the non-T2 inflammatory response was augmented by IL-17A and IL-17F. CSE significantly induced the production of IL-17F in the human epithelial cells while induced IL-17A and IL-17F production in peripheral blood mononuclear cells (132). Further, a study showed by using an ex-vivo explant culture system that CS increased IL-17A and IL-17F production from human lung parenchymal tissue (133). The production of IL-17A and neutrophil numbers were significantly increased in the bronchial mucosa of the asthmatic smokers compared to the non-smokers (134). Asthmatic smokers also demonstrated a high IL-17A

concentration in sputum supernatant compared to non-smokers with asthma (135). These studies show that CS could contribute to the non-T2 inflammatory response by inducing its related cytokine production in asthma.

1.5. Role of oxidative stress in airway inflammation in asthma

Oxidative stress is characterised by the imbalance between reactive oxygen species (ROS) production and antioxidant defences (136). Oxidative stress plays a curial role in the pathogenesis of asthma (137). Oxidative stress exacerbates airway inflammation in bronchial asthma by producing a range of pro-inflammatory mediators, triggering bronchospasm and increasing bronchial hyperresponsiveness (138).

Endogenous and exogenous ROS, like superoxide anion, hydroxyl radical, hypohalite radical, and hydrogen peroxide, as well as reactive nitrogen species, such as nitric oxide, peroxynitrite, and nitrite, contribute to airway inflammation in asthma and are predictors of the disease severity (139). Asthma is also linked to reduced antioxidant defences such as superoxide dismutase, catalase, and glutathione (139). Numerous mediators of asthma, such as lipid mediators, and chemokines, may act as triggers or promoters of ROS generation. Additionally, some environmental conditions associated with asthma, like air pollutants (for example, ozone and cigarette smoke), might lead to an increase in ROS formation in the airways (140-142). Glutathione (GSH) is the most important small molecular weight thiol produced in the lungs, which works together with its redox enzymes to provide an important antioxidant system (143).

1.5.1 Cigarette smoke and oxidative stress in airway inflammation

Oxidative stress is made up of reactive oxygen radicals, which are known as ROS and contribute to the pathogenesis of airway inflammation (144, 145). Studies have shown that CS causes oxidative stress leading to lung damage. CS induces oxidative stress in humans both directly and indirectly via the effect of free radicals that exist in the smoke or the inflammatory response resulting from smoking, respectively (146). CS induces oxidative stress (147), due to the high quantities of ROS contained in the cigarette (148). A study demonstrated that CSE caused oxidative stress in a number of alveolar epithelial cell lines and in primary human small airway epithelial cells by increasing levels of the oxidatve stress biomarker 4-hydroxy-2-nonenal and decresing levels of the intracellular antioxidant glutathione (144). CSE enhanced heme-oxygenase-1, an intracellular indicator of oxidative stress, in HASMCs [7]. Chronic CSE exposure elevated the marker of oxidative stress Mn-SOD expression in human bronchial epithelial cell line BEAS-2B [19]. Acute CSE exposure also elevated oxidative stress in A549 cells (149).

In vitro and *in vivo* studies demonstrated a reduction of GSH in lung cells after acute exposure to CS (150, 151). Changes in the levels of GSH were shown to be due to redcution by acute smoking and after that enhanced activation of gamma-glutamylcysteine synthetase (γ-GCS), which is the rate-limiting enzyme for GSH production (152, 153). Oxidative stress is a potential pathway that may mediate the CSE effect on cytokine production. For instance, a study showed that GSH inhibited CSE-induced production of IL-8 in HASMCs (112), suggesting that the effect of CSE on the production of non-T2 inflammatory response is dependent on oxidative stress. Further,

a study showed that CSE increased the intracellular levels of ROS and mediated non-T2 inflammatory response by increasing the production of IL-6 and IL-8 in bronchial epithelial cells (154). These studies illustrate that CS causes oxidative stress contributing to non-T2 airway inflammation and lung damage. However, the role of oxidative stress in the T2 inflammatory response in HASMCs is unknown.

1.6. Role of COX-2, PGE_2 and cyclic AMP in airway inflammation

Cyclooxygenase (COX) is a key enzyme in prostanoid synthesis and has two main isoforms, COX-1 and COX-2 (155). COX-1 is considered as a housekeeping gene, which is widely expressed in most cells and is responsible for prostanoid formation in physiological conditions, whereas COX-2 is inducible by a number of stimulants, including cytokines, and is responsible for the production of prostanoids at the site of inflammation (155). The COX enzyme converts arachidonic acid into prostaglandin H_2 (PGH_2), which is then metabolised into different prostanoids, such as prostacyclin (PGI_2), thromboxane A_2 (TXA_2), prostaglandin D_2 (PGD_2), prostaglandin E_2 (PGE_2), and prostaglandin F_2 (PGF_2) (156). PGE_2 is the main prostanoid produced by HASMCs, which primarily has pro-inflammatory effects but also has some anti-inflammatory effects in the airways (156, 157). For instance, studies showed that exogenous PGE_2 have pro-inflammatory effects by increasing the non-T2 inflammatory cytokine IL-6 and IL-8 production in HASMCs (158, 159) and fibroblast cells (160) while other study demonstrated that exogenous PGE_2 may have some

anti-inflammatory effects by inhibiting LPS-induced TNFα and IL-6 production in mouse and human monocytes (161).

(Figure 1.4).

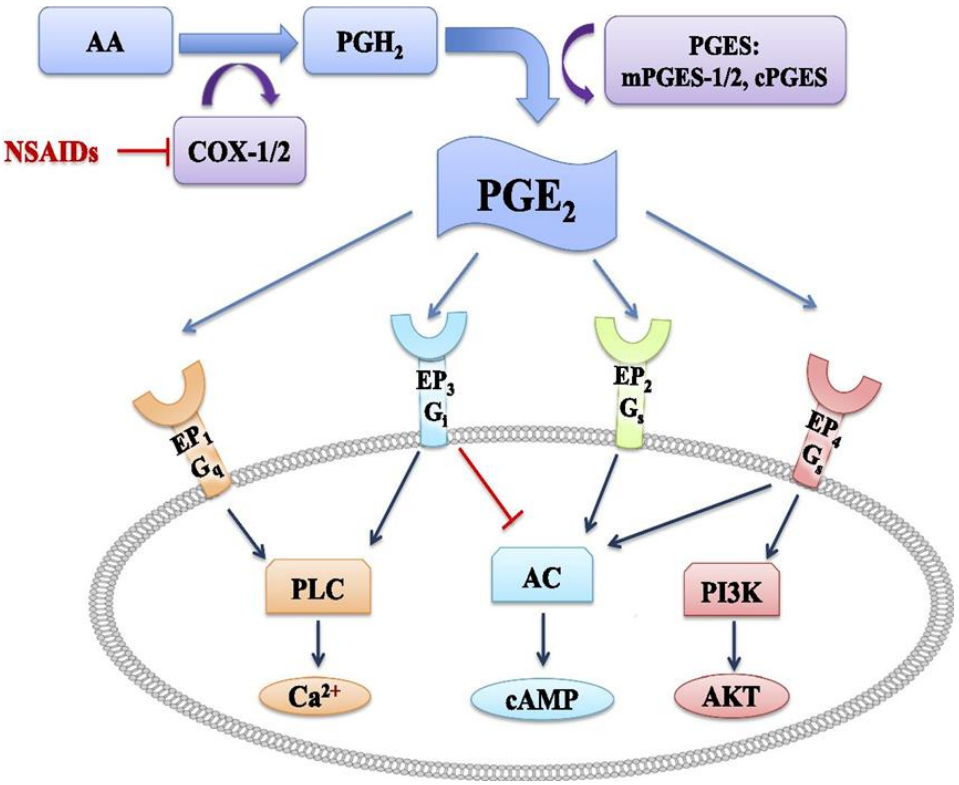


Figure 1.6. COX-2 pathway

COX-1 and COX-2 convert arachidonic acid into PGH_2. PGH_2 is converted to multiple prostanoids; PGI_2, TXA_2, PGD_2, PGE_2, and PGF_2. PGE_2 signals via four known receptors (EP_1 – EP_4). The inhibitors of COX-2 signaling pathway include nonsteroidal anti-inflammatory drugs (NSAIDs) and COX-2 selective inhibitors (NS-398). Prostaglandin EP2 and EP4 receptors are linked to the stimulation of the cyclic adenosine monophosphate (cAMP), which is responsible for major suppressive and regulatory functions of PGE2, signalling by activating adenylate cyclase. Adapted from (162).

1.6.1 COX-2

A variety of airway microenvironmental mediators have been shown to stimulate COX-2 and enhance COX-2-mediated prostanoid production in cells involved in airway inflammation. A study demonstrated that HASMCs under control conditions (untreated cells) did not express protein expression

of COX-2 (163). However, a cytokine mixture of TNFα, IL-1β and IFNγ induced protein and mRNA expression of COX-2 in HASMCs (163). Pang and Knox showed that HASMCs produced prostaglandins in response to stimulation with IL-1β and that COX-2 largely mediated this response (164). These findings suggest that HASMCs may be an important source of prostaglandins in human airways. Taken together, COX-2 may play an important role in the regulation of airway inflammation in asthma. IL-17A alone had no effect on COX-2 protein and mRNA expression in HASMCs (165).

CS causes airway inflammation which could result from enhancing the expression of COX-2 in the lung (166). A study conducted by our group showed that CSE induced COX-2 expression in human pulmonary artery smooth muscle cells (PASMCs) (167). CSE increased the expression of COX-2 in human tracheal smooth muscle cells (168). COX-2 expression and neutrophil infiltration were significantly greater in smokers and smokers with COPD compared with healthy subjects (169). These findings, to some extent, illustrate that some effects of CS in airway inflammation could be mediated by COX-2 expression. However, whether COX-2 mediate the effect of CSE on inflammatory response in HASMCs is unknown.

1.6.2 PGE_2

PGE_2, which is the main product of COX-2, is a potent inflammatory mediator (170). PGE_2 is the main prostanoid produced by HASMCs; other prostanoids are produced in much fewer amounts (156). PGE_2 signals via four known receptors (EP_1–EP_4), , Prostaglandin EP_2 and EP_4 receptors are linked to the stimulation of the cyclic adenosine monophosphate (cAMP),

which is responsible for major suppressive and regulatory functions of PGE_2, signalling by activating adenylate cyclase (171, 172).

There was an inverse relationship between PGE_2 levels and eosinophil counts in the sputum of asthmatics, which suggests that higher PGE_2 levels could protect against airway eosinophilia (173, 174). Inhaled PGE_2 reduced the change in methacholine airway reactivity and decreased the number of eosinophils in the airway after inhaled allergen challenge (175, 176), suggested that PGE_2 has some anti-inflammatory effects.

PGE_2 has diverse effects on the lung by modulating the function of many types of airway cells (177). PGE_2 has been shown to inhibit the proliferation of HASMCs (178). Exogenous PGE_2 inhibited LPS-induced production of TNFα and IL-6 in a concentration-dependent manner mediated via its EP_4 receptor in mouse and human monocytes (161).

Exposures to CS lead to airway inflammation, which could be mediated via the expression of COX-2 and PGE_2 synthesis.

Exogenous PGE_2 increased the non-T2 inflammatory cytokine IL-6 and IL-8 production in fibroblast cells (160). Further, studies demonstrated that exogenous PGE2 increased the production of IL-8 and IL-6 in HASMCs (158, 159). On the other hand, a study showed that PGE_2 reduced the production of Th2 cytokines IL-5 and IL-13 induced by IL-25, IL-33, TSLP and IL-2 in group 2 innate lymphoid cells (ILC2s) (24). However, the effect of PGE_2 on the T2 inflammatory response in HASMCs is unknown.

1.6.3 Cyclic AMP

Adenylyl cyclase (AC) is responsible for the synthesis of cAMP (179). The prostaglandin EP_2 and EP_4 receptors, which are G-protein coupled prostaglandin receptors (GPCPR), stimulate Gα protein ($G\alpha_s$), resulting in

an increase in adenylate cyclase (AC) activity and followed by an increase in cAMP levels (180, 181). Studies showed that PGE_2 elevate intracellular cAMP levels in HASMCs and can inhibit HASMCs contraction as well as other HASMCs functions such as proliferation and migration (182). The expression of numerous genes is modulated by changes in cAMP in different cell types. For instance, agents known to elevate cAMP inhibited IL-1β-induced production of IL-6 in human lung fibroblasts (183). 8-Bromo-cAMP, a cell-permeable cAMP analogue, decreased IL-1β-induced Eotaxin production and mRNA expression in HASMCs (184). Another study demonstrated that a number of cAMP stimulants such as forskolin (FSK), a direct adenylyl cyclase activator, enhanced IL-8 production in a concentration-dependent manner in HASMCs (185). The β_2-agonist, another cAMP stimulant, increased IL-8 production in HASMCs (185), suggesting a pro-inflammatory effect of cAMP in asthma. These studies suggest that cAMP can contribute to airway inflammation and could be a potential pathway that may mediate the CSE effect on inflammatory response in asthma.

1.7. Airway inflammation and glucocorticoids

1.7.1 Glucocorticoids in asthma

Glucocorticoids are anti-inflammatory medications used to treat and control patients with asthma by reducing airway inflammation, hyper-responsiveness (AHR) incidents and hospital visits (186). The use of inhalation as a route of administration significantly reduces the negative effects associated with glucocorticoid use (187, 188). Glucocorticoids function by decreasing the transcription of genes related to inflammatory cytokine production (189).

Glucocorticoids bind to glucocorticoid receptor α (GRα) which they then activate and translocate these receptors into the nucleus (189). Glucocorticoid receptor β (GRβ) is isoform that spliced form of GR and does not bind glucocorticoids but alternatively interacts with DNA and works as a potent inhibitor of the glucocorticoid effect (189). Glucocorticoids have a wide spectrum of gene targets associated with the anti-inflammatory process (189), studies have shown that responsiveness to glucocorticoids among patients with the asthma is not the same and that some group of patients may require high doses of glucocorticoid (190). Fluticasone propionate and budesonide are two types of glucocorticoids that have become the treatment of choice for chronic asthma (191).

While glucocorticoids exhibit anti-inflammatory effects, such as inhibiting the production of cytokines and chemokines in a different of cells including HASMCs cells, in some inflammatory conditions glucocorticoids not only lose their anti-inflammatory properties but can even enhance the production of certain inflammatory mediators and potentially enhance

HASMCs proliferation in the presence of some inflammatory stimuli (192). (Table1.3)

Table 1.3 Effects of glucocorticoids on airway smooth muscle cells in asthma

GC	Conditions	Paradoxical effect	References
Fluticasone propionate	IL-1β and TNFα plus epidermal growth factor (EGF)	HASMCs proliferation	(193)
	IL-1β plus EGF	HASMCs proliferation (potential)	(194)
	IL-1β plus EGF	HASMCs proliferation	(194)
Dexamethasone	Collagen-rich environment	HASMCs proliferation	(195, 196)
	TNFα and IFN-γ	Increase of proinflammatory mediators	(197)
	TNFα and IFN-γ	Pro-inflammatory	(198)
	TNFα and IFN-γ	Pro-inflammatory	(199)
Dexamethasone Budesonide		Neutrophil activity and survival	(200-202)

GC	Conditions	Paradoxical effect	References
Budesonide	TNFα	Increase of CCL20 levels in airway epithelium	(203)

1.7.2. T2 airway inflammation and glucocorticoids

T2 inflammation is known to be suppressed by glucocorticoids, and glucocorticoids have been the best available controller therapy for asthma (204). Although patients with T2 asthma are generally well responsive to glucocorticoids, there is a subgroup with this endotype of asthma have uncontrolled asthma (205-209). Glucocorticoids have been shown to reduce IL-4 and IL-5 mRNA expression in cells in bronchoalveolar lavage fluid (BALF) of asthmatic patients (210, 211). Dexamethasone also significantly inhibited IL-13 and IL-5 production in mice after ovalbumin stimulation and thereby reduced BAL and lung tissue eosinophilia (212). A study demonstrated that the production of IL-4 by human lymphocytes is markedly inhibited by small concentrations of hydrocortisone (213). TNFα induced production of Eotaxin in HASMCs was strongly inhibited by dexamethasone (214). Further, dexamethasone inhibited TNFα-induced RANTES production and mRNA expression in a time- and concentration-dependent manner in human lung epithelial cells (215). These findings suggest that T2 inflammatory cytokine production is sensitive to inhibition by glucocorticoids.

1.7.3. Non-T2 airway inflammation and glucocorticoids

Inhaled glucocorticoids remain the gold standard therapy for the majority of asthmatic patients to control their symptoms (216). Some asthmatics, especially those with a severe form of the disease, are

insensitive to glucocorticoids (216). Fluticasone and Dexamethasone minimally inhibited the production of the non-T2 cytokine GRO-α in BEAS-2B cells (97). Dexamethasone demonstrated potent anti-inflammatory effects, reducing BALF levels of IL-4 and IL-13, but not on levels of IL-8 (117). These results suggest that non-T2 airway inflammation is less responsive to glucocorticoids.

It has been shown that Th17 cytokines could also be responsible for glucocorticoid insensitivity in asthma (216). For instance, *in vitro* study showed that IL-17 and IL-22 production was significantly increased after stimulation with CD3/anti-CD28-coated microbeads (T cell-activating antibodies) in isolated CD4+ T cells from mice, and Th17 cytokines production was insensitive to dexamethasone treatment at any concentration tested (1 μM, 0.5 μM and 0.1 μM) (212). Further, IL-17 upregulates the glucocorticoid receptor-beta (GR-beta), the dominant-negative regulator of active GR-alpha, by forming inactive heterodimers, hence decreasing the glucocorticoid response. Therefore, the up-regulation of GR-beta by IL-17 induces glucocorticoid insensitivity in peripheral mononuclear cells (217). IL-17A is linked to neutrophilic inflammation by inducing the production of the neutrophil chemoattractant GRO-α from airway epithelial cells and airway smooth muscle cells, which may play a role in the neutrophilic inflammation of severe forms of asthma (95, 96). A study showed that IL-17A-induced production of IL-8 was inhibited by budesonide in a concentration-dependent manner in human bronchial epithelial cells (218). Interestingly, IL-17A pre-treatment significantly reduced the anti-inflammatory effect of budesonide on TNF-α-induced IL-8 production (218).

These results suggest that IL-17 production could drive severe forms of asthma by contributing to non-T2 inflammation, which is known to be insensitive to glucocorticoids (82).

1.7.3. Cigarette smoke and glucocorticoid insensitivity in asthma

Glucocorticoids are not as effective in asthmatic smokers as in non-smokers with asthma (219). Many clinical studies have reported that asthmatic smokers are insensitive to glucocorticoids (220-223). Asthmatic smokers who were treated with inhaled budesonide (high dose 1600ug and low dose 400ug daily) for 9 months showed no improvement in FEV1 compared with non-smokers. Another study showed that asthmatic smokers who received inhaled fluticasone (250ug twice daily) for 3 weeks had no improvement in peak expiratory flow compared with the asthmatic non-smoker group (221). A clinical trial was conducted on 86 asthmatics, 19 of them were current smokers, demonstrating that there was no significant difference in response to glucocorticoids, in term of airway function and plasma inflammatory markers, between the asthmatic smokers and patients with severe asthma; however, there are some of the smokers responded to a high dose of glucocorticoids (224). Clinical studies suggest that CS may cause glucocorticoid resistance in asthmatic patients.

An *in vitro* studies showed that CSE reduced the inhibitory effect of dexamethasone on TNFα-induced IL-8 in BEAS-2B immortalised human bronchial epithelial cells (117). CSE can also reduce the inhibition of LPS-induced IL-8 and TNFα by dexamethasone or budesonide in human alveolar macrophages by 30% (225). Exposure to CS, either *in vitro* or *in vivo*,

increases the production of pro-inflammatory cytokines such as IL-8 (112, 226) and TNFα (227). These cytokines have been implicated in the non-T2 inflammatory response and thereby may lead to glucocorticoid insensitivity (228). Some clinical and *in vitro* studies demonstrated that cigarette smoke or CSE could cause glucocorticoid insensitivity; still, there is a lack of evidence to show whether CSE can influence the inhibitory effect of glucocorticoids in HASMCs.

1.8. Summary

Asthma is a common respiratory disorder affecting around 339 million people worldwide, and airway inflammation is a hallmark pathological feature of asthma. In asthma, airway inflammation is commonly classified as T2, which responds well to glucocorticoids, or non-T2, which has a poor response to glucocorticoids, based on their cellular profiles. HASMCs express a wide range of T2 and non-T2 inflammatory cytokines and chemokines, which contribute to the inflammatory process in asthma. Evidence found in the literature showed that CSE and IL-17A contribute to non-T2 airway inflammation, which may lead to glucocorticoid insensitivity. Th2 cytokines play an integral role in the process of T2 airway inflammation in asthma and are responsible for eosinophilia, overproduction of IgE and airway hyperresponsiveness in asthma. Eosinophil chemokines such as Eotaxin, IP-10 and RANTES are playing a role in the T2 inflammatory response. However, the effect of CSE and IL-17A on T2 inflammatory response in HASMCs is largely unknown. COX-2 and its downstream product PGE_2 as well as oxidative stress may play an important role in the regulation of airway inflammation in asthma. There is evidence that CS induces COX-2 expression and PGE_2 production in different cell types, including HASMCs. However, whether COX-2/PGE_2 could mediate the effect of CSE on T2 inflammatory response in asthma remain to be explored.

1.9. Hypothesis and Aims

1.9.1 Hypothesis

We hypothesised that: 1) CSE and IL-17A can affect the glucocorticoid-sensitive T2 and glucocorticoid-insensitive non-T2 inflammatory response, contributing to glucocorticoid insensitivity in HASMCs, 2) CSE may affect the production of T2 inflammatory cytokines through COX-2 and PGE_2 pathway via oxidative stress, 3) CSE can influence the anti-inflammatory effect of glucocorticoid on IL-17A-induce production of IL-8 in HASMCs.

1.9.2 Aims

- To assess the effect of CSE and Th17 cytokine IL-17A on the production of T2 and non-T2 inflammatory cytokines and to assess the impact of CSE on the effect of IL-17A in HASMCs.
- To examine the effect of CSE on the mRNA expression of T2 inflammatory cytokines in HASMCs.
- To explore the role of oxidative stress, COX-2, PGE2 and cAMP pathway in mediating the CSE effect on the production of T2 inflammatory cytokines in HASMCs
- To investigate the influence of CSE on the inhibition by glucocorticoids on IL-17A-induced production of IL-8.

Chapter 2. Methods and Materials

2.1 Introduction

In this Chapter, the general methods used in this PhD thesis are described. All materials, reagents, kits, buffer and media recipes are listed in the appendix.

2.2 Cigarette smoke extract preparation

3R4F research-grade cigarettes (Appendix 7.2) were connected via a rubber tube to the glass tube of an 80 ml vacuum filtration flask containing 20 ml of Dulbecco's Modified Eagle's Medium- (DMEM-, Appendix 7.1). The pressure in the vacuum pump attached to the 80 ml glass bottle was adjusted to 0.2 bar to obtain a steady burning rate of the cigarettes. Once the cigarette was lit, the pump removed air from the 80 ml glass bottle, and the cigarette smoke was drawn through the media and the soluble components of smoke were extracted. Two cigarettes were burnt for every 20 ml batch of CSE. The resulting CSE was filtered using a 0.22 um filter, and its absorbance was measured at 320 nm (1.5=100%) using the FLUO star Omega microplate reader (BMG LABTECH, UK) to achieve the required concentration level and strength of CSE to be used for experiments.

2.3 Drug preparation

2.3.1 (-)-Nicotine

Nicotine was purchased from Sigma-Aldrich and prepared as instructed by the manufacturer (Appendix, section 7.3). To make the original stock of 2 mM, nicotine was diluted in ethanol, and then diluted in a cell

culture medium with a dilution factor (1:2). Nicotine was used at a concentration of 500 µM based on previous work (229).

2.3.2 MG 624

The α7 nicotinic acetylcholine receptor (nAChR) has been identified as the most nAChR expressed in HASMCs (230), so we have used the selective antagonist for α7 neuronal nicotinic receptor MG 624 in this study. MG 624 was purchased from Sigma-Aldrich and prepared as per the instructions of the manufacturer (Appendix, section 7.3). The MG 624 was dissolved in Dimethyl sulfoxide (DMSO) to achieve an original stock concentration of 0.05 M, then diluted in DMSO with a dilution factor of (1:10). A concentration of 1 µM was used to treat the cells based on previous research (231). Drug vehicle solution, DMSO, was added to 1 ml of medium (Dilution factor 1:500) in control wells to obtain a final concentration of 0.2% of DMSO.

2.3.3 L-Glutathione (GSH)

GSH is an endogenous antioxidant which plays an important role in reducing reactive oxygen species. GSH was obtained from Sigma-Aldrich and prepared as per the instructions provided by the manufacturer (Appendix, section 7.3). GSH was dissolved in deionized water to achieve an original stock of 0.05 M. GSH was dissolved in deionized water to a final concentration of 100 µM according to similar study in the literature (112). Deionised water was also added to the control group and maintained the final concertation of deionised water to be equal to 0.2%.

2.3.4 NS-398

NS-398 is considered a nonsteroidal anti-inflammatory drug (NSAID) and is a selective inhibitor of COX-2 with an IC_{50} value of 1.77 µM. NS-398 was obtained from Cayman Chemical and prepared as instructed by the manufacturer (Appendix, section 7.3). NS-398 was reconstituted in DMSO (Sigma-Aldrich) to achieve an original stock concentration of 0.05 M. NS-398 concentration of 10 µM was used to treat the cells based on previous research conducted by our group (232). DMSO was added to 1 ml of medium (Dilution factor 1:500) in control wells to obtain a final concentration of 0.2% of DMSO.

2.3.5 Prostaglandin E_2 (PGE_2)

PGE_2 is produced from arachidonic acid by the cyclooxygenase 2 (COX-2) pathway. PGE_2 was obtained from Cayman Chemical and prepared according to the manufacturer's instructions (Appendix, section 7.3). The drug was dissolved in DMSO to obtain an original stock of 0.05 M. After that, a serial dilution (Dilution factor of 1:10) in DMSO was made to obtain a final PGE_2 concentration of 10 µM based on previous research (24). Furthermore, DMSO was also added to the control cells to obtain the final concertation of 0.2%.

2.3.6 PF 04418948

PF 04418948 is a potent and selective inhibitor for prostaglandin E receptor 2 with an IC_{50} value of 16 nM. The drug was purchased from tocris bioscience and prepared as per the manufacturer’s instructions (Appendix, section 7.3). The was dissolved in DMSO to achieve an original stock of 0.05 M. After that, a serial dilution in DMSO (Dilution factor of 1:10) to obtain the final concentration of 10 µM according to previous study (233). The final

concentration of DMSO = 0.2% was added to the control cells to be consistent with the experimental cells.

2.3.7 L-161,982

L-161,982 is a prostaglandin E receptor 4 antagonist. The L-161,982 was purchased from tocris bioscience and prepared as per the manufacturer's instructions (Appendix, section 7.3). The drug was dissolved in DMSO to achieve an original stock of 0.05 M. Then, a serial dilution with a dilution factor of (1:10) was made in DMSO to get the final concentration of 1 μM (The highest concentration used and did not cause cytotoxicity). DMSO was added to 1 ml of medium (Dilution factor 1:500) in control wells to obtain a final concentration of 0.2% of DMSO.

2.3.8 8-Bromo-cAMP

8-bromo-Cyclic AMP (8-bromo-cAMP) is a cell-permeable analogue of cAMP which activates cyclic-AMP-dependent protein kinase A. The 8-Bromo-cAMP was purchased from Tocris Bioscience and prepared based on the instructions of the manufacturer (Appendix, section 7.3). The compound was dissolved in DMSO to make the (original stock) of 0.05 M, serial dilution with a dilution factor of (1:10) was made in DMSO and a concentration of 100 μM was used to treat the cells based on our group previous work (185). The drug vehicle solution DMSO was added to 1 ml of medium (Dilution factor 1:500) in control wells to obtain a final concentration of 0.2% of DMSO.

2.3.9 Salbutamol

Salbutamol, a β_2 adrenoceptor agonist, is a short-acting bronchodilator used in the treatment of asthma. Salbutamol was purchased

from Sigma-Aldrich and prepared as per the manufacturer's instructions (Appendix, section 7.3). Salbutamol was dissolved in methanol to achieve an original stock concentration of 0.05 M, then a serial dilution with (Dilution factor of 1:10) was made in methanol and a concentration of 10 μM was used to treat the cells (185). Methanol was added to 1 ml of medium (Dilution factor 1:500) in control wells to obtain a final concentration of 0.2%.

2.3.10 Fluticasone

Fluticasone propionate is an anti-inflammatory drug widely used to treat asthma patients. Fluticasone was purchased from Sigma-Aldrich and was dissolved in DMSO to achieve an original stock concentration of 0.5 M as per the instructions provided by the manufacturer (Appendix, section 7.3). In the experiments, six concentrations of fluticasone were used, ranging from 10^{-11} to 10^{-6} M. In addition, the drug vehicle solution DMSO was added to 1 ml of medium in control wells to obtain a final concentration of 0.2%.

2.4 Cell culture

The use of lung tissue samples has been approved by National Research Ethics Service (NRES) Committee East Midlands-Nottingham 1. Human airway smooth muscle cells (HASMCs) were provided by Professor Dominick Shaw (Division of Respiratory Medicine, University of Nottingham). The cells were obtained from healthy donors and were cultured in Dulbecco's Modified Eagle's Medium (DMEM)+ (see section 2.1.4). The cells were incubated in a humidified incubator at 37°C with 21% O_2 and 5% CO_2. The medium was changed every 48h. To confirm the cells were not contaminated

with mycoplasma, a mycoplasma test (Myco-Alert mycoplasma detection kit from Lonza) was conducted.

Table 2.1 List of human airway smooth muscle cells

Cell ID	Age	Gender	Ethnicity	Smoking	Health status	Medication
AZAC05	26.00	F	White	Never smoke	Healthy	None
AZAC07	39.00	F	Asian	Never smoke	Healthy	None
MMP-1H10	46.00	M	White	Never smoke	Healthy	None

2.4.1 Cell freezing

HASMCs were cultured in T-225 flasks from passage 5 to passage 6. The medium for cells was changed every 48h. When the cells reached confluence, an aspirator was used to remove the medium and the flask was washed with 10 ml of PBS. 10 ml of Trypsin-EDTA solution (see section 2.1.5) was added to the cells and incubated for 3 minutes at 37°C. The cells were detached from the flask and observed under the microscope. Trypsin was neutralised by adding 10 ml DMEM+ into the flask and the contents were centrifuged for 5 minutes at 1100 rpm. The supernatant was removed by the aspirator and 10 ml of fresh DMEM+ was added to the cells. After counting (see section 2.4.2), the cells were stored in cryogenic tubes at a density of 1×10^6 cells per ml of freezing solution (90%FBS + 10%DMSO). These tubes were then placed in a Mr Frosty Freezing container (Thermo Fisher Scientific) containing 100% isopropyl alcohol. The cells in Mr Frosty were stored at -80°C for 24h to maintain a steady cooling rate of -1°C/minute. The cryotubes were then transferred to liquid nitrogen for long-term storage.

2.4.2 Cell counting

The counting of the cells was done through a glass haemocytometer and coverslip. The cell suspension was mixed in a 1:1 ratio with Trypan blue to distinguish between live and dead cells. All the four haemocytometer squares were observed under the microscope and the number of cells in each quarter was counted. Cell count was calculated by multiplying the average number of cells per square with dilution factor and 10^4

2.4.3 Cell treatment

Cells were seeded at a density of 41.666 cells/well of a 24-well plate. Before each experiment, the cells were growth-arrested with DMEM− for 24h. Confluent and growth-arrested HASMCs were pre-treated with and without the α7nAChR antagonist MG624 (1 μM), the oxidative stress inhibitor GSH (100 μM), the COX-2 inhibitor NS-398 (10 μM), the prostaglandin EP_2 receptor antagonist PF 04418948 (10 μM) and the prostaglandin EP_4 receptor antagonist L-161,982 (1 μM) for 1h prior to incubation with or without CSE (3.5%), IL-17A (10 ng/ml), CSE+IL-17A, nicotine (500 μM), PGE_2 (10 μM) and 8-Bromo-cAMP (100 μM) for 24h. In order to assess the influence of CSE on the inhibitory effect of fluticasone on the IL-17A-induced production of neutrophilic chemokines, the cells were pre-treated with or without CSE (3.5%) for 24, and then cells were treated with or without fluticasone (10^{-11}-10^{-6} M) for 1h prior to incubation with CSE (3.5%) and/or IL-17A (10 ng/ml) for 24h. The collected medium was stored in a -20ºC freezer until required for ELISA.

2.4.4 Cell lysate preparation

After the cells were treated, the plate was washed with PBS after the medium was collected from the plates, and 150 µl of complete radioimmunoprecipitation assay (RIPA) buffer (Appendix 7.4) was added to the cells. Cell scrapers were used to scrape the cell monolayer, and the cell lysates were collected from each well into 1.5 ml Eppendorf tubes to be stored at −80°C until further use. The concentration of the protein was measured through a bicinchoninic acid (BCA) assay kit (Appendix 7.5).

2.5. Bicinchoninic acid protein assay

Protein concentration in lysates was quantified using the BCA assay kit (Thermo Fisher Scientific). Bovine serum albumin (BSA) solution (0–2000 µg/ml) was made as per the guidelines of the manufacturer. The BSA standards and protein samples (5 µl) were transferred in duplicates to a 96-well plate. The BCA solutions A and B were mixed in the ratio of 1:50 and 95 µl of this solution was added to each well containing either BSA standards or protein samples. The plate was incubated for 30 minutes at room temperature. After that, the absorbance was measured by a FLUO star Omega microplate reader (BMG LABTECH) at 562 nm. The protein concentration of samples in µg/ml were calculated from absorbance measures using the Omega analysis software.

2.6 Enzyme-linked immunosorbent assay (ELISA)

The concentration of IL8 was measured in the collected supernatants using DuoSet ELISA kits (Appendix, section 7.5). These kits were used as per the guidelines of the manufacturer.

2.6.1 Assay procedure

For the ELISA assays, a high binding 96-well plate was used. Capture antibodies were diluted to working concentrations with PBS, and 100 µl was added to all the wells. The plate was then incubated overnight at room temperature. Following this, the plate was washed thrice using 400 µl/well of wash buffer. 300 µl/well of blocking buffer was used to block the plate prior to incubation for 1h at room temperature. After washing the plate again, 100 µl of diluted standard and samples was added to the wells in duplicates. After 2h of incubation on the shaker (300–400 rpm), the plate was rewashed and then 100 µl of diluted biotinylated goat anti-human detection antibody was transferred into each well of the plate and incubated for 2h. The plate was washed again, and 100 µl of streptavidin horseradish peroxidase (HRP) was added to all the wells of the plate and incubated for 20 minutes. The final step involved washing the plate again and then adding 100 µl of substrate solution followed by 20 minutes incubation in a dark place. 50 µl of stop solution (sulphuric acid) per well was added to end the enzymatic reaction. The optical density for each well was measured through the FLUO star Omega microplate (BMG LABTECH) at 450 nm, and Omega software was used to analyse the data. The normalisation of the concentrations to the total amount of protein was done by dividing the average of the duplicated cytokine and chemokine concentrations (pg/ml) by the total amount of protein content (the protein concentrations (mg/ml) were multiplied by 0.15 ml ‘’total volume of protein lysate’’) to achieve pg/mg protein.

2.7 Bio-Plex ProTM Human Cytokine Assays

The Bio-plex ProTM Human Cytokine Assays kit (Appendix, section 7.5) was used to determine the concentration of T2 and non-T2 inflammatory cytokines in the cell supernatants. The assays were conducted according to the manufacturer's instructions. The 96-well ELISA plate was used and 50 µl of the capture antibody coated beads were transferred to each well. The plate was manually washed twice, each time with 100 µl in each well using Bio-Plex wash buffer. After that, 50 µl of standards with a dilution factor of 1:4, blank, samples and controls were transferred to the assay plate. Then, the plate was sealed and incubated on a shaker at 850 rpm for 30 min at room temperature. After that, the plate was washed thrice with 100 µl of the wash buffer, then 25 µl of the detection antibodies were added to each well and incubated on shaker at 850 rpm for 30 min at room temperature. Then, the plate was washed again and then 50 µl of streptavidin-PE (SA-PE) transferred to each well and plate was sealed and incubated on a shaker at 850 rpm for 10 min at room temperature. After incubation, the plate was washed and then to resuspend beads for plate reading, 125 µl assay buffer was transferred to each well on a shaker at 850 rpm for 30 seconds at room temperature. Finally, we slowly removed and discarded the sealing tape before placing the plate on the reader. Then, the Bio-PlexTM 200 System (Bio-Rad) was used to read the plate, and the Bio-Plex Manager software version 6.1 (Bio-Rad) was used to calculate the concentration of different samples. Data were normalised to the total amount of protein, the average of the duplicated samples (pg/ml) divided by the total volume of medium (ml)

then multiplied by the total amount of protein lysate (mg/ml) and presented as pg/mg protein.

2.8 PGE_2 ELISA

The concentration of PGE_2 in the cell culture supernatant was measured by using the PGE_2 ELISA kit (Appendix, section 7.5). This competitive assay was 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 presented in Table 2.2.

Table 2.2. Cross-reactivity of 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). Because

the PGE_2 concentration varies resent while the concentration of PGE_2 tracer remains constant, the amount of PGE_2 tracer that is able to bind with PGE_2 monoclonal antibody will be inversely proportional to the amount of PGE_2 in the sample. This antibody-PGE_2 complex after that binds to goat polyclonal anti-mouse IgG that is pre-coated to the wells. After washing the plat, to remove any unbound reagents, Ellman's reagent (containing substrate to AChE) is added to the well. The product of Ellman's reagent reaction has a distinct yellow colour. The distinct yellow colour intensity is proportional to the amount of PGE_2 tracer that is bound to the well but is inversely proportional to the amount of free PGE_2 present in the sample.

2.8.1 Method

PGE_2 ELISA kit (Appendix, section 7.5) was used to measure the concentration of PGE_2 in the cell supernatants according to the manufacturer's instructions. To prepare the PGE_2 ELISA standard, the standard was diluted in DMEM- using two-fold serial dilutions to generate an eight points standard curve ranging from 1000 pg/ml to 7.8 pg/ml. After that, 50 µl of standards, samples, blanks, AChE tracer, and PGE_2 monoclonal antibody were transferred to the wells. The plate was then covered with plastic film and incubated at 4°C for 18h. No antibody pre-coating or blocking was needed, as the pate was pre-coated with anti-mouse IgG and blocked with a proprietary formulation of proteins. After that, the plate was washed five times using washing buffer, then 200 µl of Ellman's reagent was added to each well and the plate was covered with a plastic film, to enable the palate to develop in the dark, and was incubated at room temperature for 90 minutes. A FLUOstar Omega microplate reader (BMG labBtech) was used

to measure the optical density at 420 nm. PGE_2 concentrations were expressed as pg/mg of protein after being normalised as described in section 2.8.1.

2.9 Real-time reverse transcription-quantitative polymerase chain reaction analysis

2.9.1 Principle

Real-time reverse transcription-quantitative polymerase chain reaction (RT- qPCR) analysis is widely used to detect gene expression within the cell or tissue by determining the level of messenger RNA (mRNA) which is translated to protein. Isolate total RNA from the sample using NucleoSpin RNA II kit is the first step of RT-qPCR, and this step should be performed on ice due to the natural instability of RNA. Then, the second step is to convert the mRNA into complementary DNA (cDNA) through reverse transcription (RT). The final step is the PCR itself which involves the amplification of DNA using the enzyme DNA polymerase. The cycle threshold (CT) is defined as the number of cycles needed for the fluorescent signal to cross the threshold. The increase in fluorescent signal is proportional to the increase inthe amount of cDNA in the sample.

2.9.2 Total RNA isolation

Confluent cells were treated as required in a 12-well plate. At the end of the experiment, the cell culture medium was aspirated, and cells were washed with PBS before the total RNA was extracted using the NucleoSpin RNA II Kit (Appendix, 7.5) according to the instructions of the supplier. All columns and buffers that were used to isolate total RNA were provided with

the kit. After the wells being washed with PBS, the cells were lysed with 350 µl lysis buffer RA1 (with β-mercaptoethanol (10 µl/ml)). Then, the lysed sample was filtered through NucleoSpin filters (pink ring) in a 2 ml collection tube by centrifuging the lysate at 11,000 rpm for 1 min to reduce viscosity and clear the lysate. To adjust the RNA binding condition, the filter was discarded and 350 µl of 70% ethanol was added directly to the collection tube and mixed thoroughly. The lysate was then loaded to the NucleoSpin RNA Column (light blue ring) and centrifuged at 11,000 rpm for 1 min. The column was placed in a new collection tube (2 ml) after the RNA was bound to the silica membrane of the column. Next, 350 µl membrane desalting buffer (MDB) was loaded in the column and then centrifuged at 11,000 rpm for 1 min to remove contaminating salts. Next, 95 µl deoxyribonuclease (DNase) reaction mixture (10 µl reconstituted recombinant (rDNase + 90 µl reaction buffer for rDNase) per sample was added to each column to digest any contaminating DNA on the membrane. The column was then incubated at room temperature for 15 min, washed with 200 µl RAW2 buffer and then centrifuged at 11,000rpm for 1 min. Then, the silica membrane was washed and centrifuged twice with 600 µl and 250 µl of RA3 buffer, respectively. Finally, the RNA was collected by adding 50 µl RNase-free H_2O, and centrifuged at 11,000 rpm for 1 min. The isolated RNA was either used immediately or stored at -80°C until required, as RNA is very unstable.

2.9.3 RNA quantification

A nanodrop spectrophotometer (Thermo Fisher Scientific) was used to measure the concentration and quality of the RNA. For this, 2 µl of each RNA sample was loaded onto the lower measurement pedestal. The ratio

of sample absorbance at 260 and 280 nm was used. A ratio of ~2.0 is accepted as “pure” for RNA. After RNA quantification, 2 µg total RNA of each sample was used for the RT step.

2.9.4 Reverse transcription (RT)

To convert RNA into cDNA, we used a SuperScript™ IV Reverse Transcriptase kit (Appendix, 7.5). The volume of RT mixture of components per reaction was prepared as per instructed by the supplier as the following, a mixture of 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 2.8.1) was mixed and centrifuged for 30 seconds, and then heated for 5 minutes at 65°C. The reaction mixture was made up to 13 µl with nuclease-free H2O (Table 2.3). After that, the RNA was reverse transcribed in a total volume of 20 µl including 5x SSIV Buffer, 100 mM DTT, RNase inhibitor, and SuperScript™ IV Reverse Transcriptase (200 U/µL) (Table 2.4). The reaction was heated at 55°C for 10 minutes and at 80°C for 10 minutes. Finally, cDNA was then stored at -20°C until needed.

Table 2.3. List of a SuperScript™ IV Reverse Transcriptase primers

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 2.4. 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

2.9.5 Specificity of the primers

The specificity of each primer was determine using a serial dilution of cDNA from untreated cells (1:10, 1:100, 1:1000, and 1:10000). After running RT-qPCR, dissociation curves were created. Primers were regarded as specific when dissociation curves had a single peak.

2.9.6 qPCR

Reversed transcribed cDNA was used for qPCR process. A mixture of 20 µl per reaction was prepared as the following, 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. Negative controls (nuclease-free water) were transferred in duplicate on the 96-well qPCR plate. The mixture volume was transferred to each well in the 96-well qPCR plate (except the negative control) in duplicate.

The qPCR reaction was performed in three steps: denaturation, annealing, and extension at various temperatures. The denaturation step was set at 95°C for 3 minutes in order to separate double-stranded DNA into single-stranded DNA for amplification. Then, to allow the primers to bind to the template by lowering the temperature to 65°C for 30 seconds in the annealing step. In the last step, which is the extension step, the temperature was set at 65°C for 30 seconds where the new strand of DNA was made by the DNA polymerase enzyme. Amplification of the selected target sequence was achieved by repeating these three steps for 40 cycles using a

Stratagene Mx3000P qPCR System (Agilent Technologies, USA) (Table 2.5).

Table 2.5. Summary of thermal profile of the qPCR reaction

Step	Temperature	Duration	Cycle
Denaturation	95°C	3 min	40
Annealing	65°C	15 seconds	
Extension	65°C	30 seconds	

The results of qPCR were analysed using MxPro qPCR software (Agilent Technologies) after the C_T values were obtained from both controls and samples. In current the β2M was used as a reference gene for all of the experiments as it is stable between samples and most routinely used as a housekeeping gene in our lab. Firstly, after we obtained the average of C_T values for the tested samples and housekeeping genes, the differences (ΔC_T = C_T tested-C_T β2M) were calculated. Then, the double delta C_T values ($\Delta\Delta C_T$ (ΔC_T treated-ΔC_T control) were calculated. Finally, 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 2.6. List of the target gene and their forward and reverse primer sequences

Target gene	Primer sequences
IL-4	FW: GAG AAC ATT GTC CCC CAG TG RV: AAG CTG ATC TGG GGC TCC TT
IL-5	FW: CTC TTG GAG CTG CCT ACG TG RV: TCA GTG CAC AGT TGG TGA TTT
IL-13	FW: GTG GCC ATG GGG GAT AAG G RV: CAT TGC AGA GCG GAG CCT TC
Eotaxin	FW: TCC CCA GAA AGC TGT GAT CTT CAA RV: GCA ACA CTC AGG CTC TGG TT
IP-10	FW: CCA CGT GTT GAG ATC ATT GCT RV: TGC ATC GAT TTT GCT CCC CT
RANTES	FW: GGC CAA TGC TTG GTT GCT ATT RV: AGC TCA GGC TGG CCC TTT AT
β2M	FW: AAG GAC TGG TCT TTC TAT CTC RV: GAT CCC ACT TAA CTA TCT TGG
COX-2	FW: AAG CAG GCT AAT ACT GAT AGG RV: TGT TGA AAA GTA GTT CTG GG

2.10 Western blotting

2.10.1 Principle

Western blotting is an essential technique for detecting a protein of interest across a complicated mixture of proteins extracted from cells (234). Because each protein has a molecular weight, proteins are separated using polyacrylamide gel electrophoresis (PAGE) depending on their molecular weight. An electric current is used to transfer the isolated proteins on a nitrocellulose blotting membrane (Amersham Biosciences). Following that, the membrane is blocked and treated with a primary antibody specific to the protein of interest and and horseradish peroxidase (HRP) conjugated secondary antibody. Finally, bands specific to the protein of interest are detected using an enhanced chemiluminescence (ECL) substrate for detecting HRP enzyme activity.

2.10.2 Method

2.10.2.1 Running step

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

2.10.2.2 Transferring step

The gel was taken out of the electrophoresis tank and placed in 1x transfer buffer (Appendix, section 7.4) after the running step was finished in order to transfer the protein to the nitrocellulose blotting membrane (Appendix, section 7.7).Then, a sandwich of sponge, filter, paper, gel, 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 before being placed in the electrophoresis tank. After that, the electrophoresis tank was filled with 1x transfer buffer and run at 10 constant voltages for 90 min.

2.10.2.3 Blocking and antibody incubation

After the transfer step was completed, the membrane was placed in ponceau red stain (Appendix, section 7.4) to enable visualisation of the protein bands. The membrane was then washed three times with 1x tris buffered saline with tween 20 (TBS-T) buffer (Appendix, section 7.4). After that, the membrane was incubated for 1 hour at room temperature with block buffer (Appendix, section 7.4) to reduce non-specific binding in the membrane. The membrane was then incubated with the primary antibody at the concentration 1 in 1000 in blocking buffer overnight in a room temperature of 4°C. The primary antibodies mouse anti-human COX-2 monoclonal antibody (1:1000), and rabbit anti-human β-Actin antibody (1:50000) were used in our study (Appendix, section 7.6). The following day, the membrane was washed three times with 1x TBS-T for 10 minutes each, and then it was incubated with the secondary antibody in the blocking buffer at room temperature for one hour. The polyclonal goat anti-mouse immunoglobulins/HRP antibody (1:2500) and polyclonal goat anti-rabbit immunoglobins/HRP antibody (1:2500) were used in this study as secondary antibodies (Appendix, section 7.6).

2.10.2.4 Detection step

Following incubation with the secondary antibody, the membrane was washed three times with 1x TBS-T for 10 minutes each time. The membrane was then incubated for 5 minutes with a mixture of ECL solutions (1:1) (Appendix, section 7.4). ECL enable the visualization and the detection of the protein bands on the Licor C-DiGit scanner. The protein band density on the membrane was quantified using Image Studio software. 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.

2.11. Cell viability assay

The cytotoxicity of different treatments on HASMCs was measured through 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were cultured in 24-well plates for 24h with various concentrations of CSE, and with CSE or IL-17A with and without different drugs mentioned in the cell treatment section 2.4.3. 500 µl of MTT solution (dissolved in DMEM-) at a concentration of 1 mg/ml was added to each well after removal of the DMEM− growth media. The plate was incubated for 30 minutes in the incubator (37°C) before removing the MTT solution. The plate was allowed to dry at room temperature for 6h. 500 µl of DMSO was then added to the wells and the solutions were transferred to a 96-well plate. The absorbance was measured through a FLUO star Omega microplate reader (BMG LABTECH) at 550 nm to assess the metabolic activity of living cells. Cells viability data were shown as fold change over control.

2.12. Statistical analysis and IC_{50} calculation

Statistical analysis was conducted using GraphPad Prism 9. Data were expressed as mean ± standard error of the mean (SEM) from experiments conducted in the specified number of cell lines unless otherwise stated. An unpaired Student's t test was carried out to compare two data sets. In our data analysis we cannot assess the normality due to the small number of samples. As regards the normality of group data, the one-way ANOVA can tolerate data that is non-normal with only a small effect on the Type I error rate according to GraphPad prism software statistics guide. In this study, a one-way analysis of variance (ANOVA) followed by a t-test was performed to determine whether the differences between the control and the treated cells were significant. P values less than 0.05 were considered statistically significant. Two-way ANOVA was used to compare the inhibitory effect of fluticasone on cytokine and chemokine production by CSE, IL-17A or CSE + IL-17A. Holm-Sidak multiple comparisons test was used to further compare the difference in the inhibitory effect of fluticasone at the same concentration between different groups. To calculate IC50, the results from different experiments were analysed using GraphPad Prism software. The drug concentrations were converted to log10 scales and plotted on X-axis, while the normalised response of cytokine and chemokine production (As 100%) was plotted on Y-axis. Non-linear regression analysis in the software was used to generate the curve and IC50 values.

Chapter 3. Effect of cigarette smoke extract and IL-17A on production and gene expression of T2 and non-T2 inflammatory cytokines in human airway smooth muscle cells

3.1 Introduction

Airway inflammation in asthma can be classified into two main endotypes, which are Type2 (T2) and non-Type2 (non-T2). Around 50% of patients with asthma have a T2 airway inflammation, whereas the remaining patients have a non-T2 airway inflammation or mixed eosinophil and neutrophilic inflammation, which is commonly found in severe asthma (19).

Human airway smooth muscle cells (HASMCs) by their ability to synthesise and produce a wide range of inflammatory mediators, such as cytokines, chemokines, and growth factors [81, 82]. HASMCs shown to produce some of T2 and non-T2 inflammatory cytokines such as IL-8, Eotaxin, and RANTES (68, 111, 235), and also have also been shown to produce Th2 cytokines such as IL-5 and IL-13 (32, 103). In this chapter we have assessed HASMCs response to CSE and IL-17A stimulation with wide range of inflammatory cytokines and chemokines that contribute to airway inflammation and remodelling in asthma. However, this study mainly focused on T2 and non-T2 inflammatory responses.

We know from the available literature that the increase of non-T2 inflammation in asthmatic patients positively correlates with the increase in IL-8 levels [1]. CSE and IL-17A contribute to non-T2 airway inflammation, by inducing neutrophil chemoattractant such as IL-8 (83, 236). IL17A is increased in the non-T2 endotype, and that high levels of IL17A are associated with non-T2 inflammatory response (20, 83). Studies showed that IL-17A induced the production of the non-T2 inflammatory chemokines, such as IL-8 and GROα, in airway structural cells, including HASMCs (95,

96). Cigarette smoke (CS) has also been linked to non-T2 airway inflammation in asthmatic smokers compared with non-smokers (228)

T2 inflammation is characterised by the increase in Th2 cytokines (IL-4, IL-5, and IL-13) (204) and eosinophil chemokines (Eotaxin, IP-10 and RANTES), which are associated with eosinophilic airway inflammation (65, 237, 238). Activation of eosinophils and Th2 cells amplifies the T2 inflammatory response characteristic for allergy and asthma (24). The effect of CS on the T2 inflammatory response is unclear, and studies showed a different response to CS in different experimental models and cell types. A study found that IL-17A induced production of the eosinophil chemoattractant Eotaxin in HASMCs (110), suggesting that IL-17A may also contribute to T2 inflammation. However, the effect of CSE and IL-17A on T2 inflammatory response in HASMCs is largely unknown. Therefore, in this chapter, we aim to assess the effect of CSE and IL-17A on the production Th2 cytokines and eosinophil chemokines (T2 inflammatory cytokines) and non-T2 inflammatory cytokine and whether CSE impacts HASMC response to IL-17A.

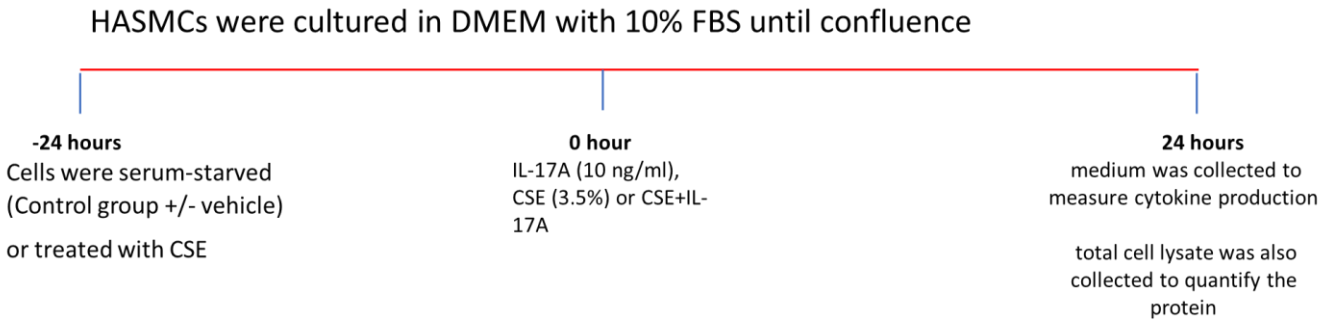
3.2 Aims

- To explore the effect of CSE and IL-17A on the production of wide range of inflammatory cytokine including T2 and non-T2 cytokines and the impact of CSE on IL-17A-induced production of T2 and non-T2 inflammatory cytokines in HASMCs.
- To investigate the individual effects of CSE and IL-17A on the mRNA expression of T2 inflammatory cytokines and whether CSE affects HASMCs response to IL-17A stimulation in HASMCs using real-time PCR.

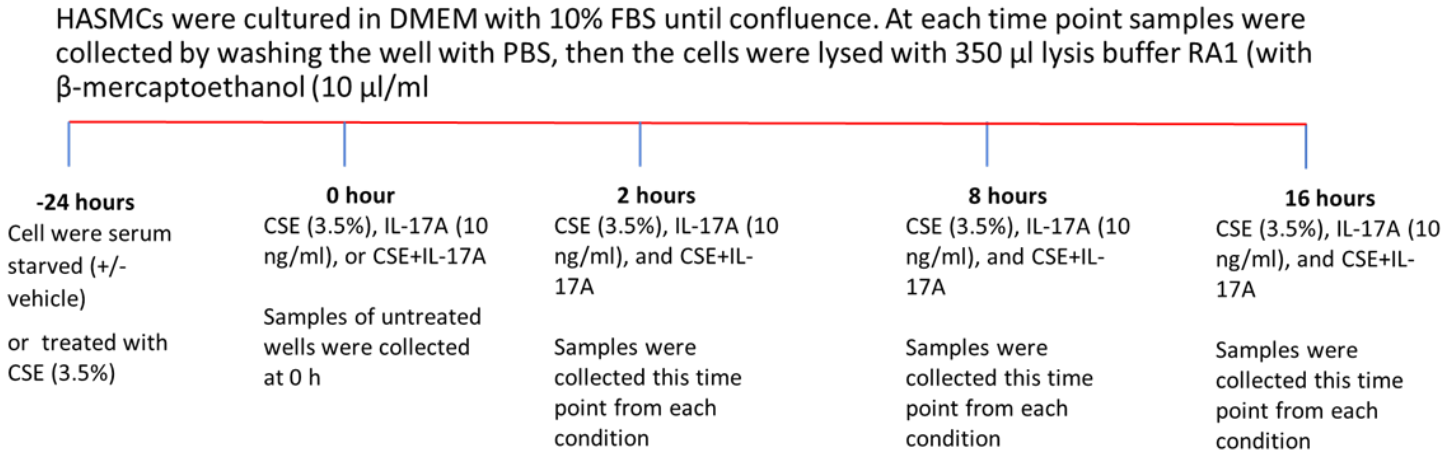
3.3 Methods

CSE was prepared from the smoke of two research cigarettes bubbled into 20 ml of cell culture medium. Confluent and growth-arrested HASMCs were pre-treated with and without CSE (3.5%) for 24h and after that, cells were treated with CSE, IL-17A (10 ng/ml) or CSE+IL-17A for 24h. Cytokines and chemokines were measured in the collected medium using the Bio-Plex Pro-Human Cytokine assay (3.3.1). RT-qPCR analysis was used to measure the mRNA expression of T2 inflammatory cytokines, and β2M was used as a reference gene for all experiments (3.3.2).

3.3.1 Timeline for experiments



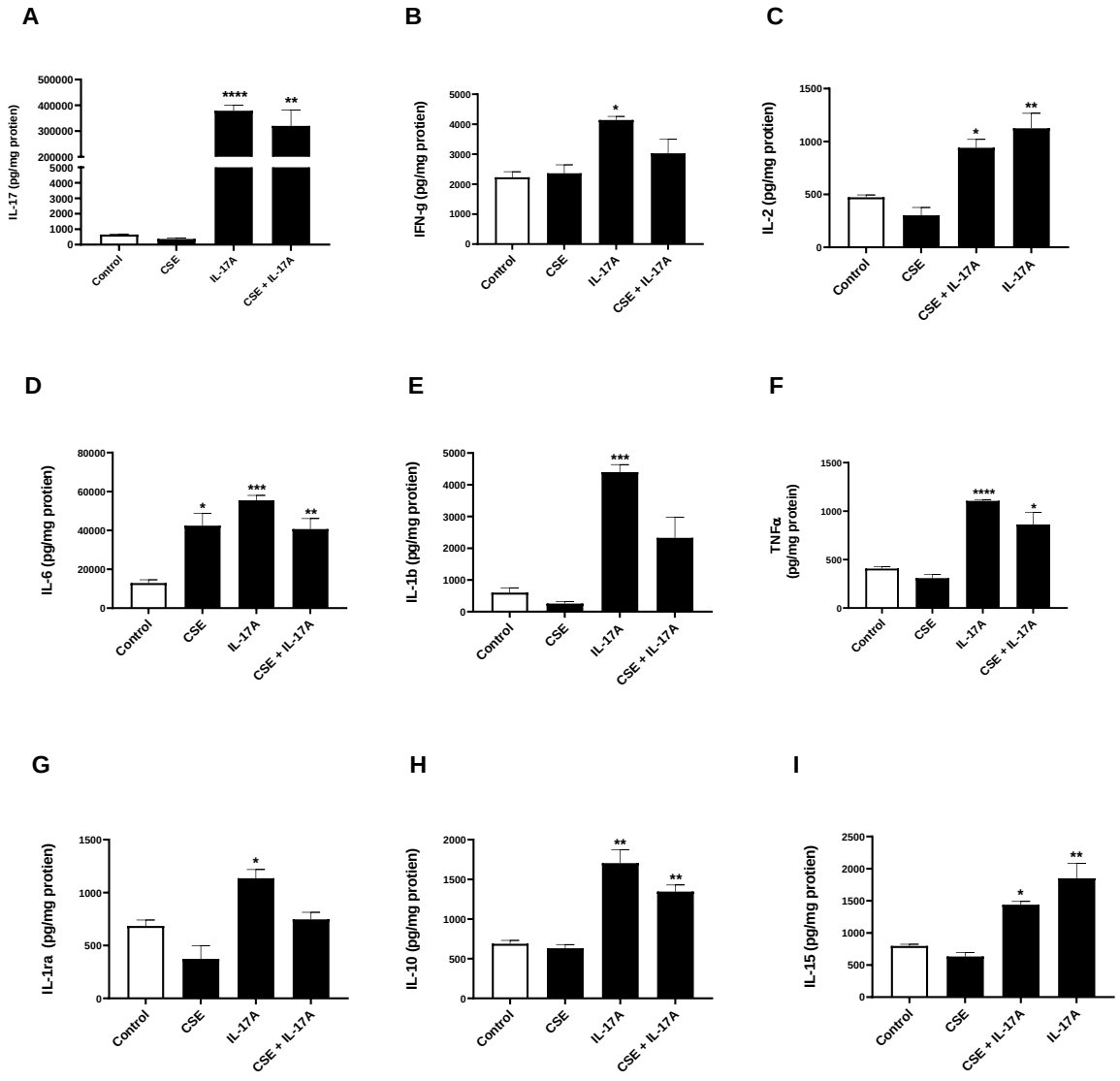
3.3.2 Timeline for experiments



3.4 Results

3.4.1 Effect of CSE on the production of different inflammatory mediators in HASMCs

In this chapter, we investigated the effect of CSE and IL-17A on the production of a wide range of different cytokines and chemokines that could play a role in airway inflammation and airway remodelling in asthma including T2 and non-T2 inflammatory cytokines. Our results demonstrated that HASMCs produced A) IL-17, B) IFN-γ, C) IL-2, D) IL-6, E) IL-1β, F) TNFα, G) IL-1ra, H) IL-10, I) IL-15, J) VEGF, K) PDGF-bb, L) FGF basic, M) MIP-1α, N) MCP-1, and O) MIP-1β basally (649.2±72.4, 2227.7±325, 472.3±38.1, 12899.1±2923.2, 602.5±261.9, 406.9±30.7, 684.7±97.2, 688.3±72.9, 796.8±37, 1373.1±89.6, 480.4±14.2, 418.6±33.7, 1490.2±149.8, 1194.7±207.1 pg/mg protein, respectively). CSE increased production of the pro-inflammatory cytokine IL-6 and the angiogenesis mediator VEGF (42417.4±11110.1 and 4214.2±1461.2 pg/mg protein, respectively, Figure 3.4.1 D and J), but it inhibited production of T2 inflammatory cytokines and increased production of the non-T2 inflammatory cytokine IL-8 (As shown in section 3.4.2, 3.4.3 and 3.4.4) and had no effect on all other tested cytokines and chemokines. IL-17A significantly increased the production of all the cytokines and chemokines that have been tested including the non-T2 inflammatory cytokine IL-8 and Th2 cytokine and eosinophil chemokines compared with untreated HASMCs.


A
IL-17 (pg/mg protien)
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400000
300000
200000
5000
4000
3000
2000
1000
0
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Control
CSE
IL-17A
CSE + IL-17A
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IFN-g (pg/mg protien)
5000
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IL-17A
CSE + IL-17A
C
IL-2 (pg/mg protien)
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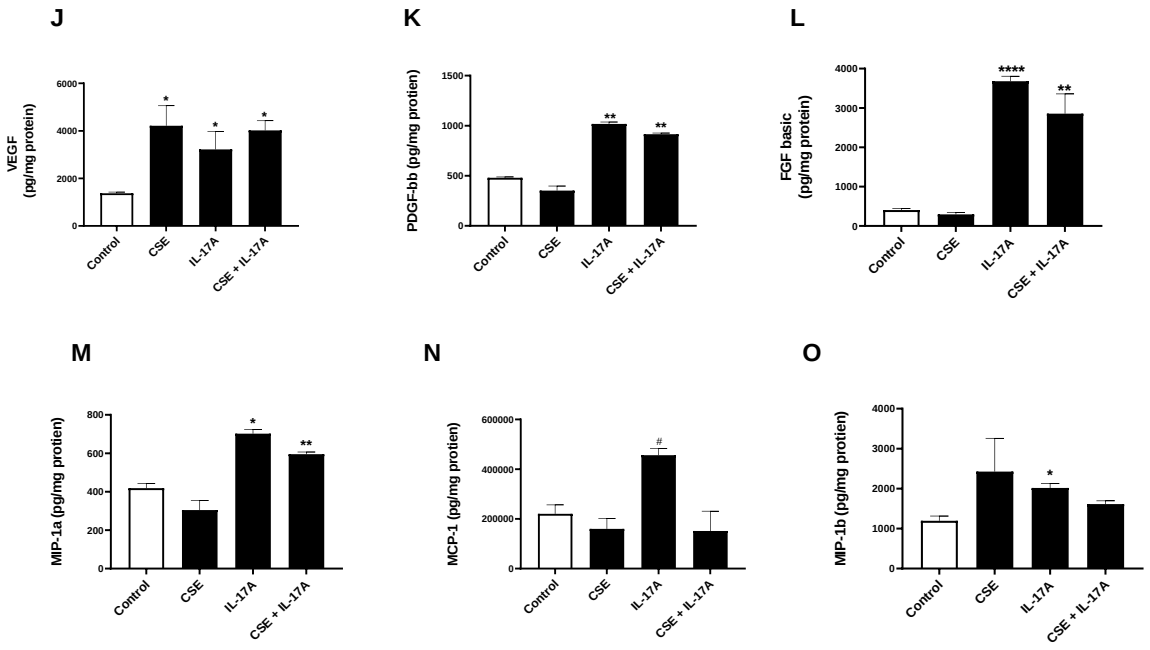


Figure 3.1 Effect of CSE and IL-17A on production of different inflammatory mediators

Confluent and serum-starved HASMCs were pre-treated with or without CSE 3.5% for 24 h, followed by incubation with CSE, IL-17A (10 ng/ml), or CSE+Il-17A for 24 h. The collected supernatants were used to measure the concentration of A) IL-17, B) IFN-γ, C) Il-2, D) IL-6, E) IL-1β, F) TNFα, G) IL-1ra, H) IL-10, I) IL-15, J) VEGF, K) PDGF-bb, L) FGF basic, M) MIP-1α, N) MCP-1, and O) MIP-1β by multiplex assay. Data were normalised to total protein. Each data point represents mean ± SEM of three experiments from one cell line. *p<0.05, **p<0.01,***p<0.001, and ,****p<0.0001 compared with control.

3.4.2 Effect of CSE and IL-17A on the production of IL-8

To explore the effect of CSE and IL-17A on the non-T2 inflammatory response, we assessed the effect of CSE and IL-17A on the neutrophilic chemoattractant IL-8 production in HASMCs. IL-8 was basally produced by HASMCs (1946.9±172.8 pg/mg protein, Figure 3.1). CSE stimulated IL-8 production by 10.5-fold (20564.9±4248.5 pg/mg protein, $p<0.05$, Figure 3.1) over the control. IL-17A increased IL-8 production by 6.1-fold compared with the control (11693.4±2630.4 pg/mg protein, $p<0.05$, Figure 3.1). A synergistic effect was observed when cells were treated with CSE and IL-17A (fold change 67.4, 131246.23±15878.1 pg/mg protein, $p<0.01$, Figure 3.1) compared with IL-17A alone. These findings suggest that CSE and IL-17A can contribute to non-T2 airway inflammation by inducing the neutrophilic chemoattractant IL-8 production in HASMCs and the combination of CSE and IL-17A have a synergistic effect on IL-8 production.

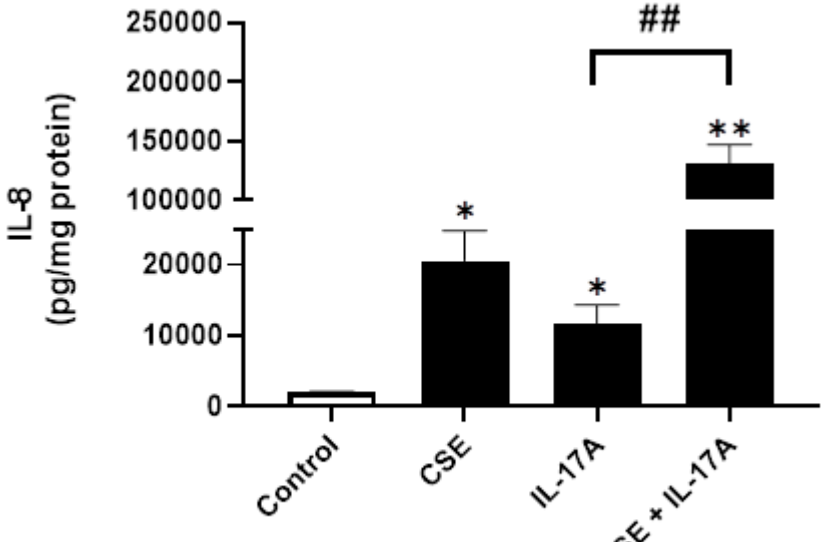


Figure 3.2 Effect of CSE and IL-17A on production of IL-8

Confluent and growth-arrested HASMCs were pre-treated with or without CSE (3.5%) for 24h and after that, cells were treated with CSE, IL-17A (10 ng/ml) or CSE+IL-17A for 24h. IL-8 concentration was measured in the collected supernatants by ELISA. Data were normalised to total protein. Each data point represents mean ± SEM from three independent experiments using cells from three different donors. *p<0.05 and **p<0.01 compared with control, and ##p<0.01 compared with IL-17A alone.

3.4.3 Effect of CSE and IL-17 on the production of Th2 cytokines

Th2 cytokines are responsible for eosinophilia, overproduction of IgE, and airway hyperresponsiveness observed in the airways of asthmatic patients. However, the effect of CSE and IL-17A on the production of Th2 cytokines in HASMCs is unclear. We assessed the effect of CSE and IL-17A on the production of Th2 cytokines IL-4, IL-5, and IL-13. HASMCs produced IL-4 (Figure 3.2), IL-5 (Figure 3.3) and IL-13 (Figure 3.4), at relatively low levels (643.5±42.2, 675.52±8.2 and 585.6±33.3 pg/mg protein, respectively). Interestingly, CSE inhibited the production of IL-4 by 57.5% (p<0.01, Figure 3.2) and IL-13 by 57.8% (p<0.01, Figure 3.4) compared with the control but had no effect of the production of IL-5 (519.6±58.5 pg/mg protein, Figure 3.3). IL-17A stimulation increased concentrations of IL-4 by 5.1-fold (p<0.01, Figure 3.2), IL-5 by 2.15-fold (p<0.01, Figure 3.3), and IL-13 by 1.5-fold (p<0.05, Figure 3.4) over the control. CSE inhibited IL-17A-induced production of IL-4 by 25.3%, IL-5 by 14.7%, and IL-13 by 61.3% compared with IL-17A alone. These findings suggest that CSE suppressed T2 inflammatory response by reducing the production of Th2 cytokines with and without IL-17A stimulation. Interestingly, IL-17A could enhance the T2 inflammatory response by stimulating the production of Th2 cytokines that are predominantly implicated in T2 airway inflammation.

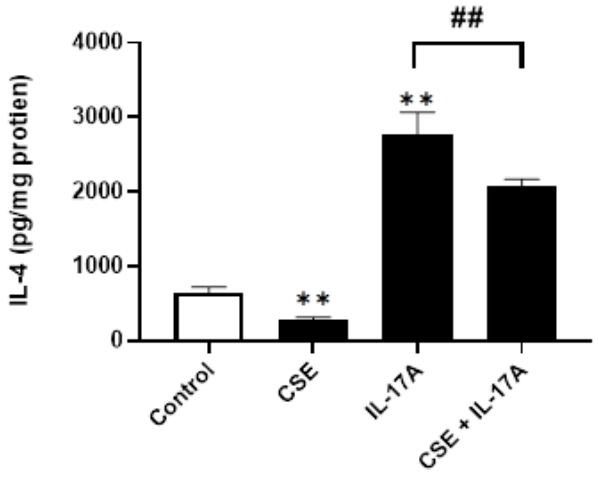


Figure 3.3 Effect of CSE and IL-17 on the production of IL-4

Confluent and growth-arrested HASMCs were pre-treated with or without CSE (3.5%) for 24h, and after that, cells were treated with CSE, IL-17A (10 ng/ml), or CSE + IL-17A for 24h. IL-4 concentration was measured in the collected supernatants using a multiplex assay. Data were normalised to total protein. Each data point represents mean ± SEM of three independent experiments using cells from three different donors. ***p*<0.01 compared with control and ##*p*<0.01 compared with IL-17A.

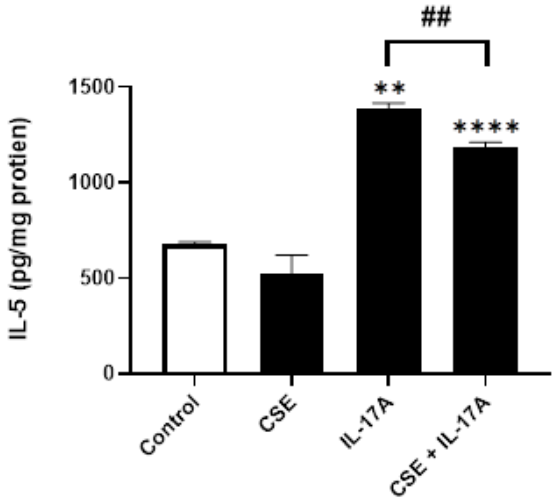


Figure 3.4 Effect of CSE and IL-17 on the production of IL-5

Confluent and growth-arrested HASMCs were pre-treated with or without CSE (3.5%) for 24h, and after that, cells were treated with CSE, IL-17A (10 ng/ml), or CSE + IL-17A for 24h. IL-5 concentration was measured in the collected supernatants using a multiplex assay. Data were normalised to total protein. Each data point represents mean ± SEM of three independent experiments using cells from three different donors. ***p*<0.01 and *****p*<0.0001 compared with control and ##*p*<0.01 compared with IL-17A.

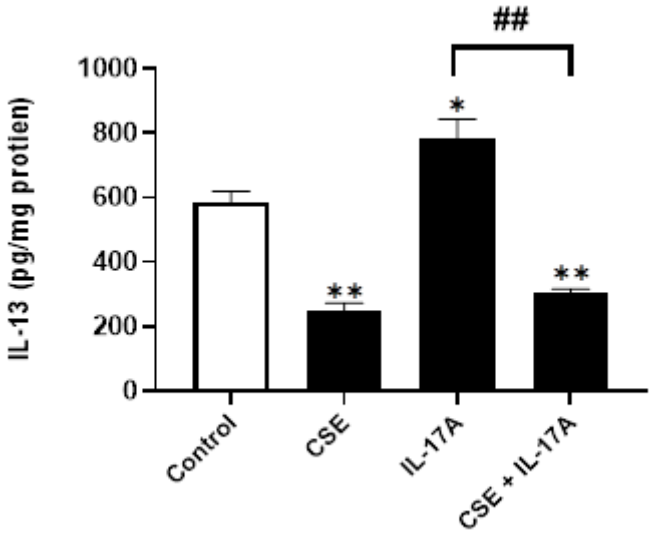


Figure 3.5 Effect of CSE and IL-17 on the production of IL-13

Confluent and growth-arrested HASMCs were pre-treated with or without CSE (3.5%) for 24h, and after that, cells were treated with CSE, IL-17A (10 ng/ml), or CSE + IL-17A for 24h. IL-13 concentration was measured in the collected supernatants using a multiplex assay. Data were normalised to total protein. Each data point represents mean ± SEM of three independent experiments using cells from three different donors. *p<0.05 and **p<0.01 compared with control and ##p<0.01 compared with IL-17A.

3.4.4 Effect of CSE and IL-17A on the production of eosinophil chemokines

Eosinophils are known to be attracted by chemokines such as Eotaxin, IP-10, and RANTES. Therefore, we sought to assess the effect of CSE and IL-17A on the production of eosinophil chemokines Eotaxin, IP-10, and RANTES in HASMCs. HASMCs basally produced eosinophil chemokines Eotaxin (57576.3±7597.2 pg/mg protein, Figure 3.5), IP-10 (783.5±43.9 pg/mg protein, Figure 3.6) and RANTES (1104.1±43.3 pg/mg protein, Figure 3.7). CSE alone significantly inhibited the production of Eotaxin by 94.7% (p<0.01), IP-10 by 58.6% (p<0.01) and RANTES by 66.9% (p<0.001) compared with the control. IL-17A increased the production of Eotaxin by 1.8-fold (p<0.05), IP-10 by 2-fold (p<0.01), and RANTES by 1.9-fold (p<0.001) over the control. CSE reduced IL-17A-induced production of Eotaxin by 89.9% (p<0.01), IP-10 by 57.8% (p<0.01) and RANTES by 62% (p<0.001) compared with IL-17A alone. Our findings strongly suggest that CSE may shift the airway inflammation from T2 to non-T2 by suppressing the production of T2 inflammatory cytokines and promoting the production of non-T2 cytokines in HASMCs. IL-17A promotes both T2 and non-T2 inflammatory responses by increasing their cytokine production in HASMCs.

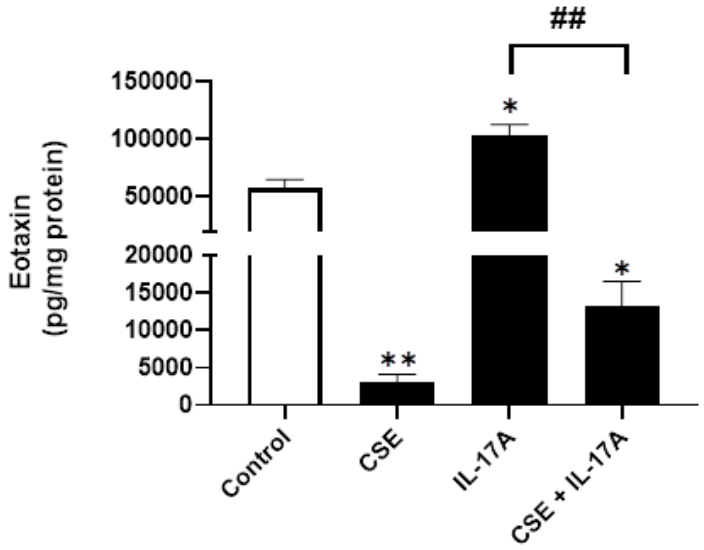


Figure 3.6 Effect of CSE and IL-17 on the production of Eotaxin

Confluent and growth-arrested HASMCs were pre-treated with or without CSE (3.5%) for 24h, and after that, cells were treated with CSE, IL-17A (10 ng/ml), or CSE + IL-17A for 24h. Eotaxin concentration was measured in the collected supernatants using a multiplex assay. Data were normalised to total protein. Each data point represents mean ± SEM of three independent experiments using cells from three different donors. *p<0.05 and **p<0.01 compared with control, ##p<0.01 compared with IL-17A alone.

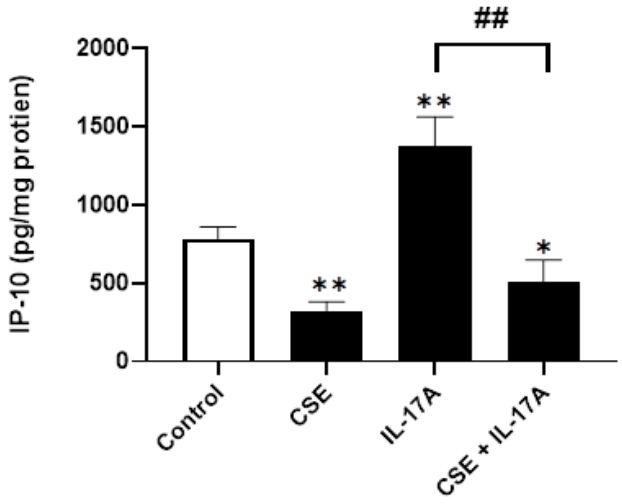


Figure 3.7 Effect of CSE and IL-17 on the production of IP-10

Confluent and growth-arrested HASMCs were pre-treated with or without CSE (3.5%) for 24h, and after that, cells were treated with CSE, IL-17A (10 ng/ml), or CSE + IL-17A for 24h. IP-10 concentration was measured in the collected supernatants using a multiplex assay. Data were normalised to total protein. Each data point represents mean ± SEM of three independent experiments using cells from three different donors. *p<0.05 and **p<0.01 compared with control, ##p<0.01 compared with IL-17A alone.

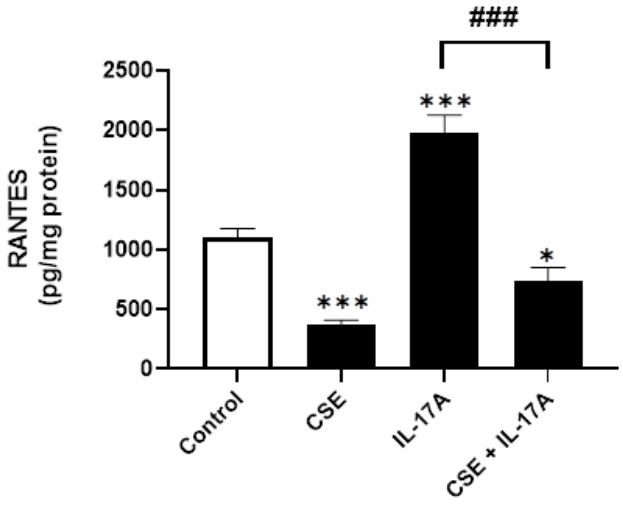


Figure 3.8 Effect of CSE and IL-17 on the production of RANTES

Confluent and growth-arrested HASMCs were pre-treated with or without CSE (3.5%) for 24h, and after that, cells were treated with CSE, IL-17A (10 ng/ml), or CSE + IL-17A for 24h. RANTES concentration was measured in the collected supernatants using a multiplex assay. Data were normalised to total protein. Each data point represents mean ± SEM of three independent experiments using cells from three different donors. *p<0.05 and ***p<0.001 compared with control, ###p<0.001 compared with IL-17A alone.

3.4.5 Effect of CSE and IL-17A on mRNA expression of Th2 cytokines

In this chapter, we have showed in 3.4.1, 3.4.2 and 3.4.3 that CSE inhibited the production of T2 inflammatory cytokine with and without IL-17A stimulation and increased the production of the non-T2 cytokine IL-8. The effect of CSE on the production of non-T2 inflammatory cytokines, particularly IL-8, is largely known. We, therefore, focused on the inhibitory effect of CSE on the production of T2 inflammatory cytokines by exploring if the changes in protein expression for T2 inflammatory cytokines are due to transcriptional regulation of the genes. So, we assessed the individual effects of CSE and IL-17A on the mRNA expression of Th2 cytokines and whether CSE affects HASMCs response to IL-17A stimulation. CSE alone had no effect on mRNA expression of Th2 cytokines at all time points. IL-17A increased mRNA expression of IL-4 (Figure 3.8), IL-5 (Figure 3.9) and IL-13 (Figure 3.10) at 2h by 2.4-, 4.4- and 1.6-fold, respectively, 8h by 1.9-, 6.4- and 1.9-fold, respectively, and 16h by 1.9-, 3- and 1.7-fold, respectively, compared with the control. Interestingly, CSE inhibited IL-17A-stimulated IL-4 mRNA expression at 2h and 16h by 42.8% and 66.4%, respectively, (Figure 3.8) IL-5 at 2h and 8h by 89.9% and 82.7%, respectively, (Figure 3.9) and IL-13 at all-time points 2h, 8h and 16h by 59.1%, 67.9% and 64.4%, respectively (Figure 3.10). Together with our results in 3.4.2, these findings strongly support that CSE may suppress T2 inflammatory response by inhibiting the production of Th2 cytokines via transcriptional regulation.

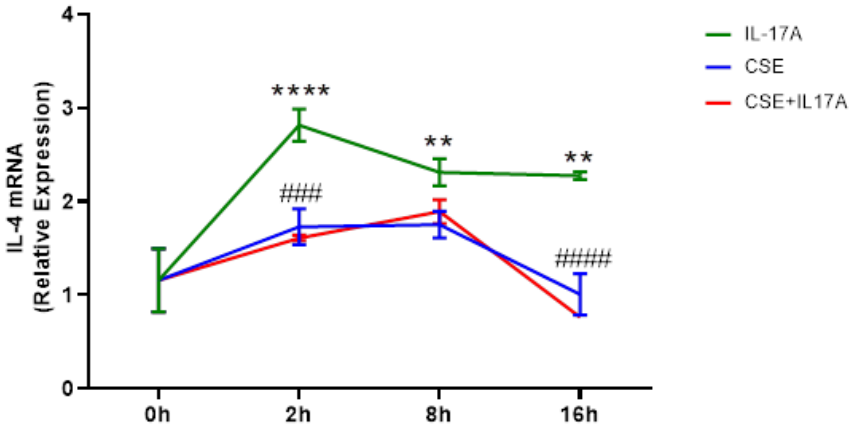


Figure 3.9 Effect of CSE and IL-17A on mRNA expression of IL-4

Confluent and growth-arrested HASMCs were treated with or without CSE (3.5%), IL-17A (10 ng/ml), or CSE + IL-17A for 2h, 8h, and 16h before total RNA isolation. The samples were assessed by RT-qPCR. Data were expressed as fold change over unstimulated cells (0h). Each data point represents mean ± SEM from three independent experiments using three cell lines. ***p*<0.01 and *****p*<0.0001 compared with control (0h), ###*p*<0.001 and ####*p*<0.0001 compared with IL-17A alone.

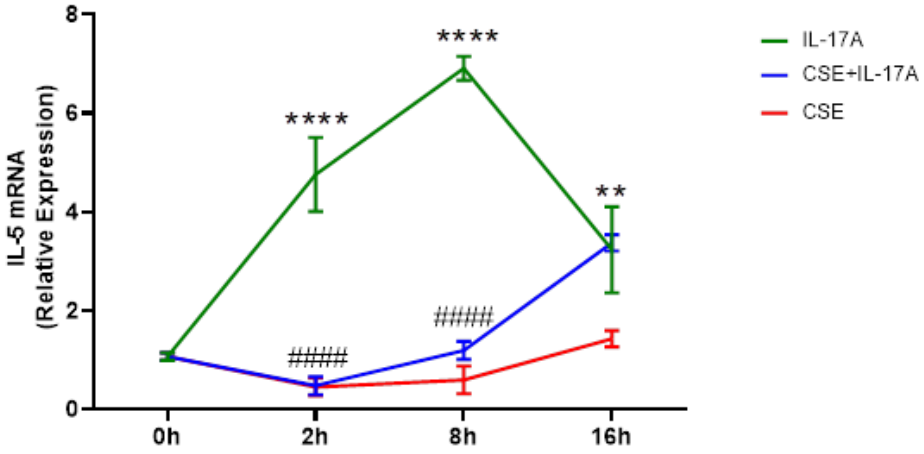


Figure 3.10 Effect of CSE and IL-17A on mRNA expression of IL-5

Confluent and growth-arrested HASMCs were treated with or without CSE (3.5%), IL-17A (10 ng/ml), or CSE + IL-17A for 2h, 8h, and 16h before total RNA isolation. The samples were assessed by RT-qPCR. Data were expressed as fold change over unstimulated cells (0h). Each data point represents mean ± SEM from three independent experiments using three cell lines. ***p*<0.01 and *****p*<0.0001 compared with control (0h), ####*p*<0.0001 compared with IL-17A alone.

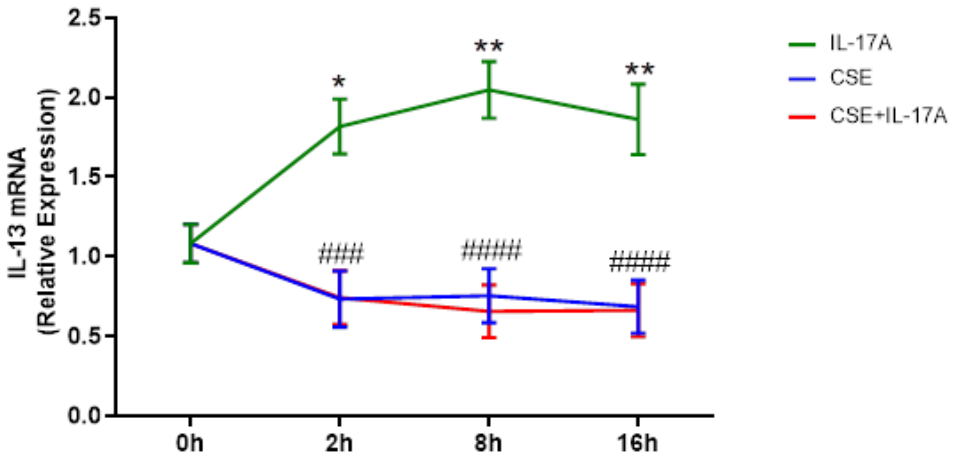


Figure 3.11 Effect of CSE and IL-17A on mRNA expression of IL-13

Confluent and growth-arrested HASMCs were treated with or without CSE (3.5%), IL-17A (10 ng/ml), or CSE + IL-17A for 2h, 8h, and 16h before total RNA isolation. The samples were assessed by RT-qPCR. Data were expressed as fold change over unstimulated cells (0h). Each data point represents mean ± SEM from three independent experiments using three cell lines. *p<0.05 and **p<0.01 compared with control (0h), ###p<0.001 and ####p<0.0001 compared with IL-17A alone.

3.4.7 Effect of CSE and IL-17A on mRNA expression of eosinophil chemokines

We investigated if the changes in protein expression for eosinophil chemokines by CSE are also due to transcriptional regulation of the genes. We assessed the effect of CSE and IL-17A on the mRNA expression of eosinophil chemokines Eotaxin, IP-10 and RANTES and whether CS affects HASMCs response to IL-17A stimulation. Our results showed that HASMCs expressed Eotaxin, IP-10 and RANTES mRNA expression at basal levels. We have demonstrated that CSE alone had no effect on mRNA expression of eosinophil chemokines Eotaxin and IP-10 but reduced mRNA expression of RANTES at all-time points 2h, 8h and 16h (53.8%, 60.2% and 45.8%, respectively) compared with the control. IL-17A stimulated mRNA expression of Eotaxin at 8h and 16h (fold 1.9 and 5.5, Figure 3.11), IP-10 at 2h and 8h (fold 5 and 3.6, Figure 3.12) and RANTES at 8h and 16h (fold 3.6 and 2.5, Figure 3.13) compared with the control. CSE inhibited IL-17A-stimulated mRNA expression of Eotaxin at 8h and 16h by 63.9 and 89.5% (Figure 3.11), IP-10 at 2h and 8h by 91.4% and 95.1% (Figure 3.12), and RANTES at 8h and 16h by 79.7% and 59.7% (Figure 3.13). The above findings suggest that CSE inhibited the production of eosinophil chemokines in HASMCs via transcriptional regulation of the genes. Together with other findings, these results strongly support that CSE can suppress T2 inflammatory response in HASMCs.

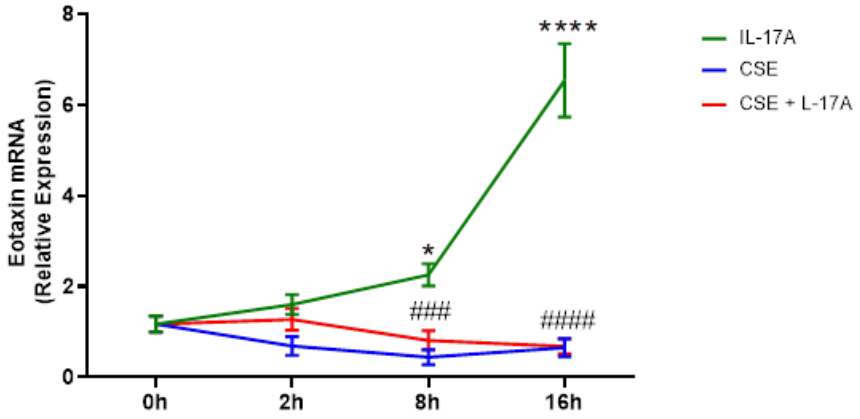


Figure 3.12 Effect of CSE and IL-17A on mRNA expression of Eotaxin

Confluent and growth-arrested HASMCs were treated with or without CSE (3.5%), IL-17A (10 ng/ml), or CSE + IL-17A over 3-time points (2h, 8h, and 16h) before total RNA isolation. The samples were assessed by RT-qPCR. Data were expressed as fold change over unstimulated cells (0h). Each data point represents mean ± SEM from three independent experiments using three cell lines. *p<0.05 and ****p<0.0001 compared with control, ###p<0.001 and #####p<0.0001 compared with IL-17A alone.

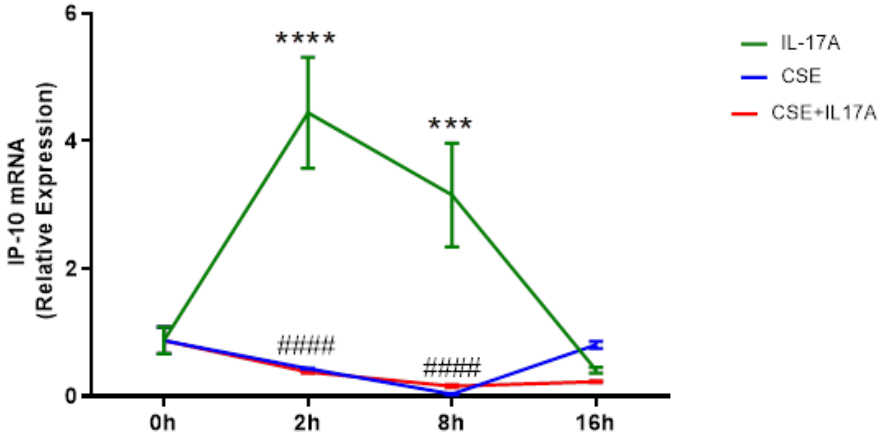


Figure 3.13 Effect of CSE and IL-17A on mRNA expression of IP-10

Confluent and growth-arrested HASMCs were treated with or without CSE (3.5%), IL-17A (10 ng/ml), or CSE + IL-17A over 3-time points (2h, 8h, and 16h) before total RNA isolation. The samples were assessed by RT-qPCR. Data were expressed as fold change over unstimulated cells (0h). Each data point represents mean ± SEM from three independent experiments using three cell lines. ***p<0.001 and ****p<0.0001 compared with control, ####p<0.0001 compared with IL-17A alone.

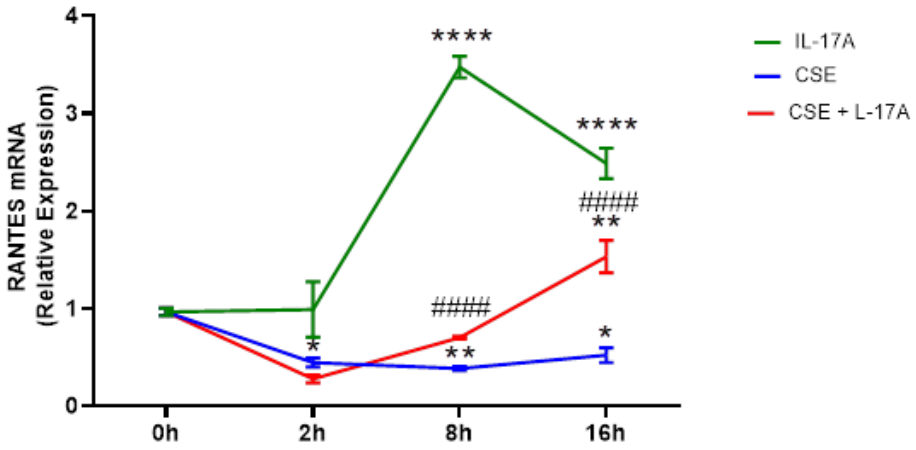


Figure 3.14 Effect of CSE and IL-17A on mRNA expression of RANTES

Confluent and growth-arrested HASMCs were treated with or without CSE (3.5%), IL-17A (10 ng/ml), or CSE + IL-17A over 3-time points (2h, 8h, and 16h) before total RNA isolation. The samples were assessed by RT-qPCR. Data were expressed as fold change over unstimulated cells (0h). Each data point represents mean ± SEM from three independent experiments using three cell lines. ***p*<0.01 and *****p*<0.0001 compared with control, ####*p*<0.0001 compared with IL-17A alone.

3.5 Discussion

This chapter showed that HASMCs can basally produce wide range of inflammatory cytokines including proinflammatory cytokines such as IL-1β, IL-6 and TNFα, anti-inflammatory cytokines like IL-1ra and IL-10, growth factors like VEGF, PDGF-bb and FGF basic, the non-T2 cytokine IL-8 and T2 inflammatory cytokines like IL-4, IL-5, IL-13, Eotaxin, RANTES and IP-10. HASMCs by their synthetic function, contractility and proliferation play important role in airway inflammation and remodeling in asthma. Surprisingly, IL-17A which known as important regulator of neutrophilic airway inflammation (non-T2) induced the production of all different inflammatory mediators that were tested in this study which may have potential wider effect on airway inflammation and remodeling in asthma. However, it is important to acknowledge that the effect of IL-17A on cytokine production shown in Figure 3.4.1 could be limited to this cell line, as the responses of one cell line may be different from others.

This chapter provides novel findings that CSE may shift the airway inflammation in asthma from T2 to non-T2 by suppressing the production of T2 inflammatory cytokines and inducing the production of non-T2 cytokine IL-8 in HASMCs. Another interesting finding in this chapter is that IL-17A can stimulate the production of Th2 cytokines and eosinophil chemokines which suggests that IL-17A could be involved in T2 airway inflammation in asthma.

Table 3.1 Summary table of the main findings of Chapter 3

Non-T2 Cytokine production	CSE	IL-17A	CSE+IL-17A vs IL-17A
IL-8	↑	↑	↑
Th2 Cytokine production	**CSE**	**IL-17A**	**CSE+IL-17A vs IL-17A**
IL-4	↓	↑	↓
IL-5	↔	↑	↓
IL-13	↓	↑	↓
Eosinophil chemokine production	**CSE**	**IL-17A**	**CSE+IL-17A vs IL-17A**
Eotaxin	↓	↑	↓
IP-10	↓	↑	↓
RANTES	↓	↑	↓
Th2 Cytokine mRNA expression	**CSE**	**IL-17A**	**CSE+IL-17A vs IL-17A**
IL-4	↔	↑	↓
IL-5	↔	↑	↓
IL-13	↔	↑	↓
Eosinophil chemokine mRNA expression	**CSE**	**IL-17A**	**CSE+IL-17A vs IL-17A**
Eotaxin	↔	↑	↓
IP-10	↔	↑	↓
RANTES	↓	↑	↓

↑ = Increased, ↓ = Reduced, ↔ = No change

HASMCs express a wide range of pro-inflammatory cytokines and

chemokines, which orchestrates the role of HASMCs in the inflammatory process in asthma disease by recruiting and activating the mast cells, leukocytes, neutrophils, and eosinophils (103). HASMCs have been shown to produce IL-8, which contributes to non-T2 inflammation, in response to inflammatory mediators, such as TNFα (111) and CSE (112). HASMCs were also shown to produce Th2 cytokines such as IL-5 and IL-13 (32), Eotaxin (43), IP-10 (44) and RANTES (45, 46) which contribute to T2 airway inflammation in asthma.

3.5.1 Role of CSE in airway inflammation in HASMCs

Studies exploring the airway inflammation of asthmatics have commonly been conducted on non-smoking asthmatics to avoid the effect of CS on the pathogenic mechanism of asthma (239, 240). Therefore, the impact of CS on airway inflammation is not very well understood (236). Cigarette smoke (CS) has been shown to contribute to non-T2 airway inflammation compared to a never-smoke patient with asthma [8]. We have demonstrated that CSE increased the non-T2 chemokine IL-8 production in HASMCs, suggesting CSE could promote non-T2 inflammatory response. In line with this finding, a study showed that CSE induced the production of IL-8 in HASMCs (112). Our findings are consistent with a study showed that CSE enhanced the IL-17A-induced production of IL-8 in human bronchial epithelial cells (BEAS-2B) (130), thereby enhancing the non-T2 inflammatory response. In support of our findings, data obtained from an *in vivo* study that showed CS significantly increased the number of neutrophils in lung tissue sections of nonallergic mice (122). Our finding showed a synergistic effect of CSE and IL-17A on IL-8 production, suggesting that

asthmatic patients who smoke would likely have increased IL-8 release leading to further neutrophilic inflammation. Our findings, together with others, may give a possible explanation for why asthmatic smokers tend to have non-T2 airway inflammation.

The main aim of this chapter is to explore the effect of CSE on the production of T2 inflammatory cytokines. Th2 cytokines are responsible for eosinophilia, overproduction of IgE and airway hyperresponsiveness (25). We presented an interesting finding that CSE alone can reduce the production of Th2 cytokines IL-4 and IL-13, but not IL-5, in HASMCs. The number of cell lines of HASMCs used in this study in addition to the relatively low basal expression of IL-5 could explain why we could not see the inhibitory effect of CSE on the production of IL-5. CSE also reduces IL-17A-mediated production of Th2 cytokines. In agreement with our findings, an *in vivo* study showed that CS attenuated house dust mite-mediated eosinophil response by reducing eosinophils in bronchoalveolar lavages (BALs) (121). Our results are also consistent with another *in vivo* research which found that CS inhibited the development of T2 inflammatory response by reducing eosinophilia and inhibiting the antigen-stimulated release of IL-4 in BALF of mice (120). In contrast with our results, a study showed that CSE (3% and 10%) increased the production of IL-4 and IL-5 in dendritic cells (124). However, the previous study (124) reported that CSE did not increase the number of eosinophils in BAL of mice. The cell-type difference, which may have different cell responses, is probably the main reason for the disparity between our findings and those in the previous study (124), and the different

cigarettes (4 full-strength Marlboro cigarettes) they used compared with the one used in our study could be another reason.

Eosinophil chemokines such as Eotaxin, IP-10 and RANTES are involved in the recruitment of eosinophils at the allergic inflammatory site (23). IL-5 was identified as a factor that promotes differentiation and proliferation of eosinophils in the bone marrow (40). Cooperation between eosinophil chemokines and IL-5 stimulates the fast build-up of eosinophils in tissues exposed to allergen (41). We have demonstrated that CSE inhibited the production of eosinophil chemokines Eotaxin, IP-10 and RANTES with and without IL-17A stimulation. Although very little was found in the literature on the effect of CSE on the production of eosinophil chemokines in HASMCs, our findings seem to be consistent with a study that found CSE inhibited Eotaxin and RANTES production in HASMCs after stimulation with TNFα but not on its own (125). In support of our findings, another study showed that CSE reduced Lipopolysaccharide (LPS)-induced production of IP-10 in an immortalised normal bronchial epithelial cell line (16-HBE) (126). In agreement with our results, *in vivo* study showed that CS significantly reduced the number of eosinophils in BAL fluid of OVA-challenged mice, while in the same study, CS increased the number of neutrophils in nonallergic animals (122). Our findings, together with others, may give a possible explanation for why asthmatic smokers tend to have non-T2 airway inflammation rather than T2 airway inflammation. It is possible that asthmatics who smoke might see a reduction of T2 inflammatory response and induction of non-T2 inflammatory response.

CSE on its own had no effect on mRNA expression of T2 inflammatory cytokines, except RANTES, but inhibited IL-17A-increased mRNA expression of T2 inflammatory cytokines in HASMCs. The reason why we could not see an effect of CSE on its own on mRNA expression of T2 inflammatory cytokines is that CSE might regulate T2 inflammatory cytokine expression through post-transcriptional regulation or post-translational modifications; however, currently there is no direct evidence to support this theory.

Our study presented an important finding that suggests CSE reduced IL-17A-induced the production of T2 inflammatory cytokines via transcriptional mechanism. Our findings are supported by an in vivo study that showed that Guinea pigs exposed to 10 cigarettes per day, 5 days a week, for 4 weeks, reduced IL-5 and Eotaxin mRNA expression (241). Both IL-5 and Eotaxin have been critical in eosinophil differentiation and activation (242, 243). Up to our knowledge from available literature, this is the first study to assess the effect of CSE on mRNA expression of T2 inflammatory cytokines in HASMCs.

Our findings, together with others, strongly suggest that CS may shift the airway inflammation in asthma from T2 to non-T2 by promoting the production of non-T2 inflammatory cytokines and suppressing the production of T2 inflammatory cytokines via transcriptional regulation which as result may lead to glucocorticoid insensitivity in asthma. Multiple potential pathways that CSE is known to affect such as COX-2 and its synthesis PGE_2 could mediate the inhibitory effect of CSE on production and transcription of

T2 inflammatory cytokines. The next chapter is going to explore the mechanism by which CSE reduced T2 inflammatory responses in HASMCs.

3.5.2 Role of IL-17A in airway inflammation in HASMCs

Th17 cells are a subgroup of CD4+ T cells associated with a severe phenotype of asthma (79). Non-T2 airway inflammation tends to show neutrophil-dominant inflammation and the production of non-T2 cytokines. Several studies have demonstrated that IL-17A plays an important role in the pathogenesis of non-T2 airway inflammation in asthma. For instance, it has also been shown that IL-17A induced the production of the non-T2 airway inflammation markers, such as IL-8 and GROα, in airway epithelial cells and HASMCs (95, 96). IL-17A also increased the production of the neutrophilic chemoattractant IL-8 and GROα from human bronchial fibroblasts (244). Clinically, blood and sputum samples from patients with asthma have shown an increase in IL-17A levels (245, 246). These findings align with our results that showed IL-17A induced production of IL-8 in HASMCs, suggesting that IL-17A may contribute to neutrophilic inflammation by inducing the production of neutrophilic chemoattractant IL-8 in HASMCs, leading to a severe form of asthma. The increase of IL-8 level in serum can be used as a biomarker to identify severity of asthma (247). Asthmatic patients with a high IL-8 level may require higher doses of glucocorticoids (247).

T2 airway inflammation in asthma is associated with eosinophil inflammation but whether IL-17A could contribute to T2 airway inflammation is unclear. Unexpectedly, we observed evidence of enhanced T2 inflammatory response by Th17 cytokine IL-17A in HASMCs. Our study

demonstrated an interesting finding that IL-17A induced the production of T2 inflammatory cytokines IL-4, IL-5, IL-13, Eotaxin, IP-10 and RANTES in HASMCs. In line with our findings, a study has shown IL-17A induced production of the eosinophil chemoattractant Eotaxin in HASMCs (110), suggesting that IL-17A could contribute to T2 airway inflammation. In agreement with our findings, it has also been shown that IL-17A increased eosinophil infiltration of the nasal polyps of chronic rhinosinusitis associated with asthma (248). To the best of our knowledge, our study is the first to explore the effect of IL-17A on the production of other T2 inflammatory cytokines in human structural cells, particularly in HASMCs.

Our study also showed the upregulation of T2 inflammatory cytokines mRNA expression by IL-17A in HASMCs, suggesting that IL-17A may upregulate T2 inflammatory cytokines gene expression via transcriptional mechanism. Unlike other tested T2 inflammatory cytokines, we have not seen an increase in mRNA expression of Eotaxin and RANTES induced by IL-17A at 2h of stimulation. In support of this finding, a study demonstrated that HASMCs stimulated with TNFα and IL-1β had no effect on mRNA expression of Eotaxin at 4h, and significant expression was seen at 8h (235). A significant mRNA expression of RANTES was observed at 12h to 24h after exposure to TNF-a (68). This could explain why we did not see an effect on Eotaxin and RANTES mRNA expression at 2h of stimulation with IL-17A. These findings, together with ours, could illustrate the critical role of the Th17 cytokine IL-17A in airway inflammation in asthma by stimulating T2 inflammatory response via transcriptional regulation.

In conclusion, this chapter provides an important finding that showed CSE may suppress the T2 inflammatory response and enhance the non-T2 inflammatory response by reducing the production of T2 inflammatory cytokines and increasing the production of T2 inflammatory cytokines. Our study provides insight that CS may switch the inflammatory responses in asthmatic smokers from T2 to non-T2 which known to be resistance to glucocorticoid. In the next chapter, we will investigate the mechanisms by which CSE inhibited the production of T2 inflammatory cytokines.

Chapter 4. Mechanisms of CSE effect on the production of T2 inflammatory cytokines in human airway smooth muscle cells

4.1 Introduction

HASMCs play an essential role in airway structure and function via regulating contractility, proliferation, and extracellular matrix production in the context of diseases such as asthma (192, 249-251). HASMCs produce inflammatory cytokines, chemokines and growth factors which contribute to airway inflammation (252, 253). CSE plays a vital role in airway inflammation in asthma, specifically neutrophilic airway inflammation (125). Chapter 3 demonstrated that CSE could suppress T2 airway inflammation while contributing to non-T2 airway inflammation by inhibiting the production of T2 inflammatory cytokines and promoting the production of non-T2 inflammatory cytokines. However, the underlying mechanisms by which CSE affects HASMCs response are still unclear. In this chapter, we sought to identify molecular mechanisms by which CSE reduced T2 inflammatory responses on its own and together with IL-17A. Nicotine is considered to be the major active component of CS which classically acts through nicotinic acetylcholine receptors (nAChRs). The α7 nicotinic acetylcholine receptor (nAChR) has been identified as the most expressed nAChR in HASMCs (230). So, we assessed the role of nicotine in mediating the inhibitory effect of CSE on the production of T2 inflammatory cytokines. It has also been shown that CS causes increased oxidative stress due to direct damage by free radical species, which enhance inflammatory responses (146). CS can stimulate reactive oxygen species (ROS), and glutathione (GSH) prevents ROS-mediated inflammation. GSH, a key antioxidant in the lungs, is reduced by cigarette smoking (254). We postulated that oxidative stress, induced by cigarette smoke, could be an important mechanism by which CSE inhibited

the production of Th2 cytokines and eosinophil chemokines and utilised the oxidative stress inhibitor to define the relative contribution of oxidative stress in the inhibitory effect of CSE. The oxidative stress inhibitor GSH, an important intra- and extracellular antioxidant in the lung, to define the relative contribution of oxidative stress in mediating the inhibitory effect of CSE.

Cyclooxygenase (COX)-2 is one of the two main isoforms of the COX enzyme, which is induced by many inflammatory stimuli such as CS (168, 255). Prostaglandin E2 (PGE_2) is the main COX product produced by HASMCs. It has been shown that CS can induce COX-2 expression and PGE_2 production in different cell types, including HASMCs (168). This chapter investigated whether the suppressing CSE effect on the T2 airway inflammation is mediated through COX-2 expression and its product PGE_2 synthesis. Furthermore, PGE_2 signals via four known receptors (EP_1–EP_4), with the cAMP signalling pathway responsible for major suppressive and regulatory functions of PGE_2 mostly through EP_2 and EP_4 receptors (256). In this chapter, we assessed whether the inhibitory effect of CSE on the production of T2 inflammatory cytokines is mediated by the prostaglandin receptors EP_2 and EP_4 through the cAMP pathway.

4.2 Aims

1- To assess the role of nicotine in mediating the inhibitory effect of CSE on the production of T2 inflammatory cytokines by applying nicotine and the α7 nicotinic acetylcholine receptor (α7nAChR) antagonist MG624.

2- To explore the role of oxidative stress in mediating the inhibitory effect of CSE on the production of T2 inflammatory cytokines by applying the oxidative stress inhibitor L-glutathione (GSH).

3- To investigate the potential role of COX2/ PGE_2 in mediating the inhibitory effect of CSE on the production of T2 inflammatory cytokines by using the COX-2 inhibitor NS-398, determining the effect of CSE on COX-2 expression and PGE_2 release using Western-blot and ELISA, respectively and lastly by assessing the effect exogenous PGE_2 on the production of T2 inflammatory cytokines in HASMCs.

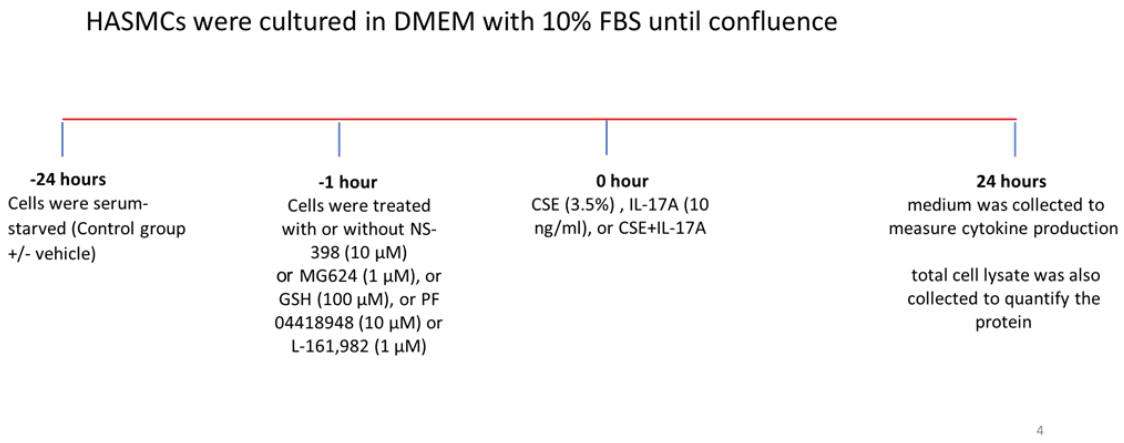
4- To assess the role of prostaglandin EP_2/EP_4 receptors in mediating the inhibitory effect of CSE on the production of T2 inflammatory cytokines by using the EP_2/EP_2 receptor antagonists.

5- To confirm the role of cAMP in mediating the inhibitory effect of CSE on the production of T2 inflammatory cytokines by applying the cAMP analogue 8-Bromo.

4.3 Methods

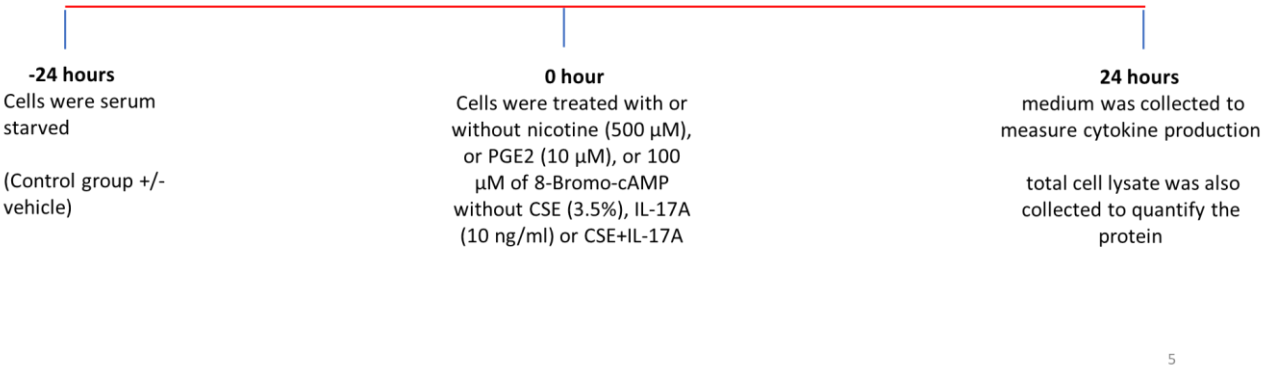
CSE was prepared from the smoke of two research cigarettes bubbled into 20 ml of cell culture medium. Confluent and growth-arrested HASMCs were pre-treated with and without the α7nAChR antagonist MG624 (1 μM), the oxidative stress inhibitor GSH (100 μM), the COX-2 inhibitor NS-398 (10 μM), the prostaglandin EP_2 receptor antagonist PF 04418948 (10 μM) and the prostaglandin EP_4 receptor antagonist L-161,982 (1 μM) for 1h prior to incubation with or without CSE (3.5%), IL-17A (10 ng/ml), CSE+IL-17A (4.3.1). Confluent and growth-arrested HASMCs were treated with or without nicotine (500 μM), PGE2 (10 μM) and 8-Bromo-cAMP (100 μM) for 24h (4.3.2). Cytokines and chemokines were measured in the collected medium using the Bio-Plex Pro-Human Cytokine assay. RT-qPCR analysis was used to measure the mRNA expression of COX-2, and β2M was used as a reference gene. Western blotting was used to measure COX-2 protein expression.

4.3 Timeline for experiments



4.3.2 Timeline for experiments

HASMCs were cultured in DMEM with 10% FBS until confluence



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4.4 Results

4.4.1 Role of nicotine on the production of Th2 cytokines

We demonstrated in chapter 3 that CSE inhibited the production of Th2 cytokines IL-4, IL-5, and IL-13 in HASMCs. In this chapter, we assessed the role of nicotine in mediating the inhibitory effect of CSE on the production of T2 inflammatory cytokines by using nicotine and the α7 nicotinic acetylcholine receptor (α7nAChR) antagonist MG624. Th2 cytokines IL-4, IL-5 and IL-13 were basally produced by HASMCs. As we showed before in chapter 3, CSE alone reduced the production of Th2 cytokines IL-4 and IL-13, but not IL-5 (Figure 4.1) compared with the control. CSE also inhibited all tested Th2 cytokines IL-4, IL-5 and IL-13 induced by IL-17A. MG624 did not reverse the inhibitory effect of CSE on the production of Th2 cytokines with and without IL-17A stimulation. M624 also had no effect on IL-17A-induced production of Th2 cytokines. Unlike the CSE, stimulation of the cells with nicotine had no effect on the production of Th2 cytokines. These results suggest that the inhibitory effect of CSE on the production of Th2 cytokines in HASMCs is not mediated by nicotine.

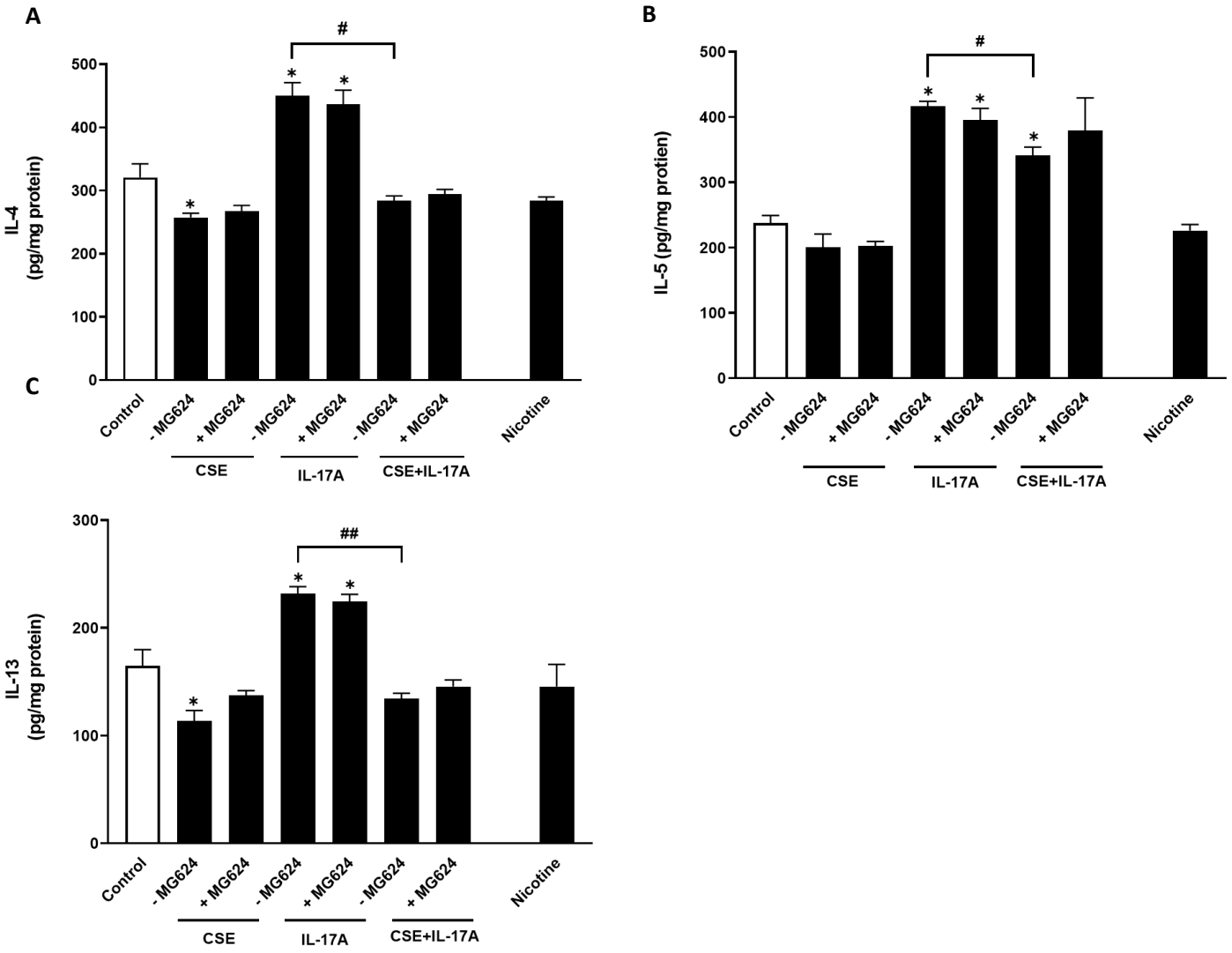

Figure 4.1 Role of nicotine on the production of Th2 cytokines

Confluent and growth-arrested HASMCs were pre-treated with or without MG624 (1 µM) for 1h prior to incubation with or without CSE (3.5%), IL-17A (10 ng/ml), CSE+IL-17A or nicotine (500 µM) for 24h. IL-4 (A), IL-5 (B) and IL-13 (C) concentrations were measured in the collected supernatants using a multiplex assay. Data were normalised to total protein. Each data point represents mean ± SEM from three independent experiments using cells from three different donors. **p*<0.05 compared with untreated cells (control), #*p*<0.05 and ##*p*<0.01 compared with IL-17A alone.

4.4.2 Role of nicotine on the production of eosinophil chemokines

Eosinophil chemokines are responsible for eosinophil recruitment at the site of inflammation. In order to assess the role of nicotine in mediating the inhibitory effect of CSE on the production of eosinophil chemokines, we used the α7nAChR antagonist MG624, the most expressed nAChR in HASMCs, as a tool. As shown before, CSE alone reduced the production of eosinophil chemokines Eotaxin, IP-10 and RANTES with and without IL-17A stimulation in HASMCs. MG624 did not reverse the inhibitory effect of CSE on the production of eosinophil chemokines and on IL-17A-induced production of eosinophilic chemokines. MG624 also had no effect on IL-17A-induced production of eosinophilic chemokines. Nicotine had no effect on the production of eosinophil chemokines (Figure 4.2). Our findings suggest that the inhibitory effect of CSE on the production of eosinophil chemokines in HASMCs is not mediated by nicotine.

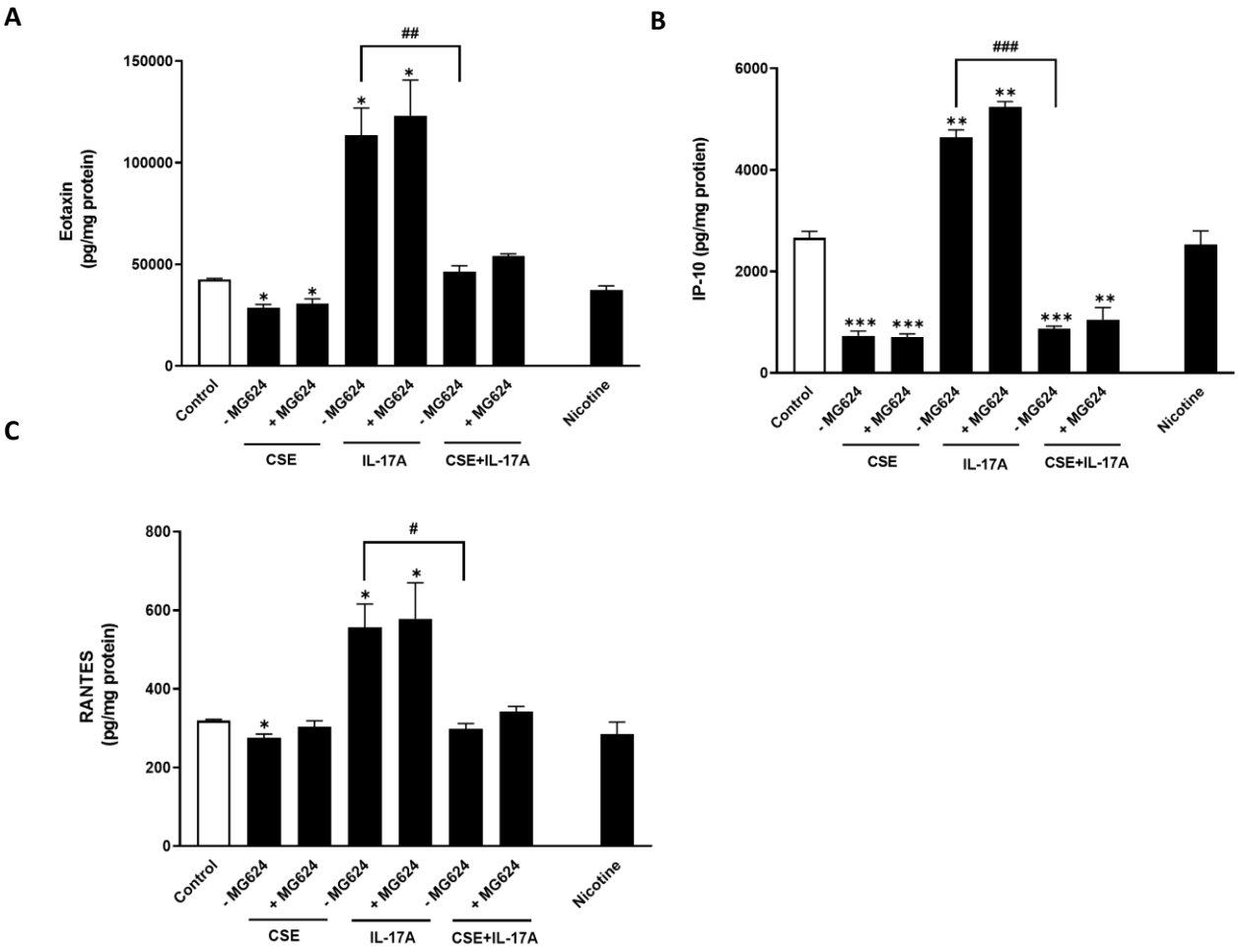

Figure 4.2 Role of nicotine on the production of eosinophil chemokines

Confluent and growth-arrested HASMCs were pre-treated with or without MG624 (1 µM) for 1h prior to incubation with or without CSE (3.5%), IL-17A (10 ng/ml), CSE+IL-17A or nicotine (500 µM) for 24h. Eotaxin (A), IP-10 (B) and RANTES (C) concentrations were measured in the collected supernatants using a multiplex assay. Data were normalised to total protein. Each data point represents mean ± SEM from three independent experiments using cells from three different donors. *p<0.05, **p<0.01 and ***p<0.001 compared with control and #p<0.05, ##p<0.01 and ###p<0.001 compared with IL-17A alone.

4.4.3 Effect of the oxidative stress inhibitor on CSE inhibition of Th2 cytokine production

In order to explore whether oxidative stress could mediate the inhibitory effect of CSE on the production of Th2 cytokines, we assessed if the oxidative stress inhibitor GSH can reverse the inhibitory effect of CSE. CSE reduced the production of Th2 cytokines IL-4 and IL-13 but not IL-5 compared with control (Figure 4.3). CSE also reduced IL-17A-induced production of IL-4, IL-5 and IL-13 (Figure 4.3). Interestingly, GSH reversed the inhibition of IL-13 production by CSE (fold change 1.88, $p<0.01$, Figure 4.3 C) but had no effect on the inhibition of IL-4 production. GSH reversed the inhibitory effect of CSE on IL-17A-induced production of IL-4 and IL-5 (fold change 1.21, $p<0.05$ and 1.56, $p<0.01$, respectively, Figure 4.3 A and B), but not IL-13. GSH had no effect on IL-17A-induced production of Th2 cytokines. These findings suggest that oxidative stress plays a role in mediating the inhibitory effect of CSE on the production of some of the Th2 cytokines in HASMCs.

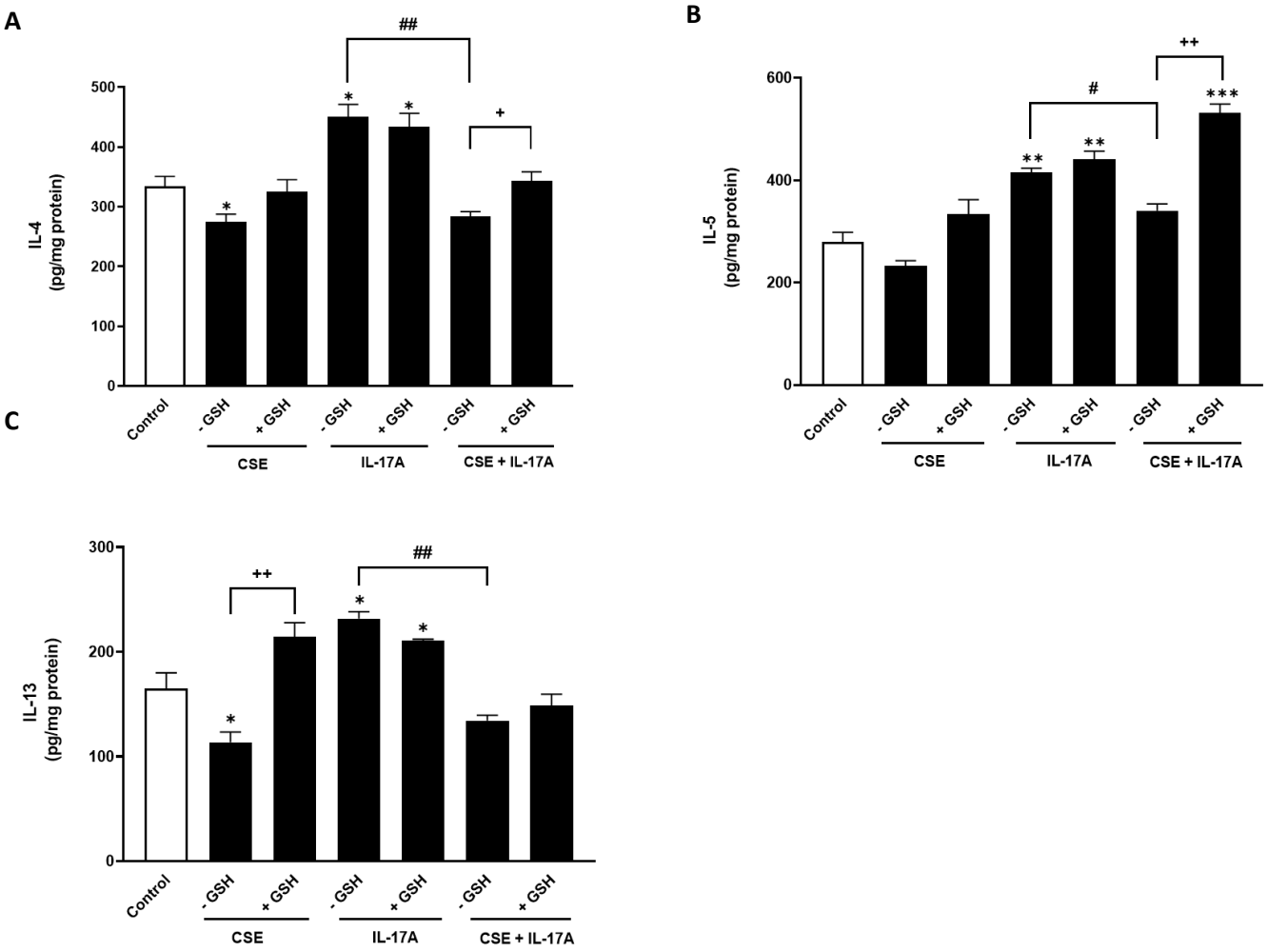

Figure 4.3 Effect of the oxidative stress inhibitor on CSE inhibition of Th2 cytokine production

Confluent and growth-arrested HASMCs were pre-treated with or without GSH (100 µM) for 1h prior to incubation with or without CSE (3.5%), IL-17A (10 ng/ml) or CSE+IL-17A for 24h. IL-4 (A), IL-5 (B) and IL-13 (C) concentrations were measured in the collected supernatants using a multiplex assay. Data were normalised to total protein. Each data point represents mean ± SEM from three independent experiments using cells from three different donors. *p<0.05, **p<0.01 and ***p<0.001 compared with control, #p<0.05 and ##p<0.01 compared with IL-17A alone and +p<0.05 and ++p<0.01 compared -GSH with +GSH.

4.4.4 Effect of the oxidative stress inhibitor on CSE inhibition of eosinophil chemokine production

We assessed the effect of the oxidative stress inhibitor GSH on the production of eosinophilic chemokines, which were inhibited by CSE on its own or together with IL-17A. As expected, CSE inhibited the production of eosinophil chemokines Eotaxin, IP-10 and RANTES on its own and after IL-17A stimulation. GSH reversed the inhibition of Eotaxin, IP-10 and RANTES production by CSE compared with CSE alone (fold change 1.6, 2.4, and 1.4, respectively, Figure 4.4). In addition, GSH also reversed the inhibitory effect of CSE on IL-17A-induced production of Eotaxin, IP-10 and RANTES (fold change 1.3, 1.3 and 1.6, respectively, Figure 4.4). Furthermore, GSH had no effect on 17A-induced production of Eotaxin, IP-10 and RANTES (Figure 4.4). These findings, together with the results described in 4.3, strongly suggest that oxidative stress plays a role in mediating the inhibitory effect of CSE on the production of T2 inflammatory cytokines in HASMCs.

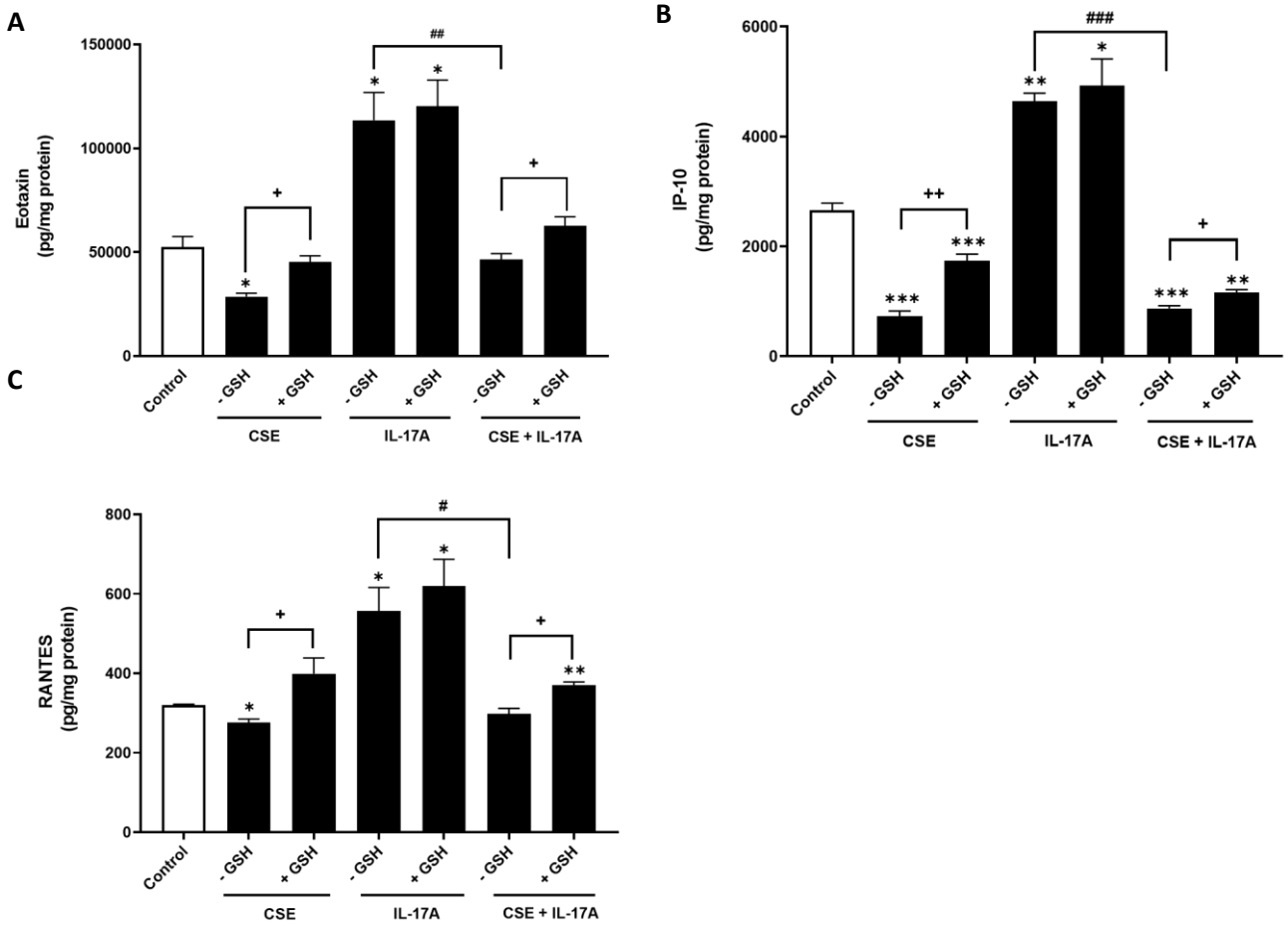

Figure 4.4 Effect of the Oxidative stress inhibitor on CSE inhibition of eosinophil chemokine production

Confluent and growth-arrested HASMCs were pre-treated with or without GSH (100 µM) for 1h prior to incubation with or without CSE (3.5%), IL-17A (10 ng/ml) or CSE+IL-17A for 24h. Eotaxin (A), IP-10 (B) and RANTES (C) concentrations were measured in the collected supernatants using multiplex assay. Data were normalised to total protein. Each data point represents mean ± SEM from three independent experiments using cells from three different donors. *p<0.05, **p<0.01 and ***p<0.001 compared with control, #p<0.05, ##p<0.01 and ###p<0.001 compared with IL-17A alone and +p<0.05 and ++p<0.01 compared -GSH with +GSH.

4.4.5 Effect of the COX-2 inhibitor on CSE inhibition of Th2 cytokine production

Studies showed that CSE induced COX-2 expression in HASMCs (168). Therefore, we investigate the potential role of COX2 in mediating the inhibitory effect of CSE on the production of Th2 cytokines by using the COX2 inhibitor NS-398. CSE inhibited the production of Th2 cytokines IL-4 and IL-13 but not IL-5, with and without IL-17A stimulation (Figure 4.5). Interestingly, NS-398 reversed the inhibition of IL-4 and IL-13 production by CSE and increased their production by 17.1% and 35.5%, respectively, compared with CSE alone ($p<0.05$ for both, Figure 4.5). NS-398 also reversed the inhibitory effect of CSE on IL-17A-induced production of IL-4 and IL-13 (Increased by 17.9%, $p<0.01$ and 14.3%, $p<0.05$, respectively, Figure 4.5), but not IL-5. NS-398 had no effect on IL-17A-induced production of Th2 cytokines. These findings suggest the inhibitory effect of CSE on the production of Th2 cytokines IL-4 and IL-13 is mediated by increased COX-2 expression and/or activity in HASMCs.

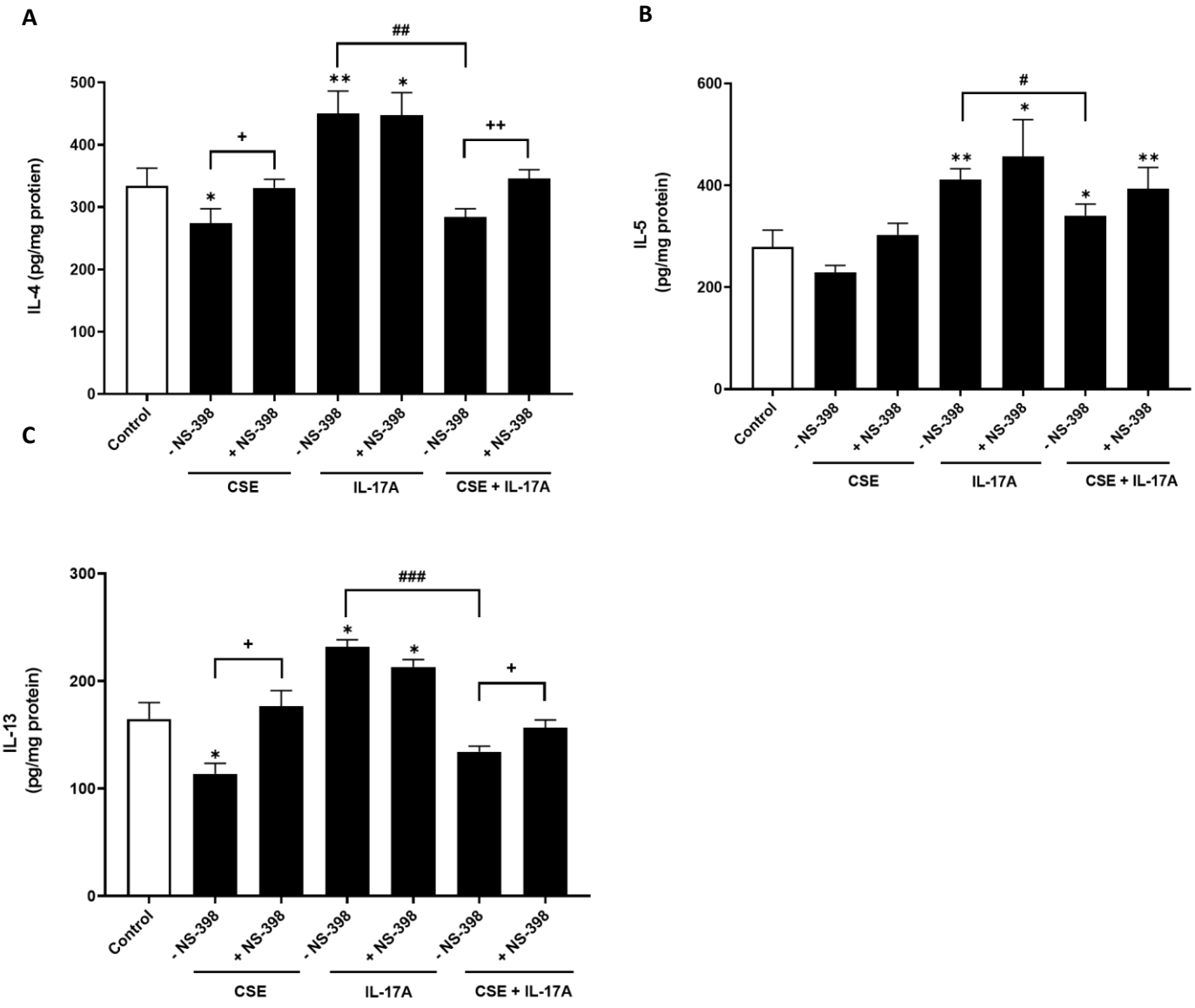

Figure 4.5 Effect of the COX-2 inhibitor on CSE inhibition of Th2 cytokine production

Confluent and growth-arrested HASMCs were pre-treated with or without NS-398 (10 µM) for 1h prior to incubation with or without CSE (3.5%), IL-17A (10 ng/ml) or CSE+IL-17A for 24h. IL-4 (A), IL-5 (B) and IL-13 (C) concentrations were measured in the collected supernatants using a multiplex assay. Data were normalised to total protein. Each data point represents mean ± SEM from three independent experiments using cells from three different donors. *p<0.05 and **p<0.01 compared with control, #p<0.05, ##p<0.01 and ###p<0.001 compared with IL-17A alone and +p<0.05 and ++p<0.01 compared -NS-398 with +NS-398.

4.4.6 Effect of the COX-2 inhibitor on CSE inhibition of eosinophil chemokine production

We showed in 4.4.5 that NS-398 reversed the inhibitory effect of CSE on the production of Th2 cytokines, and then we assessed if NS-398 could also reverse the inhibitory effect of CSE on the production of eosinophil chemokines. As shown before, CSE reduced the production of eosinophil chemokines Eotaxin, IP-10 and RANTES on its own and with IL-17A stimulation (Figure 4.6). NS-398 reversed the inhibition of Eotaxin, IP-10 and RANTES production by CSE and increased their production by 42.9% ($p<0.05$), 50.4% ($p<0.01$) and 16.2% ($p<0.05$), respectively, compared with CSE alone. NS-398 blocked the inhibitory effect of CSE on IL-17A-induced production of Eotaxin, IP-10 and RANTES and increased their production by 22.7% ($p<0.05$), 29.5% ($p<0.01$) and 32.1% ($p<0.05$), respectively, compared with CSE and IL-17A. NS-398 had no effect on IL-17A-induced production of eosinophilic chemokines. Taken together, these results suggest that the CSE effect on the production of eosinophil chemokines is mediated by COX-2 expression and/or activity in HASMCs.

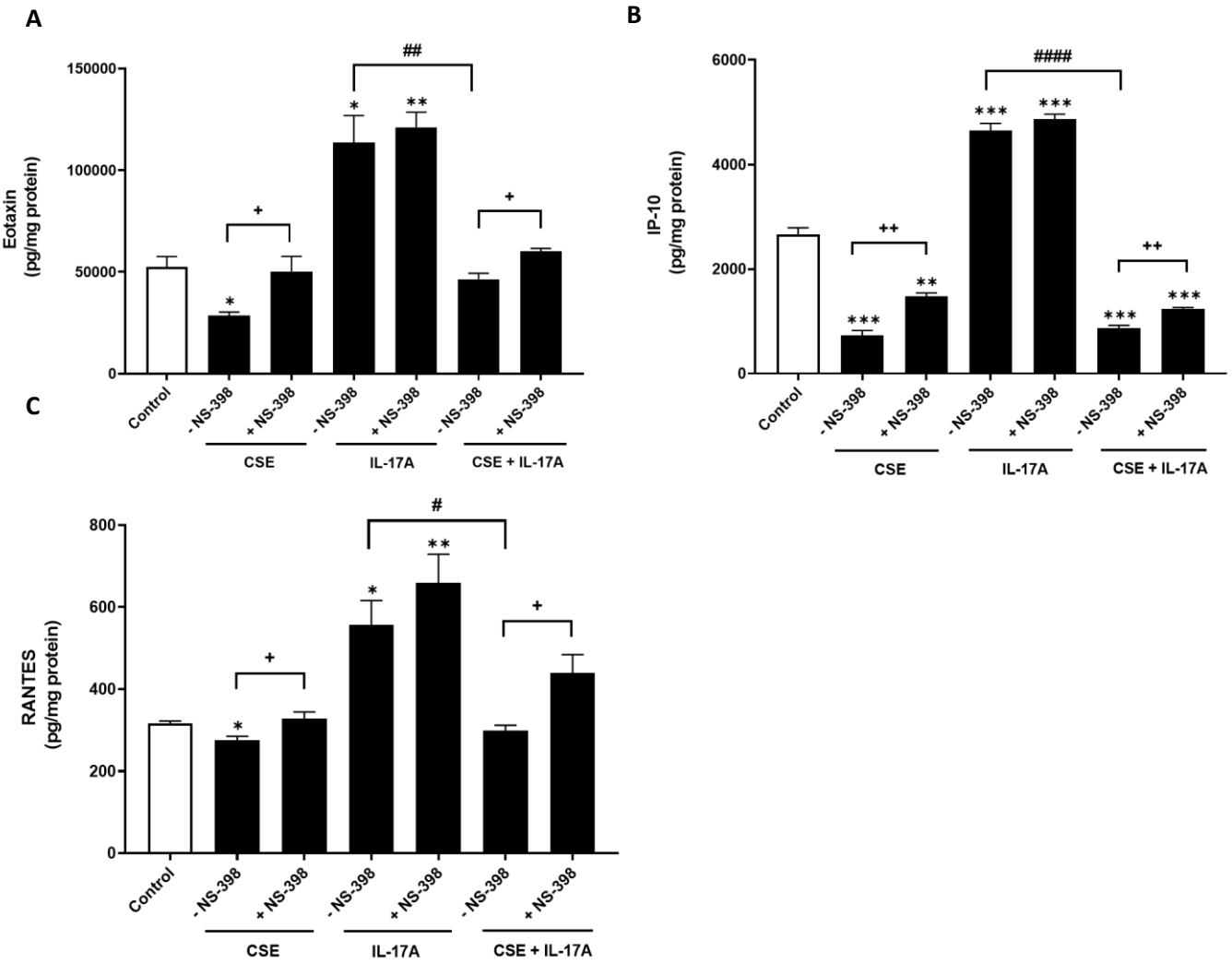

Figure 4.6 Effect of the COX-2 inhibitor on CSE inhibition of eosinophil chemokine production

Confluent and growth-arrested HASMCs were pre-treated with or without NS-398 (10 µM) for 1h prior to incubation with or without CSE (3.5%), IL-17A (10 ng/ml) or CSE+IL-17A for 24h. Eotaxin (A), IP-10 (B) and RANTES (C) concentrations were measured in the collected supernatants using multiplex assay. Data were normalised to total protein. Each data point represents mean ± SEM from three independent experiments using cells from three different donors. *p<0.05, **p<0.01 and ***p<0.001 compared with control, #p<0.05, ##p<0.01 and ####p<0.0001 compared with IL-17A alone and +p<0.05, ++p<0.01 compared -NS-398 with +NS-398.

4.4.7 Effect of CSE and IL-17A on the mRNA and protein expression of COX-2 and PGE_2 production

As we showed in 4.4.5 and 4.46, the COX-2 inhibitor NS-398 reversed CSE inhibition of T2 inflammatory cytokine production with and without IL-17A stimulation. Then we sought to assess whether the inhibitory effect of CSE on the production of T2 inflammatory cytokines could be mediated through COX-2 expression and the synthesis of its main product prostaglandin E2 (PGE_2). The protein of COX-2 was not basally expressed. CSE, but not IL-17A, significantly increased protein expression of COX-2 (fold change 2.616 ± 0.67, $p<0.01$, Figure 4.7). CSE, but not IL-17A, also increased mRNA expression of COX-2 at time point 2h and 8h (fold change 4.49 ± 0.21, $p<0.0001$ and 3.47 ± 0.41, $p<0.01$, respectively) as shown in Figure 4.8. No further increase in either protein or mRNA expression of COX-2 was observed after cotreatment of CSE and IL-17A treatment compared with CSE alone (Figures 4.7 and 4.8). Then we examined the effect of CSE and IL-17A on the production of PGE_2. PGE_2 was basally produced by HASMCs (770.5 pg/mg protein, Figure 4.7). CSE increased the production of PGE_2 by 2.3-fold ($p<0.05$), while IL-17A had no effect on the production of PGE_2 (Figure 4.9). No further increase in the production of PGE_2 was seen after cells were stimulated with CSE and IL-17A (Figure 4.4.9). These results provide direct evidence that CSE induces COX-2 expression and PGE_2 synthesis and strongly support the concept that the inhibitory effect of CSE on the production of T2 inflammatory cytokines is largely mediated by COX-2 expression and PGE_2 release in HASMCs.

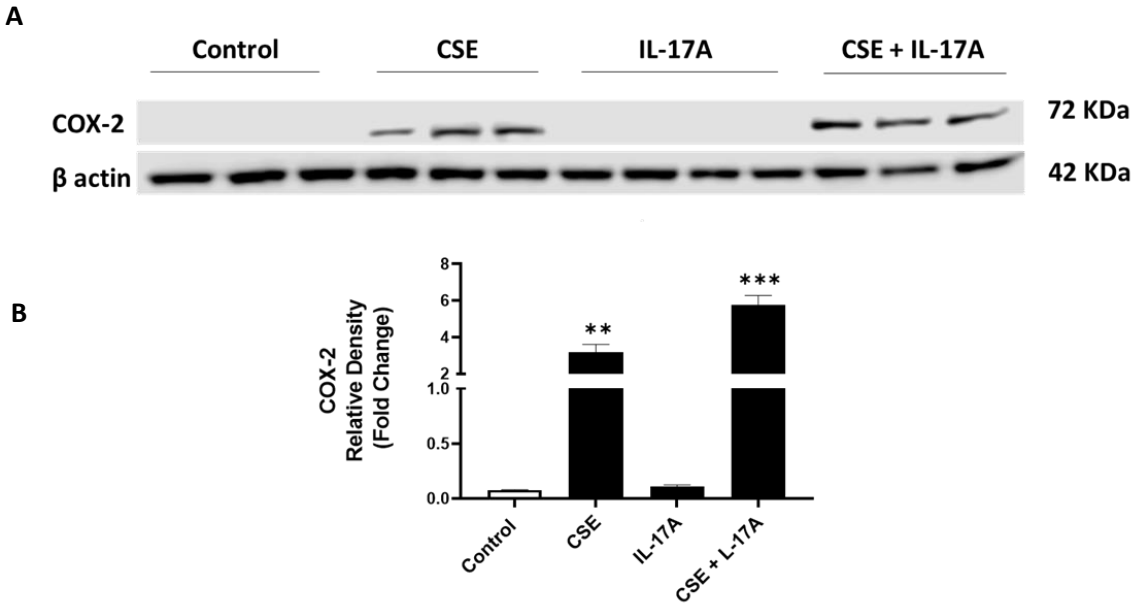

Figure 4.7 Effect of CSE and IL-17A on the protein expression of COX-2

Confluent and growth-arrested HASMCs were with or without CSE (3.5%), IL-17A (10 ng/ml) or CSE+IL-17A for 24h. Total cell lysates were collected for Western blot analysis of COX-2 protein. A, a representative Western blot showing the effect of CSE and CSE+IL-17A on COX-2 protein expression. B, optical densitometry analysis of Western blotting bands. Data were normalised with the loading control GAPDH and were expressed as fold change over untreated cells (Control). Each data point represents mean ± SEM from three independent experiments using cells from three different donors. ** p<0.01 and *** p<0.001 compared with control.

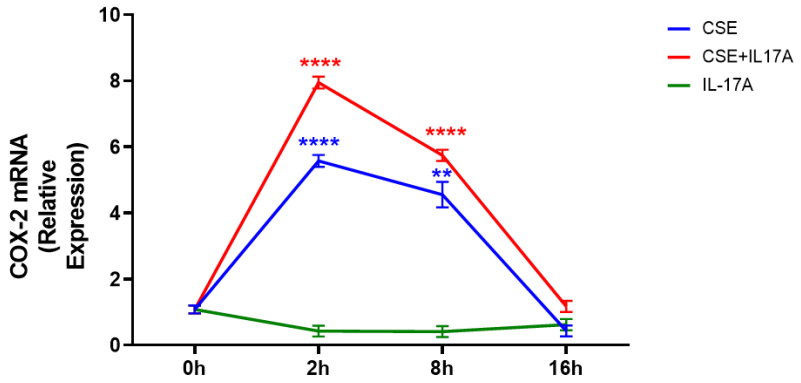

Figure 4.8 Effect of CSE and IL-17A on the mRNA expression of COX-2

Confluent and growth-arrested HASMCs were with or without CSE (3.5%), IL-17A (10 ng/ml) or CSE+IL-17A for 2h, 8h and 24h. Total RNA was isolated and then the samples were assayed for the mRNA expression of COX-2 using RT-qPCR. Data were expressed as fold change over untreated cells (Control). Each data point represents mean ± SEM from three independent experiments using cells from three different donors. $^{**}p<0.01$ and $^{****}p<0.0001$ compared with control.

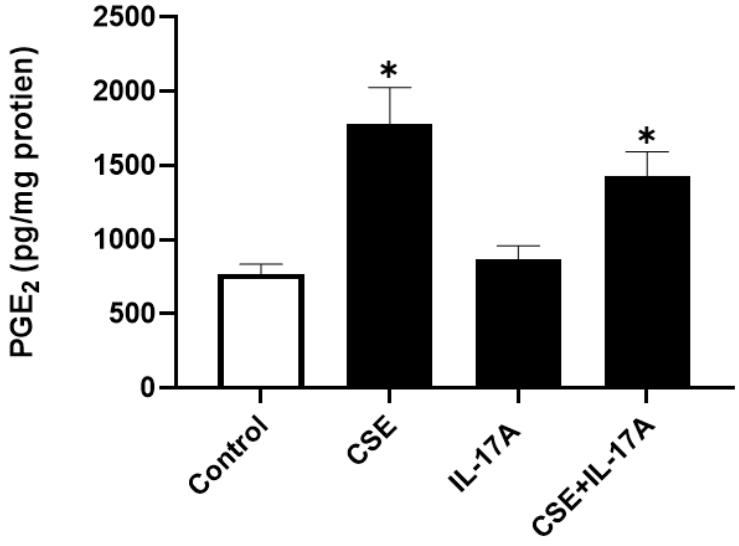

Figure 4.9 Effect of CSE and IL-17A on PGE_2 production

Confluent and growth-arrested HASMCs were treated with or without CSE (3.5%), IL-17A (10 ng/ml) or CSE+IL-17A for 24h. The collected supernatants were used to measure the production of PGE_2 by ELISA. Data were normalised to total protein. Each data point represents mean ± SEM from three independent experiments using cells from three different donors. *p<0.05 compared with control.

4.4.8 Effect of the oxidative stress inhibitor on CSE-induced protein expression of COX-2 and PGE_2 production

Our previous results showed that CSE inhibited the production of T2 inflammatory cytokines, and this inhibition was reversed by pre-treatment of HASMCs with the oxidative stress inhibitor GSH. These findings suggest that oxidative stress plays a role in the inhibitory effect of CSE on T2 airway inflammation. We also demonstrated that the inhibitory effect of CSE on the production of T2 inflammatory cytokines is largely mediated by COX-2 expression. However, whether oxidative stress plays a role in mediating CSE-induced COX-2 expression and PGE_2 release is unknown. A study conducted by our group examined the role of oxidative stress in COX-2 expression induced by CSE (unpublished observation). Our study demonstrated CSE-induced protein expression of COX-2 (fold change 12.8, $p<0.01$, Figure 4.10). Interestingly, we showed that GSH inhibited CSE-induced COX-2 protein expression in HASMCs by 86.51% ($p<0.01$, Figure 4.10). We further assessed the effect of GSH on CSE-induced production of PGE_2. CSE induced the production of PGE_2 by 2.3-fold compared with untreated cells ($p<0.05$, Figure 4.11 A), while IL-17A had no effect on the production of PGE_2 (Figure 4.11 B). GSH reduced CSE-induced production of PGE_2 in the absence or presence of IL-17A by 79.25% and 69.01% ($p<0.01$ and $p<0.01$, respectively, Figure 4.11 A and C). These results suggest that CSE-induced COX-2 expression and PGE_2 release is mediated via oxidative stress in HASMCs.

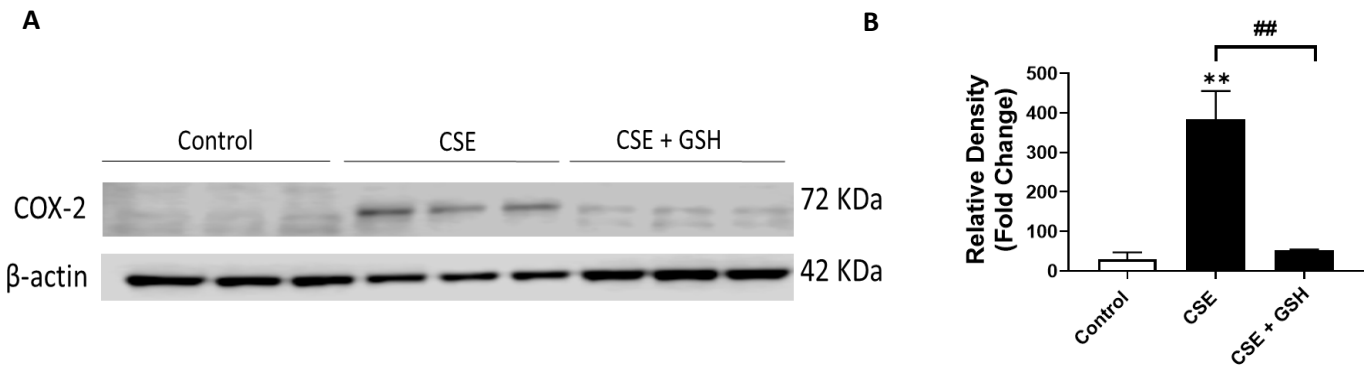

Figure 4.10 Effect of oxidative stress inhibitor on CSE induced protein expression of COX-2

Confluent and growth-arrested HASMCs were pre-treated with or without GSH (100 μM) for 1h prior to incubation with CSE (3.5%) for 24h. Total cell lysates were collected for Western blot analysis of COX-2 protein. A, a representative Western blot showing the effect of CSE with and without GSH on COX-2 protein expression. B, optical densitometry analysis of Western blotting bands. Data were normalised with the loading control GAPDH and were expressed as fold change over untreated cells (Control). Each data point represents mean ± SEM from three independent experiments using cells from three different donors. ***p*<0.01 compared with control and ##*p*<0.01 compared with CSE. This Figure was provided by our group (unpublished result)

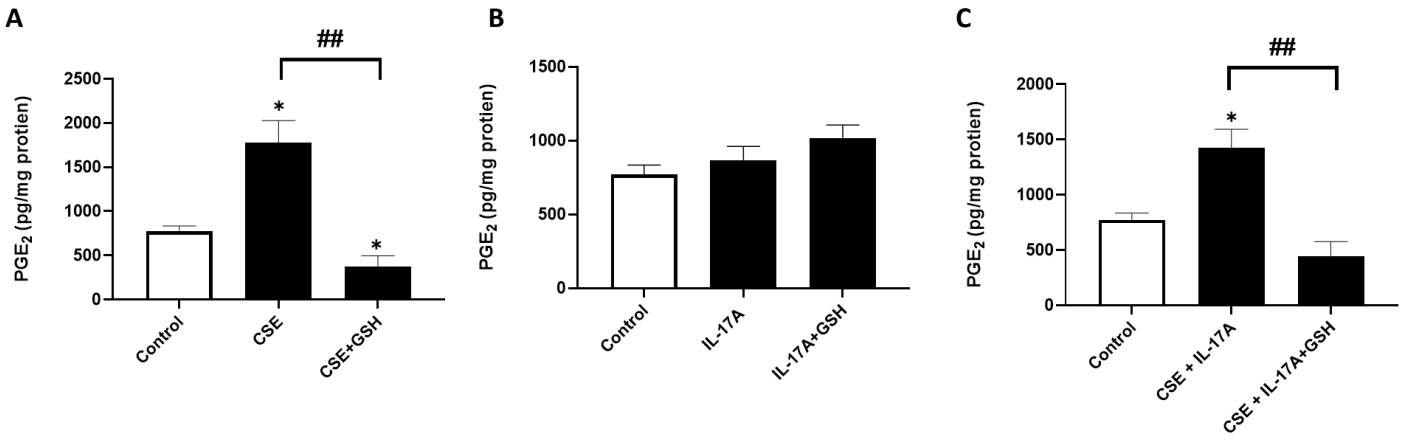

Figure 4.11 Effect of the oxidative stress inhibitor on CSE-induced PGE_2 production

Confluent and growth-arrested HASMCs were pre-treated with or without GSH (100 µM) for 1h before incubation with CSE (3.5%), IL-17A (10 ng/ml) or CSE+IL-17A for 24h. The collected supernatants were used to measure the concentration of PGE_2 by ELISA. Data were normalised to total protein. Each data point represents mean ± SEM from three independent experiments using cells from three different donors. *p<0.05 compared with control, ##p<0.01 compared to CSE alone or CSE+IL-17A.

4.4.9 Effect of exogenous PGE2 on the production of Th2 cytokines

PGE_2 is an endogenous lipid mediator of inflammation, and the rate-limiting upstream enzyme COX-2 regulates the production of PGE_2. We showed that CSE induced PGE_2 release from HASMCs (Figure 4.9). In order to further explore the role of PGE_2 in mediating the CSE effect on production of T2 inflammatory cytokines, we examined whether the exogenous PGE_2 has a similar effect as CSE on the production of Th2 cytokines in HASMCs. CSE alone reduced the production of IL-4 and IL-13 but not IL-5, while IL-17A induced the production of Th2 cytokines IL-4, IL-5 and IL-13 (Figure 4.12). CSE also inhibited IL-17A-induced production of IL-4, IL-5 and IL-13 (Figure 4.12). Exogenous PGE_2 on its own had no effect on the production of IL-4, IL-5 and IL-13 compared with untreated cells. PGE_2 treatment together with CSE did not enhance the inhibitory effect of CSE on the production of Th2 cytokines. Then we assessed the effect of PGE_2 on IL-17A-induced production of Th2 cytokines. Interestingly, PGE_2 reduced IL-17A-induced production of IL-4, IL-5 and IL-13 by 20.75%, 15.74% and 40.4%, respectively, compared with IL-17A alone ($p<0.01$, $p<0.01$ and $p<0.001$, Figure 4.12). PGE_2 did not amplify CSE inhibition of IL-17A-induced production of Th2 cytokines compared with CSE and IL-17A (Figure 4.12). Taken together, our results demonstrate that exogenous PGE_2 can mimic the effect of CSE on IL-17A-induced Th2 cytokine production and further supports that the inhibitory effect of CSE on the production of Th2

cytokines is largely mediated by COX-2/PGE_2via oxidative stress in HASMCs.

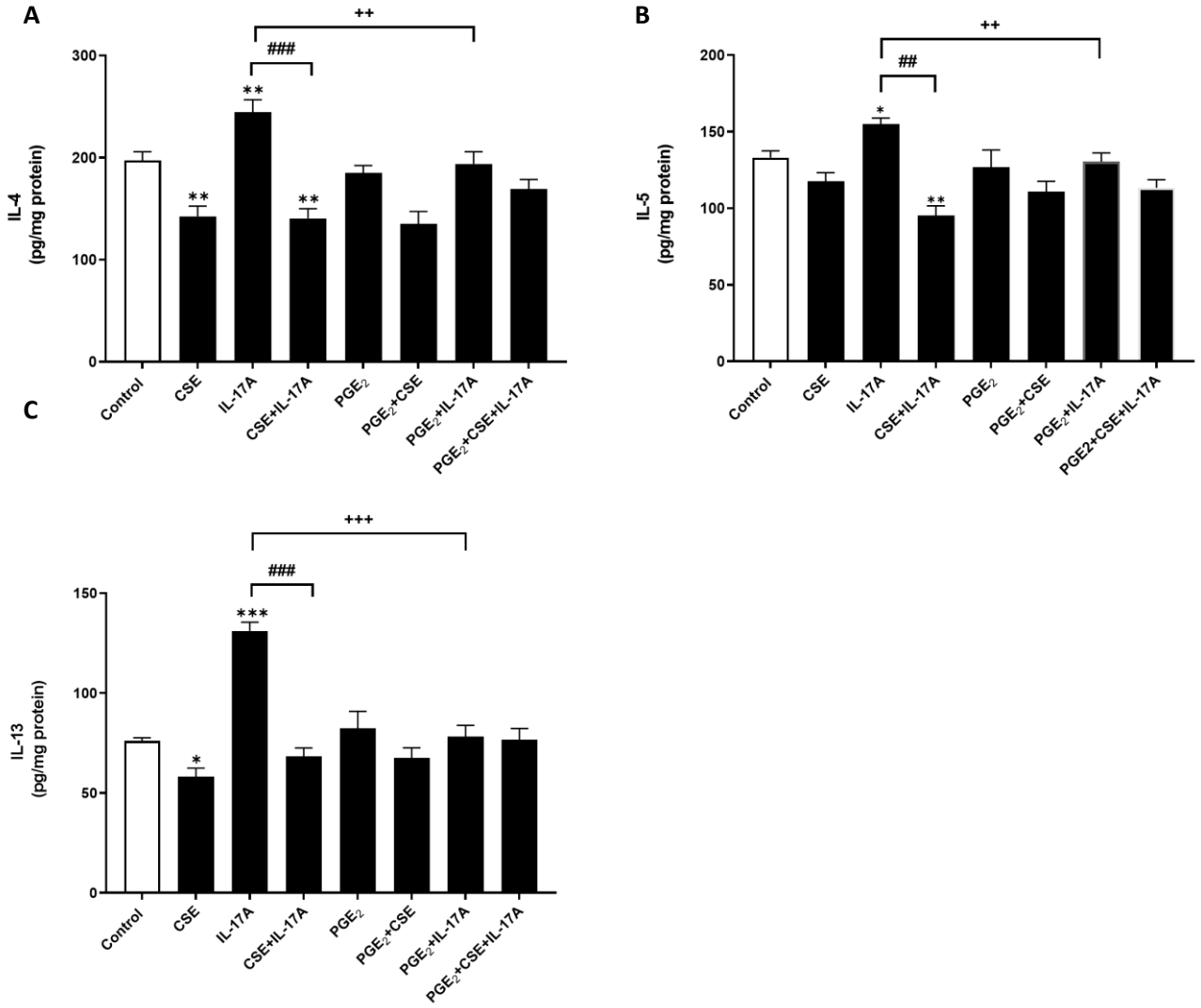

Figure 4.12 Effect of exogenous PGE_2 on the production of Th2 cytokines

Confluent and growth-arrested HASMCs were treated with or without CSE (3.5%), IL-17A (10 ng/ml), CSE+IL-17A, PGE_2 (10 µM), PGE_2+CSE, PGE_2+IL-17A or PGE_2+CSE+IL-17A for 24h. IL-4 (A), IL-5 (B) and IL-13 (C) concentration were measured in the collected supernatants using multiplex assay. Data were normalised to total protein. Each data point represents mean ± SEM from three independent experiments using cells from three different donors. *p<0.05 and ***p<0.001 compared with control, ##p<0.01 and ###p<0.001 compared with IL-17A alone and ++p<0.01 and +++p<0.001 compared IL-17A alone.

4.4.13 Effect of exogenous PGE_2 on the production of eosinophilic chemokines

To explore the role of PGE_2 in mediating the inhibitory effect of CSE, the effect exogenous PGE_2 on the production of eosinophil chemokines in HASMCs was assessed. CSE alone reduced the production of Eotaxin, IP-10 and RANTES compared with control (Figure 4.13). CSE reduced IL-17A-induced production of Eotaxin, IP-10 and RANTES (Figure 4.13). PGE_2 alone reduced the production of Eotaxin by 82.8% compared with control (p<0.01, Figure 4.13 A) but had no effect on the production of IP-10 or RANTES (Figure 4.13 B and C). PGE_2 did not enhance the inhibitory effect of CSE on the production of eosinophilic chemokines. PGE_2 inhibited IL-17A-induced production of Eotaxin, IP-10 and RANTES by 89.6% (p<0.0001), 31.4% (p<0.01) and 17.55% (p<0.01), respectively compared with IL-17A alone (Figure 4.13). PGE_2 did not enhance the CSE inhibition of IL-17A-induced production of eosinophil chemokines compared with CSE and IL-17A treatment (Figure 4.13). Together with the results described in 4.12, these findings demonstrate that exogenous PGE_2 can mimic the effect of CSE on IL-17A-induced production of eosinophil chemokines and strongly supports that the inhibitory effect of CSE on the production of eosinophil chemokines is largely mediated by COX-2/PGE_2 via oxidative stress in HASMCs

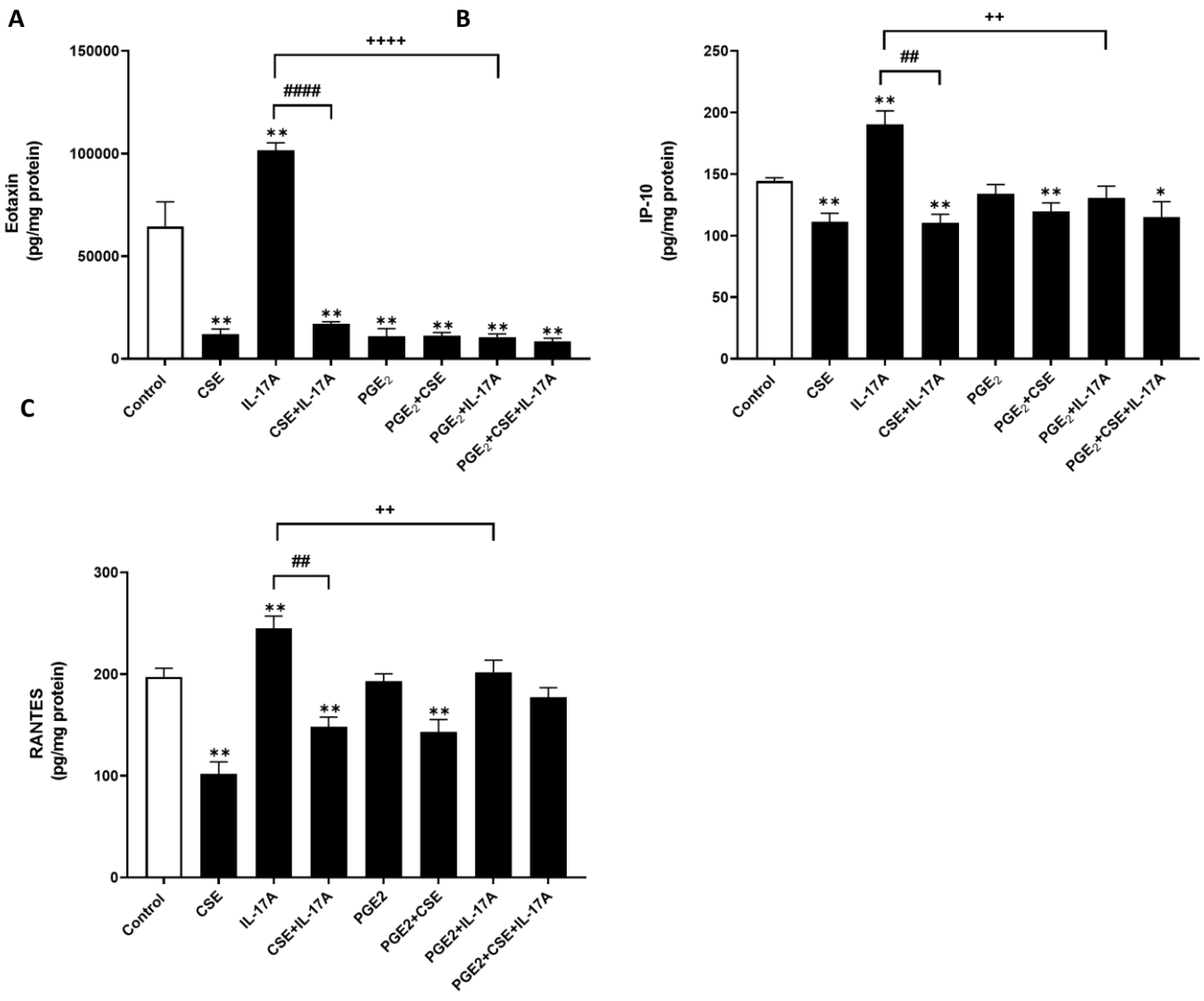

Figure 4.13 Effect of exogenous PGE_2 on the production of eosinophil chemokines

Confluent and growth-arrested HASMCs were treated with or without CSE (3.5%), IL-17A (10 ng/ml), PGE_2 (10 µM), CSE+IL-17A or PGE_2+IL-17A for 24h. The collected supernatants were used to measure Eotaxin (A), IP-10 (B) and RANTES (C) concentration by multiplex assay. Data were normalised to total protein. Each data point represents mean ± SEM from three independent experiments using cells from three different donors. **p<0.01 compared with control, ##p<0.01 and ####p<0.0001 compared with IL-17A alone, ++p<0.0 and ++++p<0.0001 compared with IL-17A alone.

4.4.10 Effect of the prostaglandin EP_2 and EP_4 receptors antagonist on CSE inhibition of Th2 cytokine production

We showed that induction of COX-2 expression by CSE (Figure 4.7) in HASMCs was accompanied by an increase in the production of PGE_2 (Figure 4.9). PGE_2 signals via four known receptors (EP_1–EP_4). PGE_2 is linked to adenylyl cyclase through its receptors EP_2 and EP_4 (257) to increase cyclic AMP production in HASMCs (258). EP_2 and EP_4 receptors were shown to be expressed in HASMCs, with EP_4 being the functionally dominant EP subtype (177). However, whether PGE2 mediates the inhibitory effect of CSE on the production of T2 inflammatory cytokines through EP_2 and EP_4 receptors. Interestingly, PF 04418948 reversed the inhibition of IL-4 and IL-13 production by CSE and increased concentrations of IL-4 and IL-13 by 1.61- (p<0.05) and 1.52-fold (p<0.01), respectively, compared with CSE alone (Figure 4.14 A and C). L-161,982 also blocked the inhibitory effect of CSE on the production of IL-4 and IL-13 and increased concentrations of IL-4 and IL-13 by 1.58- (p<0.05) and 1.45-fold (p<0.05), respectively compared with CSE alone (Figure 4.14 A and C). PF 04418948 and L-161,982 had no effect on IL-17A-induced production of Th2 cytokines. PF 04418948 blocked the inhibitory effect of CSE on IL-17A-induced production of IL-4 and IL-5 (fold change 1.1 and 1.25, respectively, Figure 4.14 A and B) but had no effect on IL-13 production. L-161,982 blocked the inhibitory effect of CSE on IL-17A-induced production of IL-4, IL-5 and IL-13 (fold change 1.5, 1.47 and 1.49, respectively, Figure 4.14). These findings confirm that PGE_2 mediates the effect of CSE on the production of Th2

cytokines through EP_2 and EP_4 receptors and suggest that downstream cAMP could play a role in mediating the inhibitory effect of CSE.

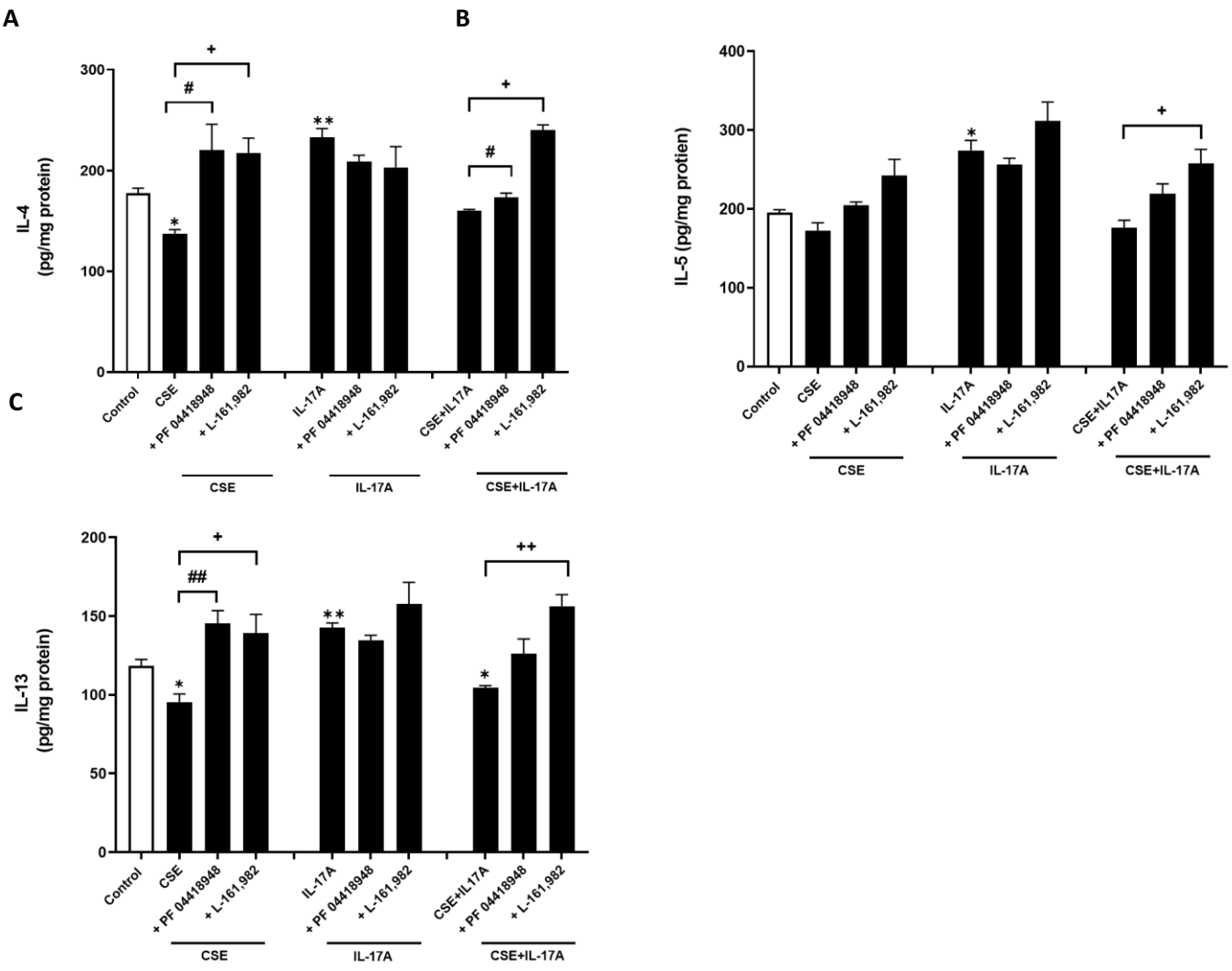

Figure 4.14 Effect of the prostaglandin EP_2 and EP_4 receptors antagonist on CSE inhibition of Th2 cytokine production

Confluent and growth-arrested 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 prior to incubation with or without CSE (3.5%), IL-17A (10 ng/ml), CSE+IL-17A for 24h. IL-4 (A), IL-5 (B) and IL-13 (C) concentrations were measured in the collected supernatants using a multiplex assay. Data were normalised to total protein. Each data point represents mean ± SEM from three independent experiments using cells from three different donors. *p<0.05 and ***p<0.01 compared with control, #p<0.05 and ##p<0.01 compared +PF 04418948 with CSE or CSE+IL-17A and +p<0.05 and ++p<0.01 compared +L-161,982 with CSE or CSE+IL-17A.

4.4.11 Effect of the prostaglandin EP_2 and EP_4 receptors antagonist on CSE inhibition of eosinophilic chemokine production

The above results (4.4.14) showed that PF 04418948 and L-161,982 reversed the inhibition of Th2 cytokine production by CSE. However, whether PF 04418948 and the L-161,982 can block the inhibitory effect of CSE on the production of eosinophil chemokines Eotaxin, IP-10 and RANTES is unknown. CSE inhibited the production of Eotaxin, IP-10 and RANTES alone and with IL-17A stimulation (Figure 4.15). PF 04418948 reversed the inhibition of Eotaxin, IP-10 and RANTES production by CSE compared with CSE alone (fold change 5.53, 1.40, and 1.21, respectively, Figure 4.15). L-161,982 also blocked the inhibitory effect of CSE and increased concentrations of Eotaxin, IP-10 and RANTES by 7.15, 1.39 and 1.72-fold, respectively, compared with CSE alone (Figure 4.15). PF 04418948 blocked the inhibitory effect of CSE on IL-17A-induced production of Eotaxin, IP-10 and RANTES (fold change 7.25, 1.18 and 1.37, respectively, compared with CSE+IL-17A, Figure 4.15). L-161,982 also blocked the inhibitory effect of CSE on IL-17A-induced production of Eotaxin, IP-10 and RANTES and increased their concentrations by 7.6, 1.5 and 1.49-fold compared with CSE+IL-17A (Figure 4.15). These findings strongly suggest that the inhibitory effect of CSE on the production of eosinophil chemokines is largely mediated by COX-2/PGE_2/EP_2/EP_4 pathway via oxidative stress and may suggest that downstream cAMP could play a role in mediating the inhibitory effect of CSE.

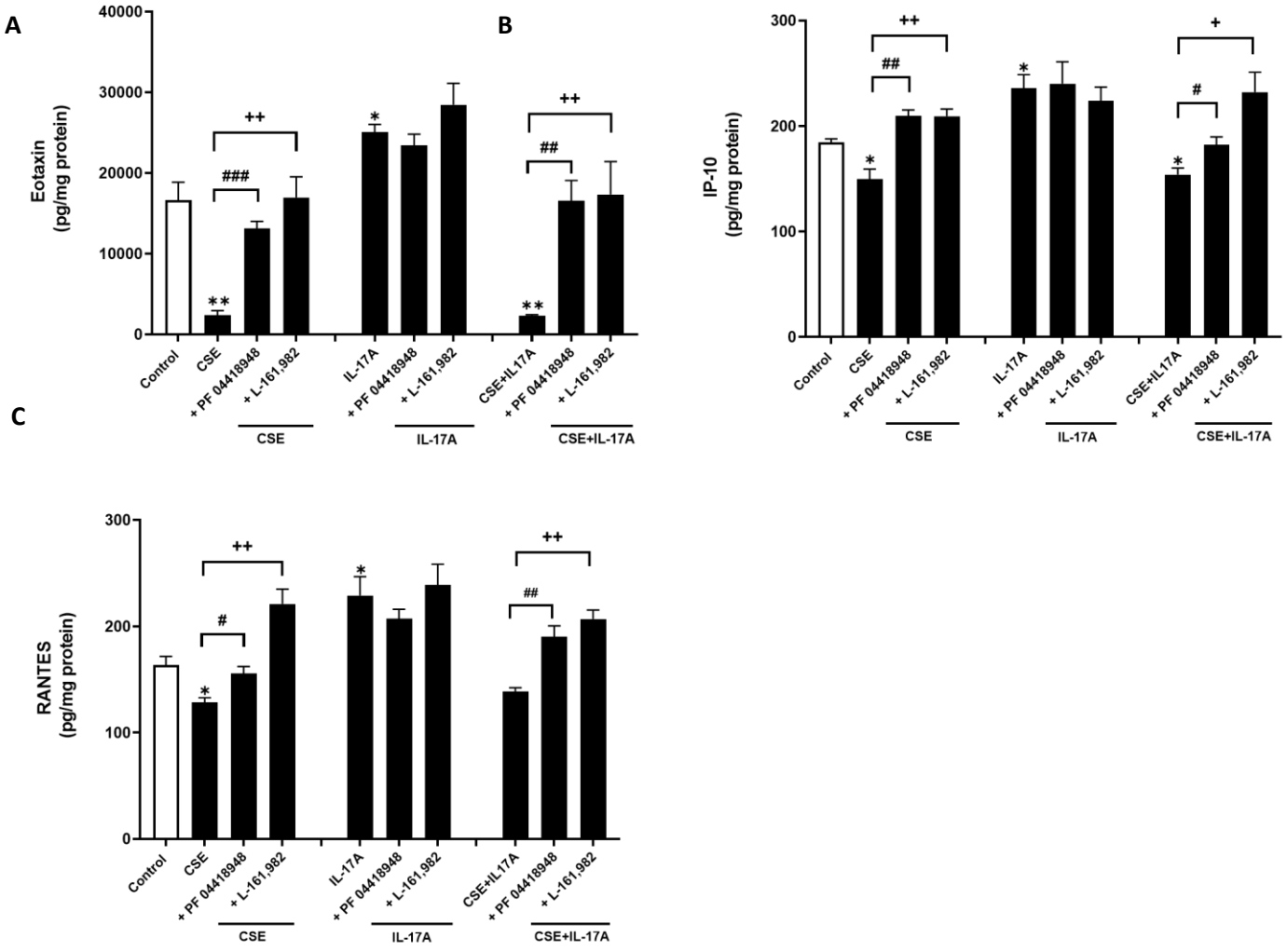

Figure 4.15 Effect of prostaglandin EP_2 and EP_4 receptors antagonist on CSE inhibition of eosinophilic chemokine production

Confluent and growth-arrested 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 prior to incubation with or without CSE (3.5%), IL-17A (10 ng/ml), CSE+IL-17A for 24h. The collected supernatants were used to measure Eotaxin (A), IP-10 (B) and RANTES (C) concentration by multiplex assay. Data were normalised to total protein. Each data point represents mean ± SEM from three independent experiments using cells from three different donors. *p<0.05 and **p<0.01 compared with control, #p<0.05 and ##p<0.01 and ###p<0.001 compared +PF 04418948 with CSE or CSE+IL-17A and +p<0.05, ++p<0.01 and +++p<0.001 compared +L-161,982 with CSE or CSE+IL-17A.

4.4.12 Effect of 8-Bromo-cAMP on the production of Th2 cytokines

We showed in 4.4.14 and 4.4.15 that PGE_2 mediates the inhibitory effect of CSE on the production of T2 inflammatory cytokines through its EP_2 and EP_4 receptors therefore we explored whether cAMP is playing a role in mediating the inhibitory effect of CSE. Therefore, we assessed the effect of 8-Bromo, a cell-permeable cAMP analogue, on the production of Th2 cytokines and IL-17A-induced production of Th2 cytokines. As expected, CSE inhibited the production of IL-4 and IL-13 compared with control, but not IL-5, on its own and with IL-17A stimulation (Figure 4.16). 8-Bromo alone had no effect on the production of Th2 cytokines compared with control (Figure 4.16). 8-Bromo did not further reduce the CSE inhibition of the production of Th2 cytokines compared with CSE alone (Figure 4.16). Interestingly, like CSE, 8-Bromo reduced the production of IL-4, IL-5 and IL-13 induced by IL-17-A by 18.64% ($p<0.05$), 17.82% ($p<0.01$) and 23.14% ($p<0.01$), respectively (Figure 4.16). 8-Bromodid not enhance the inhibitory effect of CSE on IL-17A-induced production of Th2 cytokines compared with CSE+IL-17A (Figure 4.16). Taken together, our findings suggest that the inhibitory effect of CSE on the production of Th2 cytokines is mediated through COX-2/PGE_2/EP_2/EP_4/cAMP pathway via oxidative stress.

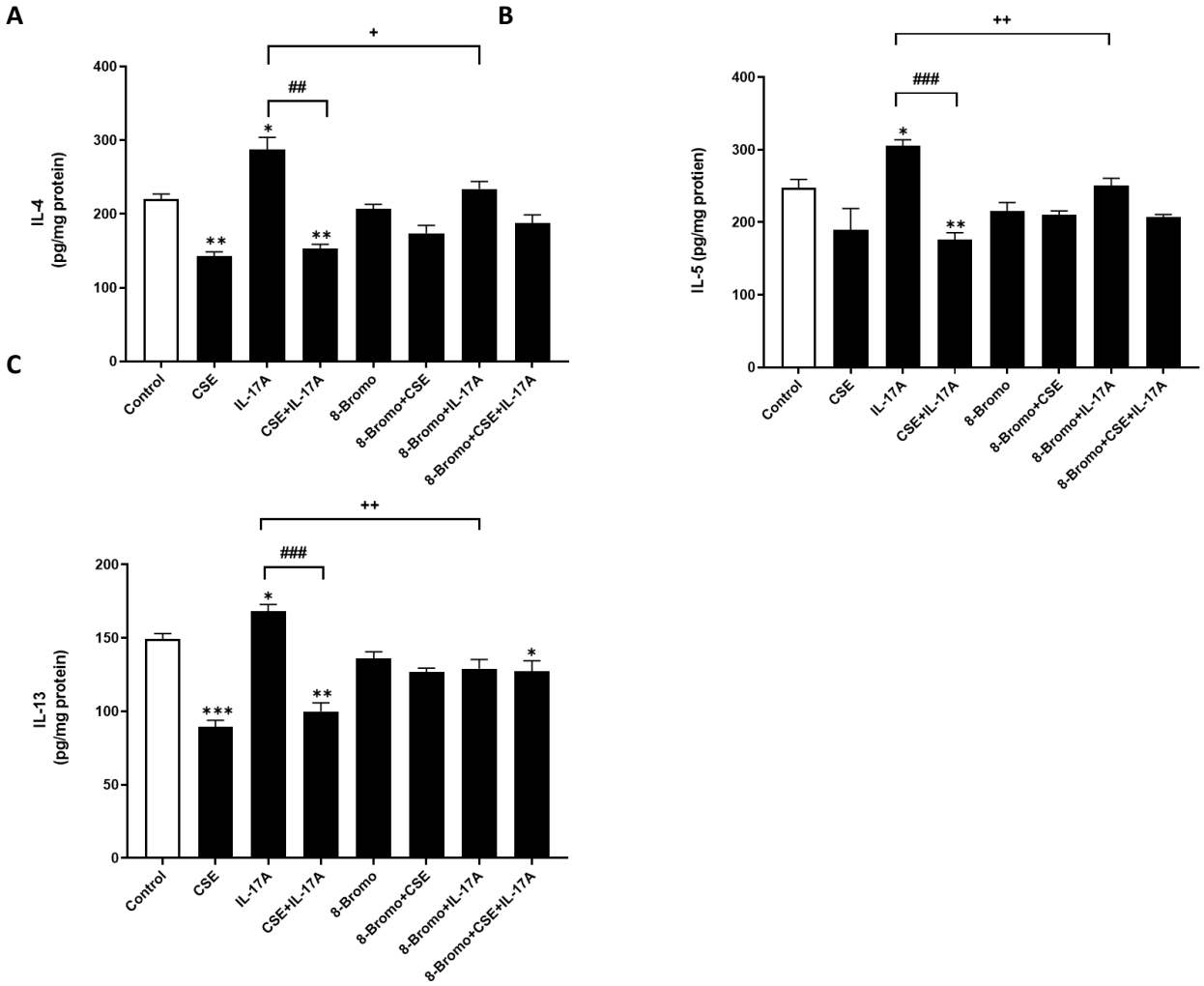

Figure 4.16 Effect of 8-Bromo-cAMP on the production of Th2 cytokines

Confluent and growth-arrested HASMCs were treated with or without 8-Bromo-cAMP (100 µM), CSE (3.5%), IL-17A (10 ng/ml), CSE+IL-17A or 8-Bromo+IL-17A for 24h. IL-4 (A), IL-5 (B) and IL-13 (C) concentration were measured in the collected supernatants using multiplex assay. Data were normalised to total protein. Each data point represents mean ± SEM from three independent experiments using cells from three different donors. **p*<0.05, ***p*<0.01 and ****p*<0.001 compared with control, ##*p*<0.01 and ###*p*<0.001 compared IL-17A alone and +*p*<0.05 and ++*p*<0.01 compared IL-17A alone.

4.4.13 Effect of 8-Bromo-cAMP on the production of eosinophil chemokines

To further confirm the role of cAMP in the inhibitory effect of CSE on the production of T2 inflammatory cytokines, we assessed the effect of 8-Bromo-cAMP on the production of eosinophil chemokines Eotaxin, IP-10 and RANTES with and without CSE and IL-17A. As shown before, CSE inhibited the production of Eotaxin, IP-10 and RANTES with and without IL-17A stimulation (Figure 4.17). 8-Bromo alone reduced production of Eotaxin, but not IP-10 and RANTES, by 91.6% compared with the control (Figure 4.17). 8-Bromo did not enhance the inhibitory effect of CSE on the production of eosinophil chemokines compared with CSE alone. Interestingly, 8-Bromo reduced IL-17A-induced production of Eotaxin, IP-10 and RANTES by 89.02% ($p<0.05$), 11.37% ($p<0.01$) and 9.47% ($p<0.01$), respectively (Figure 4.17). 8-Bromo did not enhance the CSE inhibition of IL-17A-induced production of eosinophil chemokines compared with CSE and IL-17A (Figure 4.17). Together with previous results, these findings strongly suggest that the inhibitory effect of CSE on the production of eosinophil chemokines is mediated by COX-2/PGE_2/EP_2/EP_4/cAMP pathway via oxidative stress.

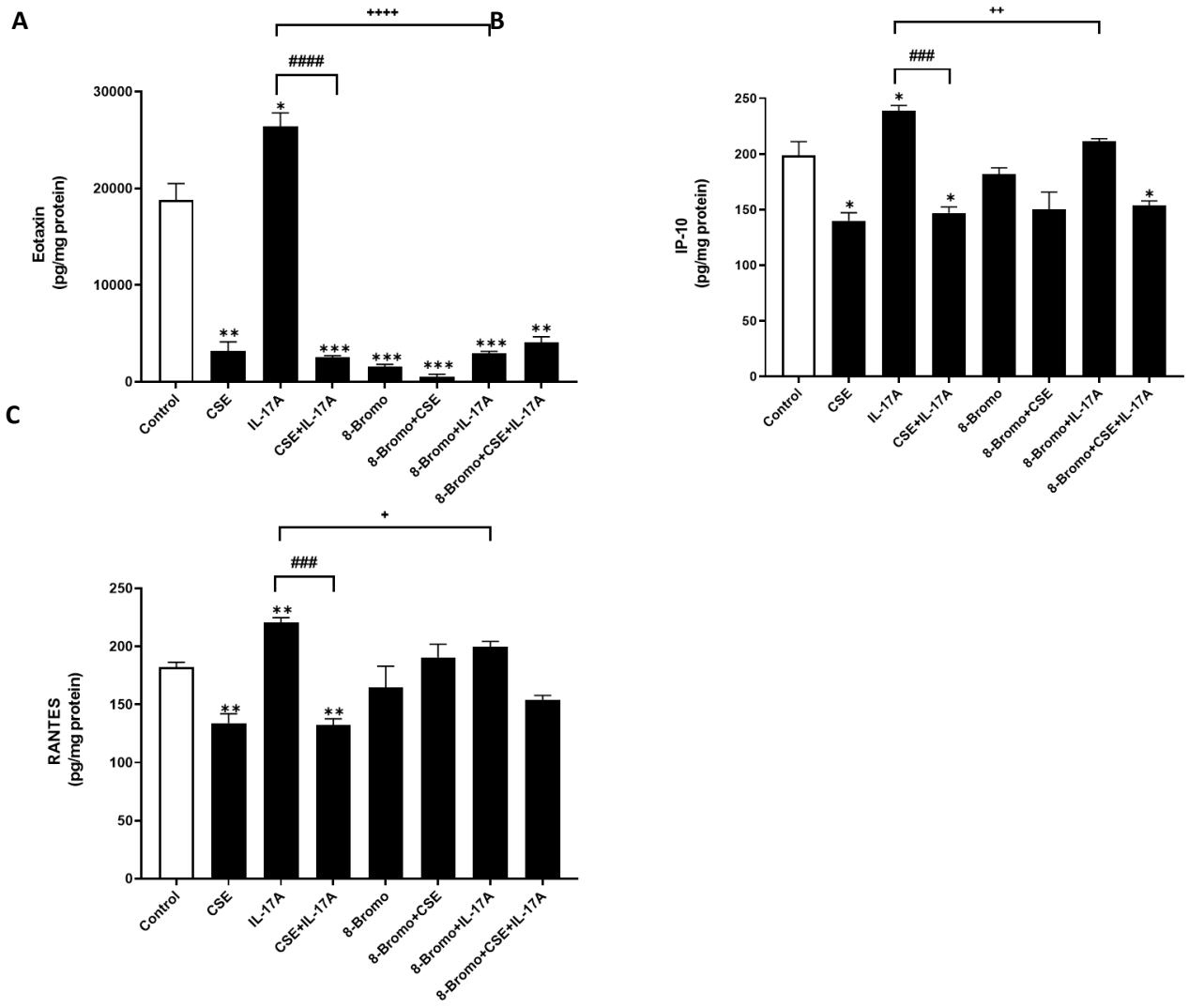

Figure 4.17 Effect of 8-Bromo-cAMP on the production of eosinophil chemokines

Confluent and growth-arrested HASMCs were treated with or without 8-Bromo-cAMP (100 µM), CSE (3.5%), IL-17A (10 ng/ml), CSE+IL-17A or 8-Bromo+IL-17A for 24h. Eotaxin (A), IP-10 (B) and RANTES (C) concentration were measured in the collected supernatants using multiplex assay. Data were normalised to total protein. Each data point represents mean ± SEM from three independent experiments using cells from three different donors. *p<0.05, **p<0.01 and ***p<0.001 compared with control, ###p<0.001 and ####p<0.0001 compared IL-17A alone and +p<0.05, ++p<0.01 and ++++p<0.0001 compared with IL-17A alone

4.5 Discussion

We showed in the chapter 3 that CSE suppressed the production of T2 inflammatory cytokines and increased the production of non-T2 inflammatory cytokines, suggesting that CSE may shift the airway inflammation from T2 to non-T2. This study investigated the molecular mechanisms by which CSE reduced the production of T2 inflammatory cytokines with and without IL-17A stimulation in HASMCs. This chapter presented evidence for the first time that the inhibitory effect of CSE on the production of T2 inflammatory cytokines is largely mediated through COX-2/PGE_2/EP_2/EP_4/cAMP pathway via oxidative stress. Table 4.1 summarised all the drugs effect on the inhibitory effect of CSE in addition to the effect of exogenous PGE_2 and 8-bromo production of T2 inflammatory cytokines.

Table 4.1 Summary for the drugs effect

Cytokine	IL-4	IL-5	IL-13	Eotaxin	IP-10	RANTES
Effect of GSH on the inhibitory effect of CSE	↔	↔	↑	↑	↑	↑
Effect of GSH on the inhibitory effect of CSE with IL-17A stimulation	↑	↑	↔	↑	↑	↑
Effect of NS-398 on the inhibitory effect of CSE	↑	↔	↑	↑	↑	↑
Effect of NS-398 on the inhibitory effect of CSE with IL-17A stimulation	↑	↔	↑	↑	↑	↑
Effect of exogenous PGE_2	↔	↔	↔	↓	↔	↔
Effect of exogenous PGE_2 with IL-17A stimulation	↓	↓	↓	↓	↓	↓
Effect of PF 04418948 on the inhibitory effect of CSE	↑	↔	↑	↑	↑	↑
Effect of PF 04418948 on the inhibitory effect of CSE with IL-17A stimulation	↑	↔	↔	↑	↑	↑
Effect of L-161,982 on the inhibitory effect of CSE	↑	↔	↑	↑	↑	↑
Effect of L-161,982 on the inhibitory effect of CSE with IL-17A stimulation	↑	↑	↑	↑	↑	↑
Effect of 8-Bromo	↔	↔	↔	↓	↔	↔
Effect of 8-Bromo with IL-17A stimulation	↓	↓	↓	↓	↓	↓

↑ = Reversed/ increased ↓ = Reduced ↔ = No change/had no effect

4.5.1 Cigarette smoke, nicotine, and oxidative stress

Nicotine is the major active substance of CS and classically acts via nicotinic acetylcholine receptors (nAChRs). It has been shown that α7nAChR is the most nicotinic receptor expressed in HASMCs, with increased expression of α7nAChR in asthmatics and smokers (230). Our study demonstrated that the inhibitory effect of CSE on the production of T2 inflammatory cytokines is not mediated by nicotine by using nicotine and the α7nAChR antagonist MG624. In contrast with our finding, an *in vivo* study showed that nicotine inhibited allergen ragweed antigen-induced mRNA expression of Th2 cytokines IL-4, IL-5 and IL-13 in rats (259). Moreover, in the BAL, the protein content of IL-4 and IL-13 was significantly less in nicotine and ragweed antigen (NRW) mice (259). In previous *in vivo* study (259) 1 mg of nicotine per kg (body weight) per day to achieve a plasma nicotine level of around 28 ng/ml which is nearly equivalent to the plasma nicotine level of a one-pack per day smoker. These differences from our findings could be justified by different experimental models (*in vitro* vs *in vivo*) and nicotine administration and exposure time (nicotine was administered for 7 days prior to exposure to an allergen). To the best of our knowledge, this is the first study to investigate the role of nicotine on the production of T2 inflammatory cytokines in airway structural cells, including HASMCs.

CS can induce oxidative stress directly by the free radicals in smoke or indirectly by the inflammatory response induced by CS (260). CSE

increased the heme-oxygenase-1, an intracellular indicator of oxidative stress, in HASMCs (125). Chronic CSE exposure significantly increased the expression of the antioxidant protein Manganese Superoxide Dismutase (Mn-SOD), a marker of oxidative stress, in human bronchial epithelial cell line BEAS-2B (261). Acute exposure to CSE also increased oxidative stress in human alveolar epithelial cell line A549 (262). These studies could illustrate that CSE causes increased oxidative stress which can contribute to airway inflammation and lung damage in smokers. Our results support the theory in the literature that oxidative stress plays an important role in smoke-related lung diseases. We demonstrated an important finding that the oxidative stress inhibitor GSH reversed the inhibitory effect of CSE on most of the production of T2 inflammatory cytokines, suggesting that oxidative stress plays a role in mediating the inhibitory effect of CSE on the production of T2 inflammatory cytokines. In fact, we showed that GSH did not reverse the CSE inhibition of IL-4 and IL-13 production on its own and after IL-17A stimulation respectively. This differences from the rest of T2 inflammatory cytokines could be generally due to the relatively low production of Th2 cytokines in HASMCs as previous study have also showed that Th2 cytokines IL-5 and IL-13 are expressed at low levels in HASMCs (32). Although we did not see a significant reversion of inhibition of CSE on these Th2 cytokines by GSH, but we observed a trend of increase in the cytokine concentration, and this could be due to the number of cell lines of HASMCs (3 cell lines) we used in this study. In contrast with our findings, a study (112) showed that GSH did not reverse the inhibition of Eotaxin and RANTES production by CSE in HASMCs. These differences can be explained by the

different cigarettes they used (Full 4 strength Marlboro cigarettes), and GSH (100 μM) was used for 30 min before cells were exposed to CSE, while in our study, we used research cigarettes (3R4F research-grade cigarettes) and the cells were pre-treated with GSH (100 μM) for 1h prior to CSE stimulation. Furthermore, the genetic and phenotypic cell-to-cell heterogeneity could be another reason for this contradiction.

CS-mediated oxidative stress enhances the inflammatory response in the airways by stimulating the release of proinflammatory cytokines (263). A study showed that GSH inhibited CSE-induced production of IL-8 in HASMCs (112), suggesting that the effect of CSE on the production of IL-8 is dependent on oxidative stress. The role of oxidative stress in mediating the inhibitory effect of CSE on T2 inflammatory cytokine production in our study and the pro-inflammatory effect of CSE on the non-T2 cytokine IL-8 production in the previous study (112) may suggest that oxidative stress caused by CSE could shift the airway inflammation from T2 to non-T2.

4.5.2 Cigarette smoke, COX-2 and PGE_2

We believe that we are the first to report that the inhibitory effect of CSE on the production of T2 inflammatory cytokines is mediated through the COX-2 pathway in HASMCs by using the selective COX-2 inhibitor NS-398. We also provide direct evidence that CSE induced mRNA and protein expression of COX-2 and PGE_2 synthesis which strongly support the concept that the inhibitory effect of CSE on the production of T2 inflammatory cytokines may be largely mediated by COX-2 expression and PGE_2 release in HASMCs. Our finding that CSE induced protein and mRNA expression of COX-2, suggesting that CSE may upregulate COX-2 gene expression via

transcriptional mechanism. In agreement with our findings, a study showed that CSE-induced COX-2 protein expression and PGE_2 production in HASMCs by CSE, and the PGE_2 induction was inhibited by pre-treatment with the inhibitor of COX-2 NS-398 (168), suggesting that CSE stimulated PGE_2 production by CSE is dependent on COX-2 expression and/or activity. In support of our finding, a study conducted by our group showed that CSE induced COX-2 expression in human pulmonary artery smooth muscle cells (PASMCs) (167). Our study is the first to link the upregulation of COX-2 with mediating the inhibitory effect of CSE inflammatory on T2 inflammatory cytokines in HASMCs.

We have further confirmed the role of PGE_2 in mediating the inhibitory effect of CSE on the production of T2 inflammatory cytokines by exposing HASMCs to exogenous PGE_2. We showed that exogenous PGE_2 can mimic the inhibitory effect of CSE on IL-17A-induced T2 inflammatory cytokine production and strongly supports that the inhibitory effect of CSE on the production of T2 inflammatory cytokine is largely mediated by COX-2/PGE2. Although COX-2/PGE2 largely mediates the inhibitory effect of CSE on the production of T2 inflammatory cytokines, The exogenous PGE_2 alone had no effect on the production of all tested T2 inflammatory cytokines except eotaxin compared with CSE. These differences between CSE and exogenous PGE_2 effect on the production of T2 inflammatory cytokines suggest that there are other mechanisms in addition to PGE2 could be involved in mediating the inhibitory effect of CSE on the production of T2 inflammatory cytokines. Since exogenous PGE_2 did not further amplify the inhibitory effect of CSE on production of T2 inflammatory cytokines with or

without IL-17A, suggesting that the induction of PGE_2 is properly high enough so even with exogenous PGE_2 we could not see further increase of inhibitory effect of CSE. In agreement with our finding that exogenous PGE_2 reduced T2 inflammatory response, an *in vivo* study showed that PGE_2 administered by intratracheal insufflation of (1µg) reduced allergen-induced airway eosinophilia in rats (264). Our hypothesis that PGE_2 reduced T2 inflammatory response is also supported by a study that showed PGE_2 suppressed Th2 cells activation and attenuated eosinophil trafficking (265). The same study (265) showed that PGE_2 had a direct inhibitory effect on the human eosinophil migration. Moreover, another study agreed with our results showed that PGE_2 reduced production of IL-5 and IL-13 induced by IL-25, IL-33, TSLP and IL-2 in Group 2 innate lymphoid cells (ILC2s) (24), suggesting that PGE_2 could suppress the T2 airway response by inhibiting the production of Th2 cytokines. Our findings are also supported by clinical study which showed that inhaled PGE_2 inhibited allergen induced T2 airway responses and inflammation, when given immediately prior to inhaled allergen. Taken together, these studies support our finding that exogenous PGE_2 has a similar inhibitory effect as CSE on the production of T2 inflammatory cytokines, and strongly supports that the inhibitory effect of CSE on the production of eosinophil chemokines is largely mediated by COX-2/PGE_2 in HASMCs.

An interesting finding by our group showed that GSH inhibited COX-2 protein expression induced by CSE and in addition to that the current study demonstrated for the first time that CSE-induced PGE_2 production is also inhibited by GSH in HASMCs, which suggest that oxidative stress plays a

role in mediating CSE-induced COX-2 expression and PGE_2 release. In agreement with this finding a clinical study demonstrated that GSH inhalation in cystic fibrosis (CF) patients reduced PGE_2 levels bronchoalveolar lavage fluid (BALF) (266).

PGE_2 signals via four known receptors (EP_1–EP_4), with the cAMP signalling pathway responsible for major suppressive and regulatory functions of PGE_2, mostly through EP_2 and EP_4 receptors (256). In our study, interestingly, EP_2 and EP_4 antagonists restored the production of T2 inflammatory cytokines inhibited by CSE alone. EP_2 receptor antagonist reversed the inhibitory effect of CSE on IL-17A-induced production of T2 inflammatory cytokines except IL-5 and IL-13, while EP_4 receptor antagonist reversed the inhibitory effect of CSE on IL-17A-induced production of all T2 inflammatory cytokines. Although we did not see a significant reversion by the EP_2 receptor antagonist of the Th2 cytokine IL-5 and IL-13 production inhibited by CSE, but we have observed a trend of increase, this could be due to number of cell lines of HASMCs (3 cell lines) we have used. Thus, increasing sample size could provide statistical power to help minimise variations among cellular responses.In support of our findings, a study showed that PGE_2 suppressed Th2 cells activation and attenuated eosinophil trafficking via EP_2 and EP_4 receptors (265). In the previous study (265), PGE_2 inhibited the mobilisation of bone marrow eosinophils induced by IL-5 and prevented the allergen-induced recruitment of eosinophils into the lungs via its EP_2 receptor. In line with our findings, blocking EP_2 and EP_4 receptors reversed the suppressing effect of PGE_2 on the production of Th2 cytokines IL-5 and IL-13 induced by IL-25, IL-33, TSLP and IL-2 in ILC2s

(267). Interestingly, in the same study (267), they have shown that activation of selective EP4 receptor have more profound suppressive effects than activation of EP2 receptor. In agreement with our finding that the EP_2 receptor antagonist did not reverse the inhibitory effect of CSE on IL-17A-induced the production of IL-5 and IL-13. Moreover, a study demonstrated that EP_2 and EP_4 receptors are functionally important in HASMCs and mediate cAMP generation and also showed that EP_4 is the predominant EP receptor subtype driving PGE_2-mediated signalling and proliferation (177). Our study findings confirm that PGE_2 mediates the effect of CSE on T2 inflammatory response through EP_2 and EP_4 receptors and suggest that downstream cAMP could play a role in mediating the inhibitory effect of CSE.

We demonstrated an important finding that cAMP analogue 8-Bromo had a similar effect as CSE on IL-17A-induced the production of T2 inflammatory cytokines, suggesting that downstream cAMP plays a role in mediating the inhibitory effect of CSE on the production of T2 inflammatory cytokines. This finding is consistent with a study that showed that 8-Bromo resulted in a decrease in IL-1β induced Eotaxin production and mRNA expression compared with IL-1β alone in HASMCs (184), suggesting that cAMP may play a role in the inhibition of production of Eotaxin. In line with our findings, another study demonstrated that 8-Bromo significantly inhibited TNF-α-induced production of Eotaxin in HASMCs (268). These findings support our results that 8-bromo had an inhibitory effect on IL-17A-induced production of T2 inflammatory cytokines, but our study is the first to link the effect of 8-bromo could mediate the inhibitory effect of CSE on production of T2 inflammatory cytokines. Our results showed that 8-Bromo had a weaker

inhibitory effect on the production of T2 inflammatory cytokines compared with CSE, suggesting that something else in addition to cAMP pathway could play a role in mediating the inhibitory effect of CSE. 8-bromo did not further increase the inhibitory effect of CSE on production of T2 inflammatory cytokines with or without IL-17A. This finding can be due that CSE produced enough PGE_2 and as result it increased cAMP to certain level which no longer can generate stronger inhibitory effect on production of T2 inflammatory cytokines. Our study provides new insights into understanding the underlying mechanisms by which CS may shift the airway inflammation in asthma from T2 to non-T2 through COX-2/PGE_2/EP_2/EP_4/cAMP pathway via oxidative stress (Figure 4.18) and may thereby induce glucocorticoid insensitivity.

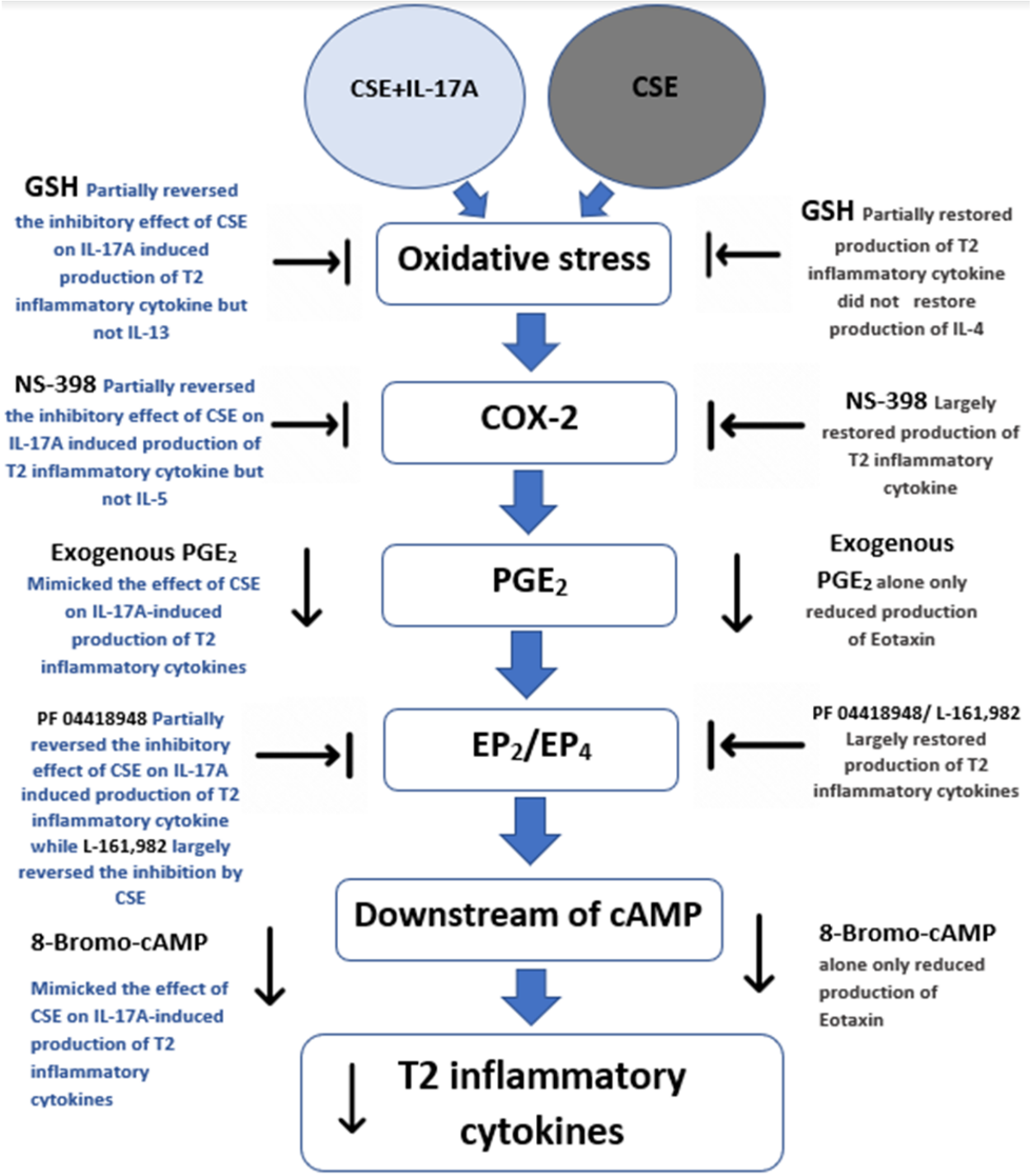

Figure 4.18 Mechanisms of CSE effect on T2 inflammatory response in HASMCs

This diagram illustrates mechanism mediating inhibitory effect of CSE on production of T2 inflammatory cytokines and on IL-17A induced production of these cytokines. We showed that oxidative stress plays a role in mediating the inhibitory effect of CSE by applying the oxidative stress inhibitor GSH. We also showed that

the inhibitory effect of CSE on the production of T2 inflammatory cytokines is largely mediated by COX-2 expression by using the COX-2 inhibitor NS-398. Then, we provide direct evidence that CSE induced COX-2 expression and PGE_2 synthesis which strongly support that the inhibitory effect of CSE on the production of T2 inflammatory cytokines is largely mediated by COX-2/PGE_2. We demonstrate that exogenous PGE_2 can mimic the effect of CSE on production of T2 inflammatory cytokines through its EP_2/EP_4 receptors by applying EP_2 and EP_4 receptor antagonist PF 04418948 and L-161,982, respectively. Finally, we showed that downstream cAMP also plays a role in mediating the inhibitory effect of CSE by using cAMP analogue 8-Bromo.

Chapter 5. Impact of cigarette smoke extract on the anti-inflammatory effect of glucocorticoids in human airway smooth muscle cells

5.1 Introduction

The non-T2 endotype of asthma is associated with neutrophilic airway inflammation as well as increased Th17 cytokines (20, 83). Cigarette smoke (CS) has been linked with non-T2 airway inflammation in asthma compared with asthmatic patients who have never smoked (236, 269). We have shown in chapter 3 that CSE may shift the airway inflammation in asthma from T2 to non-T2. CS is a significant inflammatory factor in the airways and has been shown to promote non-T2 airway inflammation in asthmatic smokers (219, 236). Th17 cytokines also play a crucial role in non-T2 airway response and the development of severe forms of asthma, which known to be insensitive to glucocorticoids treatment (270). Glucocorticoids are the main treatment for chronic inflammatory diseases including asthma (192). Studies showed that asthmatic smokers do not respond to glucocorticoids as effectively as non-smokers (220-223). We have demonstrated in chapter 3 that CSE may shift the airway inflammation from T2 to non-T2 by supressing the production of T2 inflammatory cytokines and promoting the production of non-T2 inflammatory cytokines in HASMCs which may thereby lead to glucocorticoid insensitivity. This chapter explored the role of CSE and IL-17A in non-T2 airway inflammation by inducing the production of non-T2 chemokines and their inhibition by fluticasone. The main aim of the chapter was to determine if CSE influences the inhibition of IL-17A-induced non-T2 chemokine production by glucocorticoids.

5.2 Aim

To investigate whether fluticasone can inhibit CSE and IL17A induced production of non-T2 chemokine IL-8.

5.3 Methods

CSE was prepared from the smoke of two cigarettes bubbled into 20 ml of cell culture medium. Human airway smooth muscle cells (HASMCs) were pre-treated with and without CSE (3.5%) for 24h. Cells were then treated with fluticasone (10^{-11}-10^{-6} M) for 1 hour before incubation with CSE, IL-17A (10 ng/ml), or CSE + IL17A for 24h. The non-T2 chemokines IL-8 concentration were measured in supernatants by ELISA. The results from different experiments were analysed to calculate IC_{50} using GraphPad Prism software. The drug concentrations were converted to $\log_{10}$ scales and plotted on X-axis, while the normalised response of IL-8 and GRO-α production (as 100%) was plotted on Y-axis. Non-linear regression analysis in the software was used to generate the curve and IC_{50} values.

5.4 Results

5.4.1 Effect of fluticasone on CSE- and IL-17A-induced the production of IL-8

In this study, we aimed to assess the inhibitory effect of fluticasone on CSE-induced neutrophilic IL-8 production in HASMCs. Confirming observation from Chapter 3, a 15.2-fold increase was observed in IL-8 concentration after stimulation with CSE (9627.1 pg/mg protein) compared with the control (633.3 pg/mg protein) (p<0.001, Figure 5.4.1 A). Fluticasone inhibited CSE-induced IL-8 production in a concentration-dependent manner with significant inhibition by 78.8% seen at fluticasone concentration of 10^{-9} M compared to CSE (p<0.01, Figure 5.4.1 A). Maximum inhibition was achieved with the highest concentration of fluticasone 10^{-6} M compared with the control (IL-8 concentration 683.8 and 633.3 pg/mg protein, respectively). These findings suggest that fluticasone can inhibit CSE increased IL-8 production in HASMCs.

In this study, we also investigated the inhibitory effect fluticasone on IL-17A-induced IL-8 production in HASMCs. IL-8 concentration was increased by 4.4-fold after stimulation with IL-17A (4142.2 pg/mg protein) over the control (943.5 pg/mg protein, Figure 5.4.1 B), confirming the findings in Chapter 3. IL-17A-induced IL-8 production was inhibited by fluticasone in a concentration-dependent manner with a significant inhibition of 31.1% seen at a concentration of 10^{-10} M compared to IL-17A (p<0.05). The maximum inhibition of IL-17A-induced IL-8 by fluticasone (74.1%) was seen at a concentration of 10^{-6} M (1074.2 pg/mg protein) compared with the

control (Figure 5.4.1 B). The findings suggest that fluticasone can inhibit IL-17A induce IL-8 production in HASMCs.

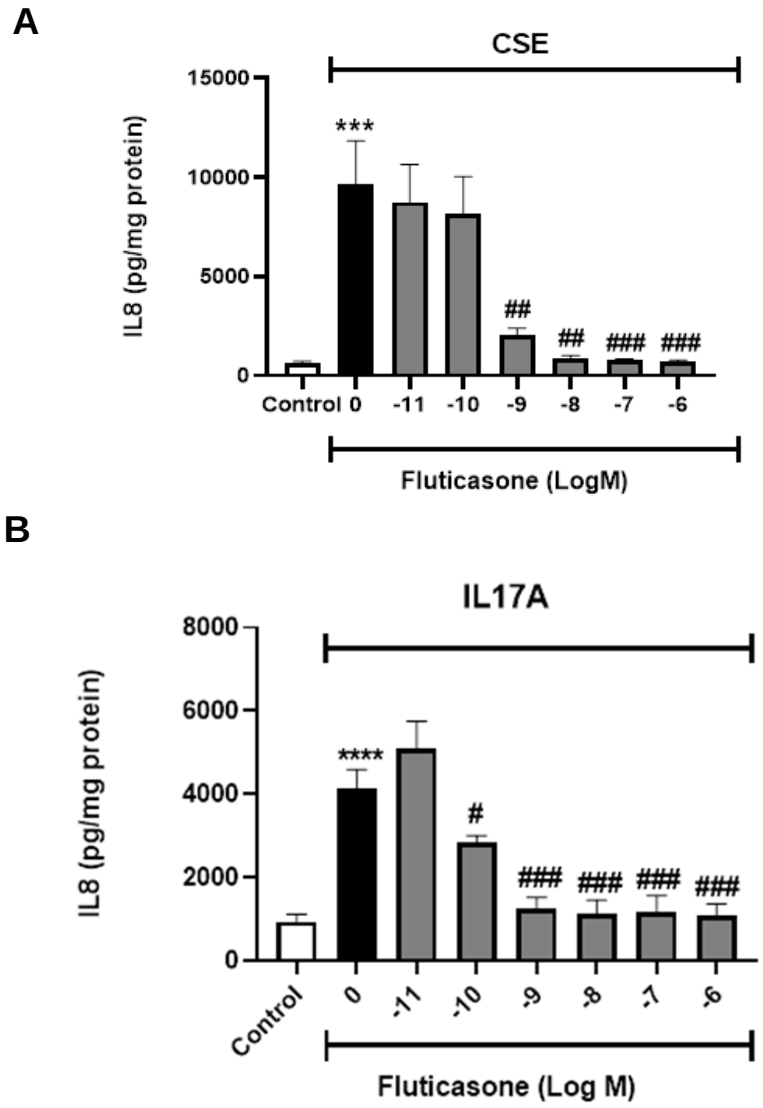

Figure 5.4.1 Effect of fluticasone on CSE- and IL-17A-induced the production of IL-8

Confluent and growth-arrested HASMCs were pre-treated with or without CSE (3.5%) for 24h prior to treatment with an increasing concentration of fluticasone (10^{-11}-10^{-6} M) for 1h and after that, cells were treated with CSE or IL-17A for 24h. The collected supernatants were used to measure IL-8 concentration by ELISA. Data were normalised to total protein. Each data point represents the mean ± SEM of three experiments carried out in triplicate using cells from three different donors. ***p<0.001 and ****p<0.0001 compared with control (unstimulated cells), #p<0.05, ##p<0.01 and ###p<0.001 compared with CSE or IL-17A.

5.4.2 Effect of fluticasone on (CSE + IL-17A)-induced the production of IL-8

We explored the impact of CSE on IL-17A-induced production of IL-8 and its inhibition by fluticasone in HASMCs. IL-8 concentration was increased by 17.5-fold after stimulation with (CSE + IL-17A) (46346.3 pg/mg protein) over the control (2701.1 pg/mg protein, Figure 5.4.2). A synergistic effect was observed on IL-8 production after (CSE + IL-17A) stimulation compared with CSE alone (9627.1 pg/mg protein, Figure 5.4.1) or IL-17A alone (4142.2 pg/mg protein, Figure 5.4.1). CSE and IL-17A (CSE+IL-17A)-induced IL-8 production was inhibited by fluticasone in a concentration-dependent manner with a significant inhibition of 62.1% seen at a concentration of 10^{-9} M compared to (CSE + IL-17A) stimulation (p<0.05). The maximum inhibition of (CSE+-IL-17A)-induced IL-8 by fluticasone (81.5%) was seen at a concentration of 10^{-6} M (8556.2 pg/mg protein) compared with the control (Figure 5.4.2). The findings suggest that CSE and IL-17A have a synergistic effect on IL-8 production, which may lead to a further increase of non-T2 inflammation, and this production is sensitive to inhibition by fluticasone.

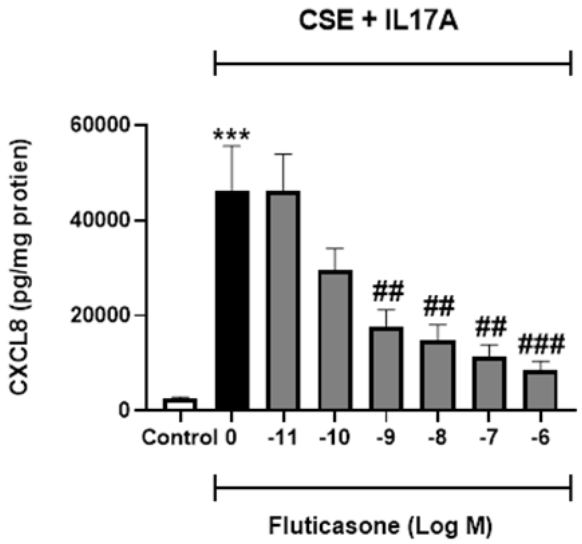

Figure 5.4.2 Effect of fluticasone on (CSE + IL-17A)-induced the production of IL-8

Confluent and growth-arrested HASMCs were pre-treated with or without CSE for 24h. Cells were then treated with an increasing concentration of fluticasone (10^{-11}-10^{-6} M) for 1h prior to incubation with CSE+IL-17A 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 carried out in triplicate using cells from three different donors. ***p<0.001 compared with control (unstimulated cells), ##p<0.01 and ###p<0.001 compared with CSE+IL-17A alone.

5.4.3 CSE impact on the inhibitory effect of fluticasone on IL-17A-induced IL-8 production

Clinical and *in vitro* studies have shown that cigarette smoke or CSE enhance to non-T2 airway response, which contribute to sever form of asthma and may lead to glucocorticoid insensitivity. However, there is a lack of evidence that CSE can influence the inhibitory effect of glucocorticoids in cellular studies. Therefore, we aimed to explore whether CSE influences the inhibitory effect of fluticasone on IL-17A-induced production of the neutrophilic chemoattractant IL-8 in HASMCs. IL-17A-induced production of IL-8 and (CSE and IL-17A)-induced production of IL-8 were inhibited by fluticasone in a concentration-dependent manner with IC_{50}= 0.051 nM and IC_{50}= 0.616 nM, respectively as shown in Figure 5.4.3. Complete inhibition of IL-17A-induced IL8 production by fluticasone was observed at a concentration of 10^{-6} M but no complete inhibition by fluticasone was achieved in (CSE and IL-17A)-induced IL-8 production even with the highest concentration of 10^{-6} M. A significant difference was observed in the inhibition of (CSE and IL-17A)-induced IL-8 production compared with IL-17A-induced IL-8 production (Two-way ANOVA analysis, p<0.0001, Figure 5.4.3). There was a significant reduction in the inhibitory effect of fluticasone at 10^{-9} and 10^{-8} M concentrations on (CSE and IL-17A)-induced IL-8 production compared with IL-17A-induced IL-8 production (p<0.01, Figure 5.4.3). The results suggest that CSE reduce the inhibitory effect of fluticasone on IL-17A-induced IL-8 production in HASMCs, which may lead to glucocorticoid insensitivity.

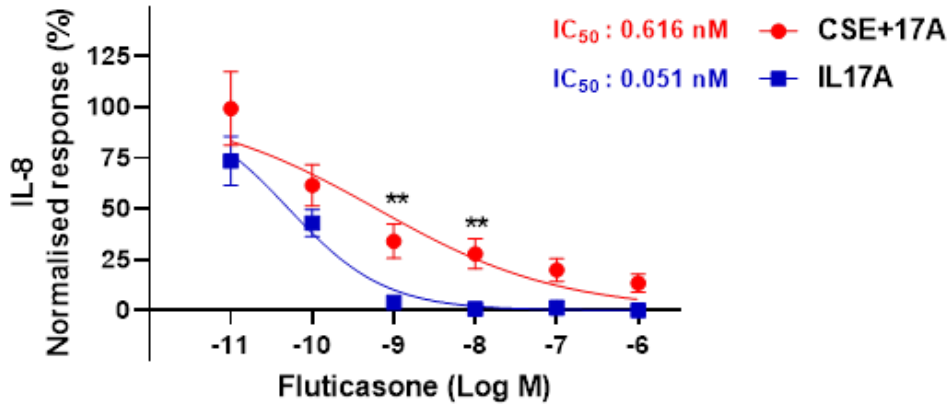

Figure 5.4.3 Comparison of the inhibitory effect of fluticasone on (CSE and IL-17A)- and IL-17A-induced IL-8 production

Confluent and growth-arrested HASMCs were pre-treated with fluticasone (10^{-11}-10^{-6} M) for 1 hour prior to incubation with IL-17A (10 ng/ml), CSE (3.5%) or CSE+IL-17A for 24 hours. The collected supernatants were used to measure IL8 concentration by ELISA. Data were normalised to total protein and then expressed as a percentage against either IL-17A alone or CSE+IL-17A (100%). Each data point represents mean ± SEM of one experiment carried out in triplicate. ***p*<0.05 compared with control. Two-way ANOVA was used to compare the inhibitory effect of fluticasone on IL-8 production by CSE+IL-17A and IL-17A alone.

5.5 Discussion

We found that CSE significantly induced the production of IL-8 but had no effect on GRO-α production in HASMCs and CSE-induced production of IL-8 was sensitive to inhibition by fluticasone. Studies supporting our findings have previously shown that CSE increased the production of IL8 and in HASMCs (129, 271), suggesting that CSE may contribute to non-T2 inflammatory response. Taken together, our findings along with other studies suggest that CSE enhanced the non-T2 inflammatory response by inducing the neutrophilic chemoattractant IL-8 production.

We then evaluated whether IL-17A could contribute to non-T2 inflammation by stimulating the production of neutrophilic chemokine IL-8 and GRO-α in HASMCs. We found that fluticasone inhibited IL17A-increased the production of IL-8. In agreement with our findings, studies showed that IL-17A induced the production of IL-8 in airway epithelial cells and HASMCs (95, 96). Our findings with others strongly suggest that IL-17A may contribute to non-T2 inflammation by inducing neutrophilic chemoattractant IL-8 and GRO-α production in HASMCs, which may lead to severe forms of asthma.

Glucocorticoid insensitivity is a main therapeutic challenge in managing severe asthmatic patients. A study reported that asthmatic smokers' glucocorticoid insensitivity is associated with increased IL-17A levels (272). Although, in the previous study, increased IL-17 concentrations suggested a potential role in the poor response to glucocorticoids of smokers

with asthma, the effect of CSE on IL-17A-induced production of non-T2 chemokines is unknown. With the same inhaled glucocorticoid dosage, heavy smokers showed more severe airflow limitation than light smokers and never smokers, indicating glucocorticoid insensitivity but less blood eosinophil count, indicating a distinct phenotype (273). These data suggest that CS leads to glucocorticoid insensitivity in asthmatic airways. The reduction in enzyme histone deacetylase 2 (HDAC2), which is required for glucocorticoids to exhibit their maximum effects in terms of pro-inflammatory cytokine suppression, is an important mechanism that could explain the relative glucocorticoid insensitivity in asthmatic smokers (274). An increased number of neutrophils in the airway of asthmatic smokers has also been associated with glucocorticoid insensitivity (275). The overexpression of the GR-β is thought to inhibit the action of the GR-α, through which the anti-inflammatory effects of glucocorticoids are mediated (276-278), 54, 55. Hence neutrophils have a higher amount of GR-β; this may provide an explanation for the glucocorticoid insensitivity seen in asthmatic smokers.

Our study is the first to report that CSE reduced the inhibitory effect of fluticasone on IL-17A-induced production of IL-8 in HASMCs. In agreement with our findings, CSE reduced the anti-inflammatory effect of dexamethasone on TNFα-induced IL-8 production in BEAS-2B cells by impairing HDAC2 activity. (An immortalized cell line isolated from normal human bronchial epithelium) (117). Another study also supported our finding showed that fluticasone inhibited IL-8 production induced by CSE, TNFα or

cotreatment of cigarette smoke extract and TNFα in a dose-dependent manner. However, while IL-8 production in the presence of CSE alone was completely inhibited by fluticasone, IL-8 production induced by CSE and TNFα was only partially inhibited by fluticasone in HASMCs, suggesting that increased levels of pro-inflammatory cytokines such as TNFα in airway inflammation may reduce the anti-inflammatory effects of glucocorticoid (279). In support of our results, a study demonstrated that CSE and IL-1β-induced production of IL8 and were completely resistant to inhibition by glucocorticoids (Budesonide and prednisolone up to 10 μM) in human monocytes (239). The synergistic effect of CSE and IL-17A on IL-8 production which might require higher concentrations of glucocorticoid, and this could therefore cause the reduction in the inhibitory effect of fluticasone in our study.

As mentioned before, these findings have obvious clinical relevance that the non-T2 inflammation (smoking-related inflammation) is generally insensitive to the therapeutic effects of glucocorticoids. Our findings suggest that CSE may contribute to non-T2 airway inflammation by inducing the production of neutrophil chemokine and enhancing IL-17A-induced production of the non-T2 inflammatory cytokine IL-8. CSE may also reduce the anti-inflammatory effect of glucocorticoids on IL-17A-stimulate non-T2 airway inflammation in HASMCs.

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

7.1 General discussion

This study is the first to report that CSE can shift the inflammatory response from T2 to non-T2 by inhibiting the production and mRNA expression of T2 inflammatory cytokines IL-4, IL-13, eotaxin, IP-10, and RANTES but increasing the production of non-T2 inflammatory cytokines IL-8 in HASMCs. Our findings suggest that CS could modify the inflammatory response in asthmatic smokers. Identifying the mechanisms through which CS modifies the inflammatory response is essential to developing new treatments. Therefore, this study provides evidence that the effect of CSE on the production of T2 inflammatory cytokines was largely mediated through the COX-2/PGE_2/EP_2/EP_4/cAMP pathway via oxidative stress. In addition, CSE reduced the anti-inflammatory effect of fluticasone on IL-17A-induced IL-8 production in HASMCs, suggesting that CS could contribute to glucocorticoid insensitivity. Interestingly, the Th17 cytokine IL-17A, which is known to be involved mainly in non-T2 inflammation, promoted both T2 and non-T2 inflammatory responses in HASMCs.

Based on our findings, although CSE reduced T2 and induce non-T2 inflammatory response in HASMCs, which may appear to be a positive effect, shifting the airway inflammation from T2 and non-T2 could be an important driver of asthma exacerbation in smokers. Unlike T2 airway inflammation, non-T2 inflammation is associated with a poor response to glucocorticoids in asthma. Our study findings may explain why smokers with asthma tend to have non-T2 inflammation and, therefore, may encounter poorly managed asthma, a poor response to glucocorticoid treatments and

accelerated deterioration in lung function (280). Our study not only showed that CSE could increase non-T2 inflammatory response but also showed the synergistic effect of CSE and IL-17A on IL-8 production, suggesting that asthmatic patients who smoke would likely have increased IL-8 release leading to further non-T2 inflammation. On the other hand, T2 inflammation is the most well-defined asthma endotype and responds well to glucocorticoids and also has an approved biological therapy such as anti-IgE omalizumab (71), anti-IL-5 mepolizumab, reslizumab and benralizumab (55) and anti-IL-4/IL-13 dupilumab (73). These biological therapies have been shown to minimise asthma exacerbations and improve lung function in patients with severe T2 airway inflammation. Understanding how CS causes airway inflammation is critical to exploring potential therapeutic options for asthmatic smokers. This thesis highlighted important mechanisms that play an important role in the inhibitory effect of CSE on the production of T2 inflammatory cytokines in HASMCs. Oxidative stress caused by CS has been found to be responsible for airway inflammation and lung damage in smokers (146). Studies reported that CSE could induce COX-2 expression and PGE_2 in HASMCs (168, 281). Our study is the first to demonstrate that the COX-2/PGE_2/EP_2/EP_4/cAMP pathways presented in this thesis mediated the inhibitory effect of CSE on the production of T2 inflammatory cytokines via oxidative stress in HASMCs. Thus, it is possible that inhibitors targeting COX or prostaglandin EP_2 and EP_4 may prevent the shift from glucocorticoid sensitive T2 to glucocorticoid insensitive non-T2 inflammation. However, it is worth noting that nonsteroidal anti-inflammatory drugs (NSAIDs) such as COX inhibitors may cause bronchoconstriction in some asthmatic patients,

commonly referred to as aspirin-exacerbated respiratory disease (AERD) (282). Reduced production of PGE_2 caused by COX inhibitors, along with an increase in cysteinyl leukotrienes (CysLT), enhances the bronchoconstriction caused by NSAID ingestion (283, 284). Therefore, further research investigating the dual effect of COX inhibitors and anti-leukotrienes such as zafirlukast and montelukast in asthmatic smokers is needed.

Our study demonstrated that CSE increased non-T2 inflammatory response, which is supported by clinical studies demonstrating that asthmatic smokers respond less effectively to glucocorticoids than non-smoker asthmatics (220-223). It has been observed that ex-smokers display an increased number of eosinophils in their blood count (285, 286). Thus, smoking cessation is an important therapeutic approach as It is possible that smoke cessation may help reduce non-T2 airway inflammation and shift to T2 airway inflammation, which may improve asthma control. In support of our claim, a study found that after 6 weeks of quitting smoking, asthmatic patients who stopped had significantly improved lung function and decreased sputum neutrophil counts compared to subjects who continued smoking (287). Ex-smokers showed a higher eosinophil blood counts compared with current smoker (288). These studies suggest that smoking cessation may help modify the airway inflammation form the non-T2 to T2 by reducing neutrophil counts and increasing eosinophil counts. Our study showed that CSE reduce the anti-inflammatory effect of fluticasone on production of IL-8, contributing to glucocorticoids insensitivity. It is also possible that quitting smoking may reduce glucocorticoid insensitivity since

a study demonstrated that ex-smokers who had quit smoking for at least a year showed a better response to oral prednisolone than current smokers (223).

7.2 Conclusion

In conclusion, the findings outlined in this thesis provide evidence for the first time that CSE can modify the inflammatory responses in HASMCs, promoting a shift from glucocorticoid sensitive T2 inflammatory responses to glucocorticoid insensitive non-T2 inflammatory responses, thereby contributing to glucocorticoid insensitivity. Our study presented for the first time that Th17 cytokine IL-17A can enhance T2 and non-T2 inflammatory responses by stimulating their cytokine production in HASMCs. Our results demonstrated that CSE suppresses T2 inflammatory response largely through a COX-2/PGE2/EP2/EP4/cAMP pathway via oxidative stress. Our findings suggest that targeting this pathway may be of great potential for the development of novel treatments for asthmatic smokers.

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7.2 Limitations and future directions

- The findings demonstrated in this thesis are obtained from in vitro experiments using healthy HASMCs. However, it is unknown whether the inhibitory effect of CSE on T2 inflammatory response can also be observed in diseased HASMCs. Therefore, our future studies need to use HASMCs from asthmatic smokers and non-smokers, as well as precision-cut lung slices from patients and animals, to validate key findings.

- In this study, HASMCs from three different healthy donors were used to explore the effect of CSE on T2 and non-T2 cytokine production, which may explain why we did not see a significant reversion of inhibition of CSE on some Th2 cytokines by GSH, although we observed a trend of increase in the cytokine concentration, and this could be due to the number of cell lines of HASMCs we used in this study. Therefore, increasing the number of donors will provide statistical power which could minimise the variations in the results.

- This study was conducted using single airway structural cells type (HASMCs), but CSE and IL-17A may have a different effect on the human bronchial epithelial cells (HBECs). Thus, it would be interesting to explore the effect of CSE on HBECs as it plays an important role in the initiation of allergen induce T2 inflammatory response, as well as it is the first layer of the airways that gets exposed to CS in asthma. Further, it is also a good idea to use a co-

culture of epithelial and smooth muscle cells model to explore the effect of CSE on the interactions in both cell types.

- We exposed HASMCs to CSE for a quite short exposure time (up to 48 hours) compared to asthmatic smokers who smoke for a long period, and the effect we presented in our study may differ from asthmatic smokers. Thus, further research using patient samples such as bronchoalveolar lavage fluid (BALF) and assessing the T2 and non-T2 cytokine levels are required to confirm our findings.
- We were not able to quantify the nicotine level as we had no access to the high-performance liquid chromatography (HPLC), it would be great to quantify nicotine level in the future work.

Chapter 7. Appendix

7. Tables of Materials

7.1 Materials for cells culture and MTT cell viability assay

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

DMEM+ supplemented with 10% 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 cigarette smoke extract material

3R4F research-grade cigarettes One cigarette contains 9.4mg of tar, 0.7mg of nicotine, 12mg of carbon monoxide	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 IL-17A (Human Cell-expressed) Protein Mass: 25 ug Molecular weight: 31.3 kDa	R&D Systems

Original stock of 25 mg was diluted in 1ml of Dulbecco's Modified Eagle's Medium (DMEM) (0.5% of FBS) for a final concentration of (25000 ng/1 ml)	
Recombinant Human IL-13 Protein Mass: 5 ug Molecular weight: 12.6 kDa Original stock of 5 µg was diluted in 1ml of Dulbecco's Modified Eagle's Medium (DMEM) (0.5% of FBS) for a final concentration of (5000 ng/1 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. Mass: 5 mg. Molecular weight: 451.39. To make original stock of 0.05 M, dissolve in 251.5 µL of DMSO.	Sigma Aldrich
L-Glutathione (GSH). Mass: 10 mg. Molecular weight: 307.32.	Sigma Aldrich

To make original stock of 0.05 M, dissolve in 650.6 µL of Water.	
(-)-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
8-Bromo-cAMP, sodium salt Mass: 10 mg. Molecular weight: 430.09. To make the original stock of 0.05 M, dissolve in 470 µL of DMSO.	Tocris Bioscience
Salbutamol. Mass: 25 mg. Molecular weight: 239.31. To make original stock of 0.05 M, dissolve in 2,088 µL of methanol (MeOH).	Sigma-Aldrich
Prostaglandin E2 (PGE2). 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.	Tocris bioscience

Molecular weight: 409.41. To make original stock of 0.05 M, dissolve in 488.4 µL of DMSO.	
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-HCl) and 150 mM NaCl.	Sigma Aldrich
Bovine serum albumin (BSA)	Thermo Fisher Scientific.
Block buffer for 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 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 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 IL-8 DuoSet ELISA Kit	R&D Systems
Human GRO-α DuoSet ELISA kit	R&D Systems
Bio-Plex Pro Human Cytokine 27-plex Assay Kit	Bio-Rad Laboratories
Prostaglandin E2 ELISA-Monoclonal 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
Goat Anti-Mouse Immunoglobulins/HRP	Dako
Goat Anti-Rabbit Immunoglobulins/HRP	Dako
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 Cell viability

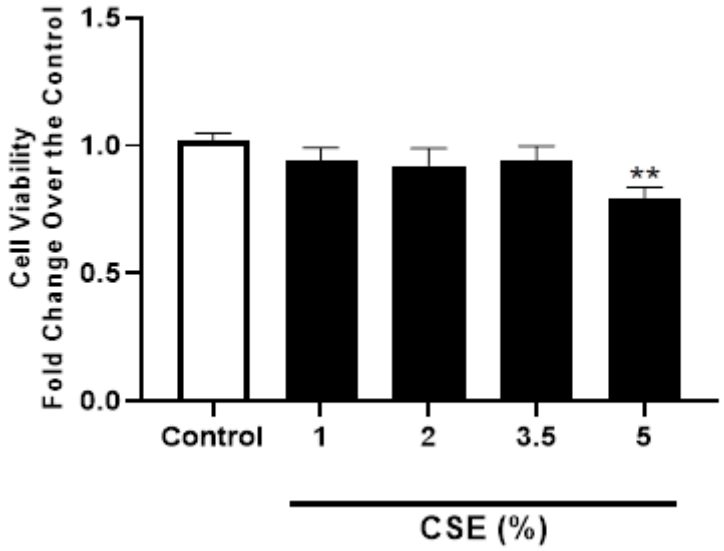

Figure 7.8.1 Effect of CSE on Cell Viability

Confluent and growth arrested HASMCs were treated with CSE (1%, 2%, 3.5% and 5%) for 24h. Cell viability was assessed by MTT cell viability assay. Each data point represents mean ± SEM of one experiment carried out in triplicates. ** $p<0.01$ versus control.

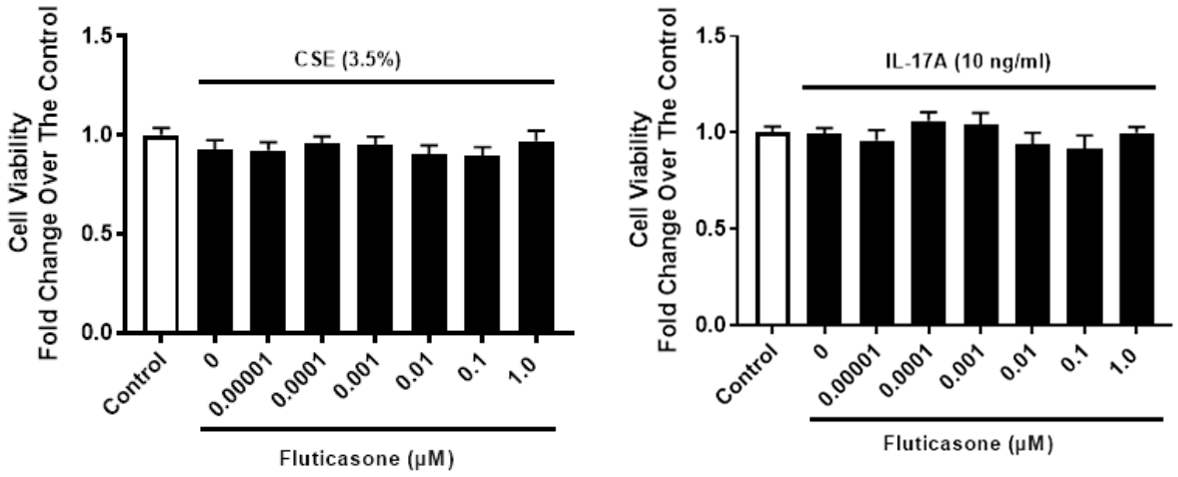

Figure 7.8.2 Effect of fluticasone with CSE and IL-17A on Cell Viability

Confluent and growth-arrested HASMCs were treated with fluticasone (10^{-11}-10^{-6} M) for 1h prior to incubation with CSE (3.5%) or IL-17A for 24h. The cell viability was assessed by MTT assay. Each data point represents mean ± SEM of one experiment carried out in triplicates.

7.9 Housekeeping gene β2M

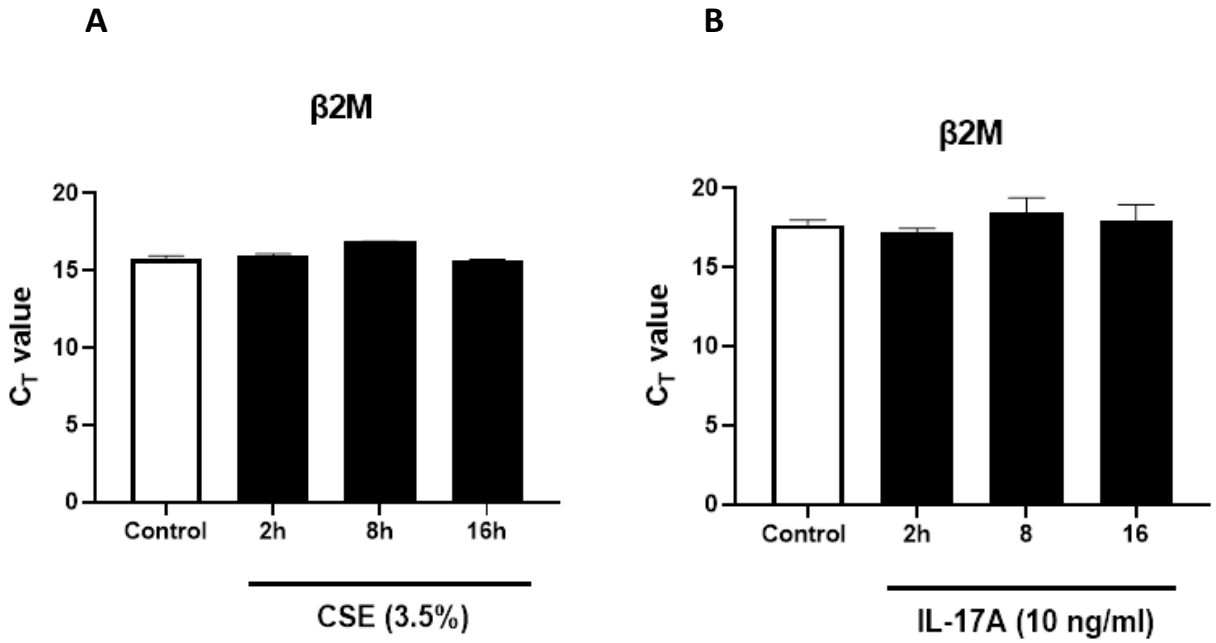

Figure 7.9.1 Effect of CSE and IL-17A on the mRNA expression of the housekeeping gene β2M in HASMCs

Confluent HASMCs were stimulated with concentrations of CSE (3.5%, A) or IL-17A (10 ng/ml, B) for 2, 8 and 16h 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.

7.11 Effect of β_2-agonist (Salbutamol) on T2 inflammatory response in HASMCs

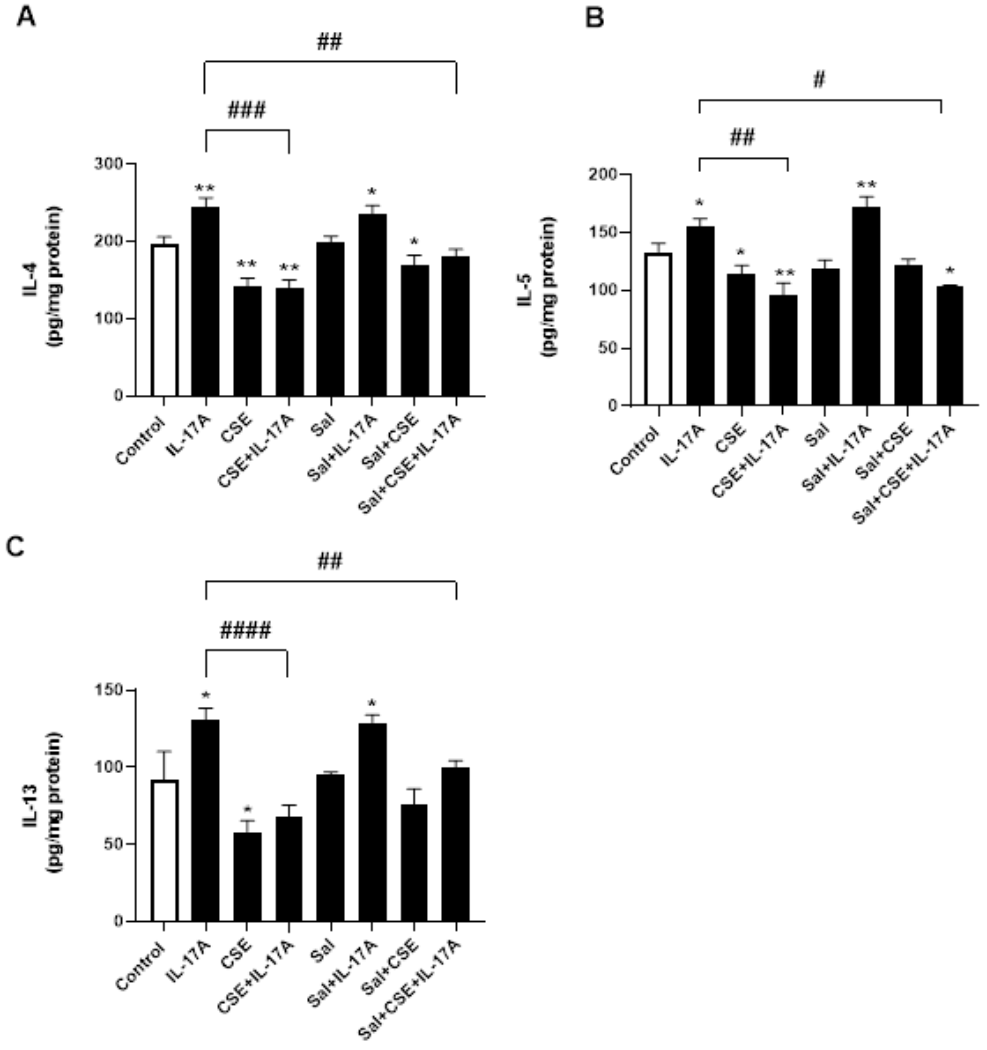

Figure 7.11.1 Effect of salbutamol on the production of Th2 cytokines

Confluent and growth-arrested HASMCs were treated with salbutamol (Sal, 10 µM), CSE (3.5%), IL-17A (10 ng/ml), Sal+CSE, Sal+IL-17A, CSE+IL-17A or Sal+CSE+IL-17A for 24h. IL-4 (A), IL-5 (B) and IL-13 (C) concentrations were measured in the collected supernatants using a multiplex assay. Data were normalised to total protein. Each data point represents mean ± SEM from three independent experiments using cells from three different donors. *p<0.05 and **p<0.01 compared with control. #p<0.05, ##p<0.01, ###p<0.001 and ####p<0.0001 compared with IL-17A alone.

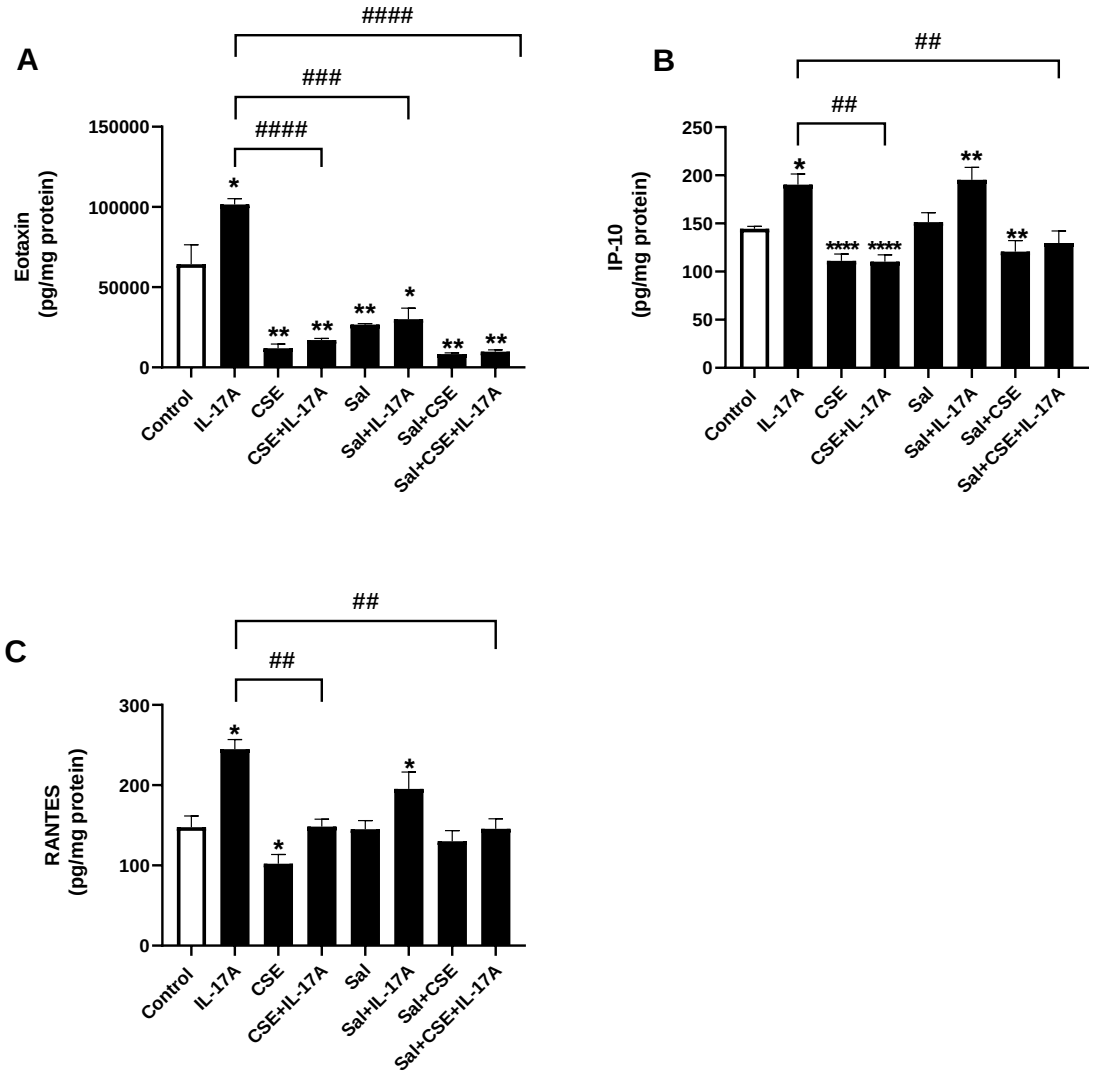

Figure 7.11.2 Effect of salbutamol on the production of Th2 cytokines

Confluent and growth-arrested HASMCs were treated with salbutamol (Sal, 10 µM), CSE (3.5%), IL-17A (10 ng/ml), Sal+CSE, Sal+IL-17A, CSE+IL-17A or Sal+CSE+IL-17A for 24h. Eotaxin (A), IP-10 (B) and RANTES (C) concentrations were measured in the collected supernatants using a multiplex assay. Data were normalised to total protein. Each data point represents mean ± SEM from three independent experiments using cells from three different donors. *p<0.05, **p<0.01 and ****p<0.0001 compared with control. ##p<0.01, ###p<0.001 and ####p<0.0001 compared with IL-17A alone.

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