The University of Nottingham

UNITED KINGDOM · CHINA · MALAYSIA

Cigarette smoke extract reduces the anti-inflammatory effect of fluticasone in human airway smooth muscle cells

Abdulrhman Saad G Alghamdi

Division of Respiratory Medicine

Thesis submitted to the University of Nottingham for the degree of Master of Science by Research in Respiratory Medicine

Supervisors:

Prof. Linhua Pang

Dr. Amanda Tatler

April 2019

Abstract

Background: Airway inflammation is a hallmark of asthma disease, and cytokines play a major role in the inflammation process. Human airway smooth muscle cells (HASMCs) are considered as an important source of inflammatory cytokines and chemokines in Asthma. Inhaled corticosteroids (ICS) are used with asthmatic patients as anti-inflammatory therapy to control the severity of the disease and maintain lung function. Clinical studies suggest asthmatic smokers do not respond to steroid as well as non-smokers, but whether cigarette smoke can reduce steroid sensitive *in vitro* is unclear. We hypothesise that cigarette smoke extract (CSE) reduces the anti-inflammatory effect of fluticasone in HASMCs.

Methods: CSE was prepared from the smoke of two cigarettes bubbled into 20 ml of cell culture medium. Confluent and growth-arrested HASMCs were pre-treated with and without CSE (3.5%) for 24 or 48 hours and then were treated with fluticasone (10^{-11}-10^{-6} M) for 1 hour prior to incubation with TNFα (1 ng/ml), CSE (3.5%), or TNFα + CSE for 24 hours. Cytokines and chemokines were measured in the collected medium by ELISA. MTT assay was used to assess the cell viability. IC_{50} of fluticasone was calculated using GraphPad Prism.

Results: We found that TNFα induced CXCL8 and IL-6 production, but not IL-5, IL-13 and VEGF production, in HASMCs. CSE also induced CXCL8 and IL-6 production. TNFα- and CSE-induced CXCL8 was similarly inhibited by fluticasone in a concentration-dependent manner (IC_{50} 0.2135nM vs 0.5253nM). Co-treatment of TNFα and CSE reduced the inhibitory effect of fluticasone significantly on CXCL8 production compared with TNFα alone ($p<0.0001$ by two-way ANOVA, IC_{50} 2.914nM vs 0.2135nM). CSE pre-treatment for 24 and 48 hours prior to TNFα treatment significantly reduced the inhibition of CXCL8 production by fluticasone compared with TNFα alone ($p<0.0001$, IC_{50} 3.438nM vs 0.2135nM and $p<0.0001$, IC_{50} 2.956nM vs 0.2135nM, respectively). CSE-induced IL-6 was more sensitive to inhibition by fluticasone than TNFα-induced IL-6 ($p<0.0001$, IC_{50} 0.0209nM vs 0.5646nM). But co-treatment of TNFα and CSE and CSE pre-treatment for 24 and 48 hours prior to TNFα treatment had no significant effect on the inhibition of IL-6 production compared with TNFα alone (IC_{50} 2.719nM, 0.7341nM, 0.7758nM and 0.5646nM, respectively).

Conclusion: Cigarette smoke selectively reduces the anti-inflammatory effect of fluticasone on TNFα-induced production of the neutrophil chemoattractant CXCL8 in HASMCs.

Acknowledgement

I would first like to give my sincere appreciation and warm thanks to my supervisor Prof Linhua Pang for giving me the opportunity to take my MSc in Respiratory Medicine under his supervision, for his patience and help throughout my study. The door to Prof Linhua Pang office was always open whenever I had a question about my research project or thesis writing. I would also give my warm thanks to my second supervisor Dr Amanda Tattler for her valuable guidance, support and patience during my study.

I want to express my thanks to all the members of the Respiratory Medicine department for their support throughout my study journey. Special thanks to the PhD student Mr Abdullah Alqarni for his help and advice. Mr Abdullah was there to help me whenever I need help or had a question; I could not be any more grateful.

Appreciative thanks to my sponsor King Saud University (Saudi Arabia/Riyadh) for allowing me to continue my education and for their generous support. Also, warm thanks to the Saudi government and for the Saudi Arabian Cultural Bureau in London for their help and support to finish my study

Finally, but most importantly, I must express my very profound gratitude to my Mom and Dad, thank you for providing me with endless support, encouragement and prayers, your sacrifices meant a lot for me, and this accomplishment would not have been possible without you. I extend my thanks to my amazing family; I want to thank you for all that you have done for me. Thank you.

ABBREVIATIONS

A

Activator protein 1 (AP-1)

Airway hyperresponsiveness (AHR)

B

Bicinchoninic acid (BCA)

Bovine serum albumin (BSA)

Bovine serum albumin (BSA)

Bronchoalveolar lavage (BAL)

C

Chronic obstructive pulmonary disease (COPD)

Cigarette smoke (CS)

Cigarette smoke extract (CSE)

D

Dimethyl sulfoxide (DMSO)

Disability-adjusted life years lost (DALYs)

Dulbecco's Modified Eagle's Medium (DMEM)

E

Enzyme-linked Immunosorbent Assay (ELISA)

F

Fetal bovine serum (FBS)

Forced expiratory volume per second (FEV_1)

Forced vital capacity of lungs (FVC)

G

Global Initiative for Asthma (GINA)

H

Human airway smooth muscle cells (HASMCs)

Human bronchial epithelial cells (BEAS-2b)

L

Lipopolysaccharide (LPS)

M

Monocyte chemotactic protein-1 and -3 (MCP-1 and -3)

N

National Health Service (NHS)

Nuclear factor κB (NF-κB)

P

Peak expiratory flow (PEF)

Phenylmethylsulphonyl Fluoride (PMSF)

Phosphate-buffered saline (PBS)

Protease Inhibitor Cocktail (PIC)

R

Radioimmunoprecipitation assay (RIPA)

Regulated upon Activation, Normal T cell Expressed, and Secreted (RANTES),

S

Sodium Dodecyl Sulphate (SDS)

T

Tris-buffered saline (TBS)

Tumour necrosis factor (TNFα)

V

Vascular endothelial growth factor (VEGF)

W

World health organisation (WHO)

Table of Contents

Chapter 1: Introduction

1.1 Asthma Definition, Diagnosis and Treatment

1.1.1. Definition

Asthma is defined by the Global Initiative for Asthma (GINA) as a heterogeneous disease, usually characterised by chronic airway inflammation [1]. Its clinical symptoms include wheezing, shortness of breath, chest tightness and cough that vary over time and in intensity, along with variable expiratory airflow limitation [2].

1.1.2. Diagnosis

Spirometry is the gold standard diagnostic technique to evaluate patients with asthma. It is used to assess lung function by measuring the ratio of forced expiratory volume per second (FEV1) to the forced vital capacity of the lungs (FVC). Patients with asthma have a lower FEV1 (<80% predicted) associated with a reduction in FEV1/FVC ratio (<75% predicted) [2, 3], confirming the diagnosis of airflow limitation. Once asthma has been diagnosed, a treatment plan should be set to control the disease and prevent exacerbation.

1.1.3. Asthma Prevalence, Mortality and Morbidity

Asthma is a very common disorder affecting many people worldwide. In 2004, around 300 million people suffered from asthma [1, 4]. There was a significant increase in the global prevalence of the disease, as it reached 358 million in 2015 [5]. It is estimated that the number of patients with asthma will increase worldwide by 100 million in 2025 [6]. In the UK, it is estimated that there are 5.4 million people diagnosed with asthma, of which, there are 1.1 million children and 4.3 million adults [7]. Asthma affects 1 in every 11 people and 1 in every 5 households in the UK [7].

Asthma is a major cause of death worldwide. According to the world health organisation (WHO), 383,000 people died because of asthma in 2015 [8]. In the following year, the number of deaths increased to 420,000 [9]. In the UK, 1,410 people died from asthma in 2016, and it is estimated that 3 people die every day because of an asthma attack [7].

The burden of asthma on health care systems and patients is considerable. In 2016, the estimation of the disability-adjusted life years lost (DALYs) due to asthma across all ages was 23.7 million worldwide [9]. Moreover, asthma is not only among the top-ranked causes of years lived with a disability but also a major leading cause of disease burden [9]. In the UK, it is reported in 2018 that there is at least one hospital admission due to asthma attack every 8 minutes and the estimated annual cost on the National Health Service (NHS) to treat and manage asthma is more than £1.1 billion [7].

1.1.4. Treatment

Treatment of asthma involves the use of short-acting β2-adrenoreceptor agonists such as salbutamol and long-acting β2-adrenoreceptor agonists such as salmeterol [2, 10]. Both short- and long-acting β2-adrenoreceptor agonists are used as a bronchodilator and therefore relieve acute symptoms. Short-acting β2-adrenoreceptor agonists are used as a quick reliever and can last between 3-4 hours, but the bronchodilation effect of long-acting β2-adrenoreceptor agonists lasts for at least 12 hours [1, 10, 11].

In addition to treatments that relieve symptoms, many asthmatic patients require drugs to treat chronic airway inflammation. Inhaled glucocorticoids are the most effective anti-inflammatory medication currently available [10, 12]. Inhaled glucocorticoids reduce airway inflammation, improve lung function and bronchial hyperresponsiveness [13]. These effects are achieved by the down-regulation of inflammatory genes and up-regulation of anti-inflammatory genes [14]. A step-wise approach is used to treat asthma patients, where treatment is started at the step most appropriate to the degree of asthma severity [10]. For example, A mild asthmatic may only take reliever medications (short-acting β2-adrenoreceptor agonists), whereas a severe asthmatic is likely to take all three types of the drug on a daily basis (short and long-acting β2-adrenoreceptor agonists and glucocorticoids) [10, 15].

There are some alternative controller therapies such as leukotriene receptor antagonists, sodium cromoglicate, nedocromil sodium and theophyllines. These alternatives therapies are less effective preventer therapies, but it can be used with patients who only take short-acting β2-adrenoreceptor agonists alone [1, 10].

1.2. Airway Inflammation

Airway inflammation is a hallmark of asthma, characterised by epithelial destruction and an airway inflammatory infiltrate containing eosinophils, neutrophils, T lymphocytes, basophils and mast cells [16-18]. Chronic airway inflammation in patients with asthma could lead to structural changes of the epithelium, smooth muscle and vasculature which is known as airway remodelling [19].

Airway inflammation is asthma can be classified into two clinical inflammatory phenotypes (eosinophilic and neutrophilic). Increased eosinophils counts in the sputum, airway and blood refer to eosinophilic airway inflammation [20]. The preferred method to determine eosinophilic asthma is by sputum cellular profiles. Sputum eosinophils are reported as a percentage of total cells, and eosinophilic asthma is defined if eosinophils levels are greater than 2-3% [20]. Eosinophils could also be detected in the biopsies of the bronchial wall. There is no cut-off numbers of eosinophil in the airway wall that define eosinophilic asthma, but Wenzel and colleagues demonstrated that eosinophilic asthma subjects showed an average of 20 eosinophils/mm2 of tissue (interquartile range, 16-31 eosinophils/mm2) [21]. Eosinophilic asthma is characterised by IL-4 and IL-13 upregulation in early-onset atopic asthma and predominance of IL-5 in late-onset atopic asthma or non-atopic asthma (early-onset asthma is developed during childhood, and late-onset develops at adulthood) [22]. IL-5 plays a very significant role in recruiting eosinophils to the inflammation site, which then triggers the release of inflammatory mediators from infiltrated eosinophils including IL-13. IL-13 is implicated in bronchial hyperresponsiveness and sets a cascade of the inflammatory response that is self-sustained and no longer requires external stimulus [23-25].

Non-eosinophilic airway inflammation demonstrates low eosinophils counts. Further, individuals considered to have non-eosinophilic can be distinguished into paucigranulocytic, mixed granulocytic or neutrophilic inflammation which defined based on the values of sputum neutrophilia ranging from as low as >40% to as high as ≥70% [20]. Neutrophilic asthma specifically involves the upregulation of CXCL8 via NF-κB pathway [26]. CXCL8 is responsible for the recruitment of neutrophils to the site of inflammation, which then produces an inflammatory environment in the airways [27]. TNFα, C reactive protein and IL-6 are other key inflammatory mediators implicated in neutrophilic asthma [28]. Among these, IL-6 is also involved in airway remodelling and smooth muscle cell proliferation [29]. Airway inflammation is a common phenomenon, and is critical for determining disease prognosis, regardless of the type of asthma.

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 (Th) 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. Furthermore, Th2 cells are divided into two molecular phenotypes: Th2 high and Th2 low [30]. Asthmatic with neutrophilic and eosinophilic inflammation demonstrate a relatively Th2-low and Th2-high inflammatory milieu cytokine, respectively [20] (Figure 1.1).

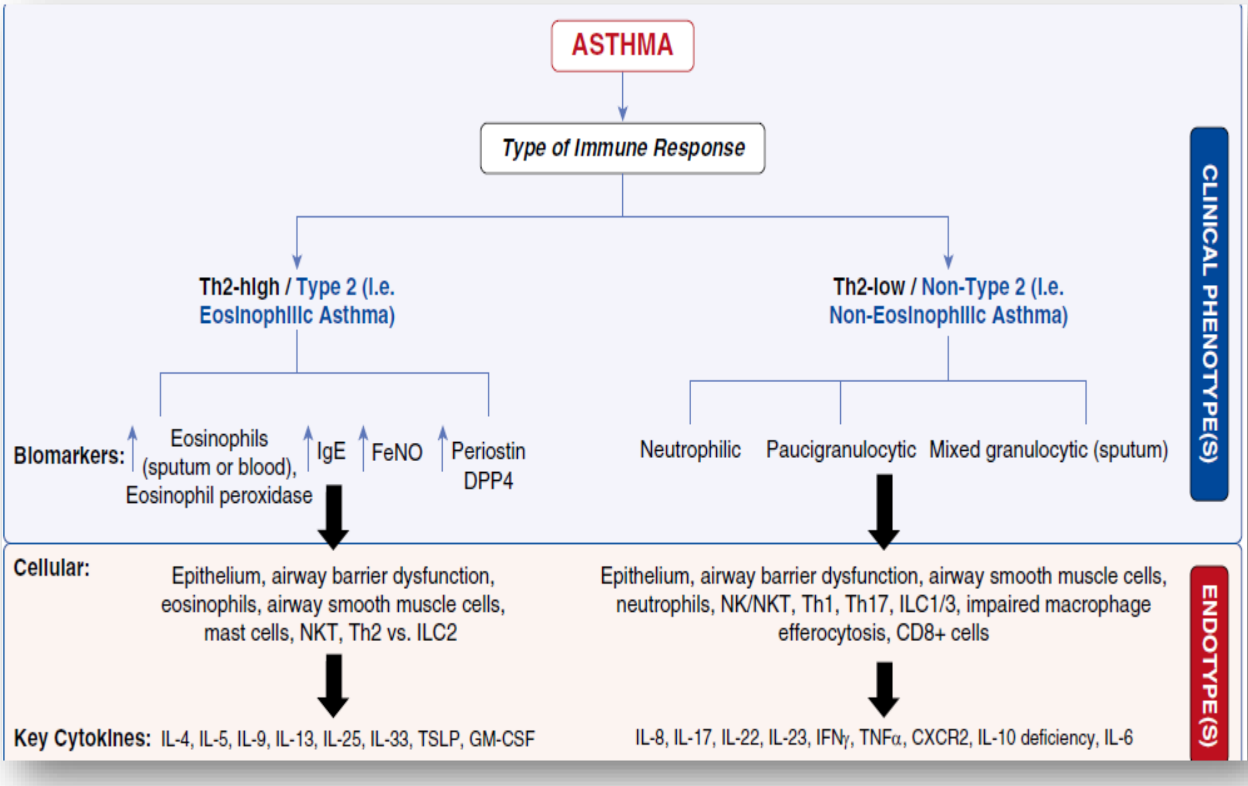

Figure 1.1 This schematic diagram demonstrates the clinical phenotypes of asthma (Th2-high and Th2-low), the biomarkers for each type (ex: eosinophils and neutrophils) and the key cytokines for each type [20].

1.3. Cytokines and Chemokines Associated with asthma

Cytokines are extracellular signalling proteins (<80 kD in size), which participate in cell-to-cell interactions [31]. Cytokines play an important role in regulating chronic inflammation in asthma by activating, recruiting and promoting inflammatory cells in the airways [32]. There are many types of cytokines can be classified into lymphokines (secreted by T cells), pro-inflammatory cytokines (amplifies inflammatory process), growth factors (promotes cell survival and causes structural changes in the airways) and chemokines (chemotactic for inflammatory cells) [32].

Chemokines are chemotactic cytokines (8–10 kDa) that are responsible for recruiting inflammatory cells [31, 33]. There are many examples of chemokines that are involved in asthma such as CXCL8, Regulated upon Activation, Normal T cell Expressed, and Secreted (RANTES), monocyte chemotactic protein-1 and -3 (MCP-1 and -3) [32, 34]. CXCL8 is an 8.5-kDa CXC chemokine that is a neutrophil chemoattractant. It plays an important role in the initiation and maintenance of inflammatory responses in the lung [35]. An increase in neutrophilic inflammation in patients with asthma appears to correlate with an increase of CXCL8 production [36]. CXCL8 mediates influx of mast cells via CXCR1 receptors [37] and also contributes to angiogenesis regulation which is a crucial pathological feature of asthma [38].

Interleukin-6 (IL-6) is a pleotropic cytokine that acts as a pro-inflammatory mediator (REF). IL-6 is primarily produced by cells of the innate immune system but has also been reported to be produced by structural cells such as HASMCs, endothelial cells, fibroblasts and epithelial cells [39, 40]. There are several studies demonstrating the elevation of IL-6 in serum and BAL fluid of asthmatic patients, specifically in an activated disease state [17, 39]. IL-6 was detected even in the induced sputum of mild asthmatic patients without excessive airway inflammation in comparison to healthy counterparts [41]. Additionally, IL-6 is also over-expressed independently in bronchial epithelial cells from asthmatic individuals [42].

Interleukin-5 (IL-5) is a Th2 cytokine that plays an important role in initiating and maintaining eosinophilic inflammation since it is involved in the differentiation of eosinophils from bone marrow precursor cells and can prolong eosinophil survival [32, 43]. Elevated levels of IL-5 have been reported in asthmatic serum, BAL fluid and sputum [44-46]. In addition, activated T-cells in asthmatic lung tissue has been shown to exhibit high expression of IL-5 mRNA, which correlates with heightened severity of disease as well as a number of lung eosinophils [47-49]. A recent study demonstrated the successful use of anti-IL-5 monoclonal antibodies to treat eosinophilic asthma and subsequent reduction in asthma exacerbations and improved lung function [50].

Interleukin-13 (IL-13) is also a Th2 cytokine that contributes to airway hyperresponsiveness and acts as a central mediator of allergic asthma [51]. IL-13 is overexpressed in peripheral blood, bronchial submucosa, induced sputum and airway smooth muscle bundle of asthmatic patients [52-55]. IL-13 levels are elevated in asthmatic BAL fluid following an allergen challenge [56]. Furthermore, both atopic and non-atopic asthma bronchial biopsies have elevated levels of IL-13 mRNA compared to non-asthmatic counterparts [57]. In addition, a comparative study between steroid responsive and resistant groups of asthmatic patients showed that IL-13 mRNA remained elevated in bronchial biopsies of the resistant group even after 1 week of prednisolone treatment [58]. IL-13 polymorphisms have been linked to asthma susceptibility and airway hyperresponsiveness in a genome-wide association study [59]. Due to the prevalence of IL-13 expression in a steroid-resistant group of asthmatic individuals, blockade of IL-13 and its receptor have been extensively investigated to be used as an adjuvant treatment strategy in uncontrolled asthma [60-63]. Persistent elevation of bronchial IL-13 could be a significant cause of refractory treatment in asthma and therefore suggesting that anti-IL-13 therapies may be effective in these patients [64].

Vascular endothelial growth factor (VEGF) is a cytokine that plays an essential role in the formation of new blood vessels (angiogenesis) [65]. Increased expression of VEGF is associated with increased vascularity in asthmatic airways [66] resulting in one of the features of airway remodelling. Bronchial biopsies from asthmatic patients display higher expression of VEGF and its receptors (flt-1 and flk-1) compared to healthy control specimens. This finding was also observed to negatively correlate with airway hyperresponsiveness [67]. VEGF plays a crucial role in Th2 inflammation by inducing eosinophilic stimulation, mucus metaplasia, dendritic cell activation, sub-epithelial fibrosis, myocyte hyperplasia and airway hyperresponsiveness [68]

1.4. Airway Inflammation and Glucocorticoids

Glucocorticoids are hormones produced by the adrenal cortex and excreted in the bloodstream [69]. Glucocorticoids bind to a specific cytoplasmic glucocorticoid receptor (GRs) to exert their effects [69]. Glucocorticoids suppress inflammation by inhibiting inflammatory genes (e.g. cytokines and chemokines) that are activated by pro-inflammatory transcription factors such as nuclear factor κB (NF-κB) and activator protein 1 (AP-1) [14]. GRs interact with corepressor molecules to inhibit NF-κB-associated activity, hence inverting acetylation and chromatin remodelling actions. A reduction in acetylation is associated with the recruitment of histone deacetylase (HDAC) 2 to the activated inflammatory genes by activated GRs, therefore resulting in suppression of activated inflammatory genes [14].

Glucocorticoids can activate several anti-inflammatory genes, but the main action of glucocorticoid in the airways of asthmatic patients is believed to be suppression or inhibition of the inflammatory cells and pro-inflammatory mediators and cytokines [14, 70]. For instance, fluticasone and dexamethasone can inhibit the production of CXCL8 in HASMCs and human bronchial epithelial cells (BEAS-2b), respectively [70-72]. Additionally, budesonide and dexamethasone can inhibit lipopolysaccharide (LPS)-induce CXCL8 production in alveolar macrophages [73].

Glucocorticoids are also shown to inhibit the transcription of some cytokines and chemokines that are involved in asthma, such as IL-5, IL-6 and IL-13 [74].

Glucocorticoids suppress Th2 inflammation and have long been the most effective controller therapy for asthma [70, 75]. However, not all patients respond to steroid treatment, some respond poorly to high-dose, and some are completely refractory [76]. Additionally, some studies suggest that there is a difference in response to glucocorticoids between patients with Th2 ‘’high’’ inflammation and Th2 ‘’low” inflammation in asthma. For instance, one study examined the response of 55 subjects to a high dose of oral prednisolone (25 mg, twice daily) for 10 days. The results show that 46% of patients responded to prednisolone and that the responder subgroup was characterised by eosinophilic airway inflammation (which is associated with the production of Th2 high cytokines) [77]. These results support the concept that patients with Th2 high airway inflammation are more responsive to glucocorticoids than the Th2 low group.

1.5. Airway Smooth Muscle Cells in asthma

Airway smooth muscle cells (ASMCs) are derived from the mesenchyme and contribute to stabilisation and homeostasis of the airways system via their structural and contractile properties [78]. ASMCs play an important role in the pathophysiology of asthma in several ways. They are largely involved in airway hyperresponsiveness, by an exaggerated response to bronchoconstrictor stimuli, and contribute to airway inflammation by producing cytokines, chemokines and growth factors [79, 80].

ASMCs also contribute to airway remodelling and airflow obstruction by their ability to proliferate, undergo hypertrophy, and migrate [81]. Furthermore, increased ASMC mass and altered deposition of extracellular matrix in chronic asthma are a feature of airway remodelling [82]. Moreover, ASMCs obtained from asthmatic patients are shown to hyper-secrete VEGF, which play an important role in the regulation of angiogenesis and airway remodelling [83, 84]. These factors (airway inflammation and remodelling) will eventually lead to asthma symptoms (Figure 1.2). ASMCs are an important source of inflammatory cytokines and chemokines [85]. For instance, human airway smooth muscle cells (HASMCs) isolated from asthmatic patients show a higher level of CXCL8 compared to non-asthmatics in the basal level [12, 35].

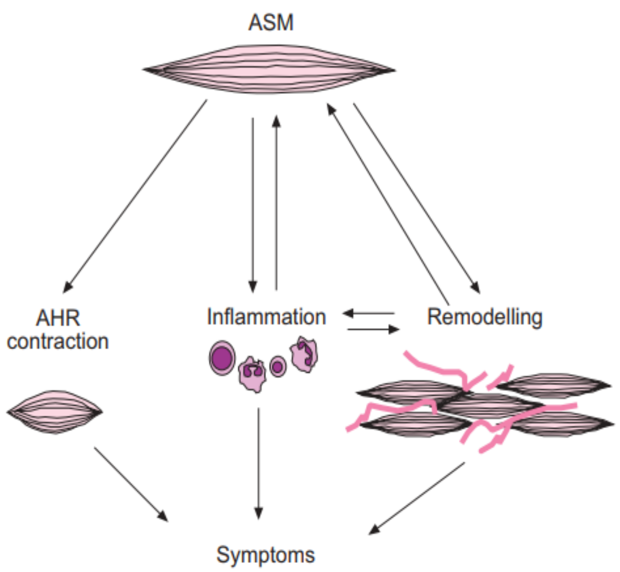

Figure 1.2. This schematic diagram depicts ASMCs cells that are involved in airway hyperresponsiveness (AHR), airway remodelling and airway inflammation and these three features lead to symptoms of asthma [80].

1.6. Association of Cigarette Smoke with Asthma

Asthmatic smokers represent 25% - 35% of total patients diagnosed with asthma worldwide [86]. It is well-documented that asthmatic smokers are at high risk of more severe disease and other asthma-related morbidities [86]. Asthmatic patients who are exposed to cigarette smoke for prolonged periods are prone to impaired lung function [86]. A 15-year study in Copenhagen revealed that the average annual decline in FEV1 was higher in male asthmatic smokers than non-smokers [87]. Furthermore, the 6-year mortality rate following a near-fatal asthma attack is higher in smokers than non-smokers [88]. It has also been reported that asthmatic smokers have poorly controlled disease and are susceptible to developing pathological features similar to chronic obstructive pulmonary disease (COPD) [89, 90]

Cigarette smoke not only affects patients who are already diagnosed with asthma, but active smokers are also more likely to develop asthma [91]. The onset of non-atopic asthma (non-atopic asthma shows negative results to skin allergy test and a normal immunoglobulin E level) is strongly associated with cigarette smoke [91]. Asthma-like symptoms and bronchial hyperresponsiveness to methacholine were associated with active smoking in a cohort of asymptomatic teenagers over 6 years [92]. Inhalation of smoke is directly associated with the lowering of FEV1. In line with this, a study showed that asthma developed before smoking was linked to atopy, whereas asthma developed after the onset of smoking was associated with low FEV1 [93]. Hence, cigarette smoke and asthma have a combined effect on the accelerated decline of lung function.

1.7. Cigarette Smoke and Airway Inflammation

Cigarette smoke (CS) is an important cause of airway inflammation. Studies have shown that CS and Cigarette smoke extract (CSE) induce inflammatory gene production in various experimental designs.

Cigarette smoke induce neutrophilic and eosinophilic airway inflammation in asthmatic smokers [94]. It has been shown that cigarette smoke can induce the production of some pro-inflammatory cytokines and chemokines [90] such as IL-4, IL-6, CXCL8 and TNFα in different cell types in vitro studies which then contribute to airway inflammation in asthma [90, 95, 96]. Furthermore, a study conducted by our group showed that CSE induced the production of CXCL8, IL-5, IL-6, VEGF and MCP-1 in HASMCs (unpublished study). These studies illustrate an important role of cigarette smoke in inducing airway inflammation.

1.8. Cigarette Smoke and Glucocorticoid Insensitivity in asthma

Glucocorticoids do not seem to be as effective in asthmatic smokers, as in asthmatic non-smokers [86]. Several clinical studies have demonstrated glucocorticoid insensitivity in asthmatic smokers. For instance, Pedersen and his colleagues show that asthmatic smokers show no improvement in FEV1 after treatment with budesonide for 9 months compared with asthmatic non-smokers [97]. Similarly, asthmatic smokers showed no improvement in peak expiratory flow (PEF) compared with asthmatic non-smokers while receiving inhaled fluticasone (250 µg twice daily) for 3 weeks [98]. Steroid insensitivity has also been reported in a study on 50 asthmatics smokers who have been treated with prednisolone 40 mg for 14 days [99]. In contrast to these reports, a clinical trial conducted on 86 asthmatic,19 of them are active smokers, showed that there was no difference between asthmatic smokers and non-smokers in response to inhaled glucocorticoids generally, but some of those smokers responded to a high dose of glucocorticoids [100]. Although clinical studies have demonstrated the effect of cigarette smoke on glucocorticoid sensitivity, the mechanisms driving it are currently unclear.

Cigarette smoke extract (CSE) can reduce the inhibitory effect of dexamethasone on TNFα-induced CXCL8 in BEAS-2B transformed human bronchial epithelial cells [72]. Moreover, in vitro study showed that inhibition of lipopolysaccharide (LPS)-induced CXCL8 production by dexamethasone and budesonide was reduced by CSE compared with LPS alone [73]. Although in general, it is increasingly clear that glucocorticoids are not as effective in asthmatic smokers as it is in non-smokers in clinical settings, there is a lack of direct evidence that cigarette smoke can reduce the anti-inflammatory effect of steroids in cellular studies.

1.9. Summary

Airway inflammation is a hallmark of asthma and is driven by inflammatory mediators such as cytokines, chemokines, and growth factors. Inhaled glucocorticoids are commonly used in asthmatic patients as anti-inflammatory therapies that can inhibit airway inflammation. However, they are not effective in all patients, and some asthmatics are refractory to steroid treatment. Cigarette smoke can induce pro-inflammatory cytokines and chemokines which contribute to airway inflammation in asthma. Several clinical studies have suggested that asthmatic smokers do not respond to glucocorticoids as effectively as non-smokers. However, the mechanisms through which cigarette smoke induces glucocorticoids insensitivity in airway structural cells remain unclear.

1.10. Hypothesis and Aims

1.10.1 Hypothesis

Cigarette smoke extract (CSE) reduces the anti-inflammatory effect of the glucocorticoid fluticasone in human airway smooth muscle cells.

1.10.2 Aims

The aims of this study are:

1- To assess whether CSE has a similar effect as TNFα in inducing the secretion of inflammatory cytokines and chemokines (CXCL8 and IL-6) in HASMCs

2- To determine whether CSE reduces the anti-inflammatory effect of fluticasone in HASMCs by comparing the inhibitory effect of fluticasone on TNFα-induced cytokine and chemokine production (CXCL8 and IL-6) with the inhibitory effect of fluticasone on the following:

- CSE alone
- CSE + TNFα
- 24h and 48h CSE pre-treatment + TNFα

Chapter 2: Materials and Methods

2.1 Tables of Materials

2.1.1 Drug

Fluticasone Propionate, F94288-5MG Mass: 5mg Molecular weight: 500.57M Dissolved in 19.98µL Dimethyl sulfoxide (DMSO) to obtain 0.5M stock	Sigma Aldrich
Recombinant Human tumour necrosis factor (TNFα) Mass: 5mg Molecular weight: 17.5 kDa Original stock of 20 µg was diluted in 1ml of Dulbecco's Modified Eagle's Medium (DMEM) (0.5% of FBS) for a final concentration of (20,000 ng/1 ml)	R&D Systems

2.1.2 ELISA Kits

Human IL-5 ELISA DuoSet kit	DY205-5, R&D Systems
Human IL-6 ELISA DuoSet kit	DY206-5, R&D Systems
Human CXCL-8 (CXCL8) ELISA DuoSet kit	DY208, R&D Systems
Human IL-13 ELISA DuoSet kit	DY213-5, R&D Systems
Human VEGF ELISA DuoSet kit	DY293B-05, R&D Systems

2.1.3 ELISA Buffers and Reagents

Phosphate-buffered saline (PBS)	Thermo Fisher Scientific
Tween 20	Sigma Aldrich
Tris-buffered saline (TBS) 20 mM Trizma® hydrochloride (Tris-HCl) 150 mM NaCl	Sigma-Aldrich
Bovine serum albumin (BSA)	Thermo Fisher Scientific.
Wash buffer 0.05% (1 ml) Tween and 5 tablets of PBS in each 1000 ml of distilled water (PH 7.2-7.4)	
Block buffer for (IL-5, IL-6, CXCL8, IL-13 and VEGF). 1% BSA in PBS Filtered through 0.02-µm pore syringe filter (PH 7.2–7.4)	
Substrate solution 1:1 mixture of Substrate Reagent A (H2O2) and Substrate Reagent B (Tetramethylbenzidine)	Thermo Fisher Scientific
Radioimmunoprecipitation assay (RIPA) buffer 50mM Tris-HCL, pH 8.0 0.5% Sodium Deoxycholate 150mM Sodium Chloride 0.1% Sodium Dodecyl Sulphate (SDS) After RIPA buffer preparation, the following materials were added before use: 2mM Phenylmethylsulphonyl Fluoride (PMSF) 1mM Sodium Orthovanadate 1nM Protease Inhibitor Cocktail (PIC)	

2.1.4 Materials for Cells Culture and MTT Cell Viability Assay

Dulbecco's Modified Eagle's Medium (DMEM)	Sigma Aldrich
Amphotericin-B 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	

2.1.5 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

2.2 Cigarette Smoke Extract Preparation

3R4F research grade cigarettes (Section 2.1.5) were connected via rubber tube to the glass tube of an 80 ml vacuum filtration flask containing 20 ml of DMEM. The pressure in 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 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

Fluticasone Propionate was dissolved in *Dimethyl sulfoxide* (*DMSO*) (description in section 2.1.1) as per the guidelines provided by the manufacturer. In the experiments, six concentrations of fluticasone were used ranging from 10^{-11} to 10^{-6} M. In addition, 2 μl of drug vehicle solution (DMSO) was added to 1 ml of medium (Dilution factor 1:500) in control wells to obtain final concentration of 0.2% of DMSO.

2.4 Cell Culture

Human airway smooth muscle cells (HASMCs) were provided by Professor Dominick Shaw (Division of Respiratory Medicine, University of Nottingham). The cells were obtained from a non-asthmatic donor and were cultured in 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.

2.4.1 Cell Subculture

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 in -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 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 average number of cells per square with dilution factor and 10^4.

2.4.3 Cell Treatment

Cells were seeded at a density of 40,000 cells/well of a 24 well plate. Before each experiment, the cells were growth-arrested with DMEM− for 24h. The cells were pre-treated with or without CSE (3.5%) for 24 and 48h, then cells were treated with fluticasone (10^{-11}-10^{-6} M) for 1h prior to incubation with TNFα (1 ng/ml), CSE (3.5%) or TNFα + CSE for 24h. The collected medium was stored in -20ºC freezer until required for ELISA.

2.4.4 Cell Lysate Preparation

After cells were treated, the plate was washed with PBS after the medium was collected from the plates, and 150 µl complete RIPA buffer (see section 2.1.3.) 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 (see section 2.5).

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)

Concentrations of IL-5, IL-6, CXCL8, IL-13 and VEGF were measured in the collected supernatants using DuoSet ELISA kits (R&D Systems). 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 (ng/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 ng/mg protein.

2.7. 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 TNFα with and without fluticasone (10^{-11}-10^{-6} M). 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.

2.8. Statistical Analysis and IC_{50} Calculation

Statistical analysis was conducted using GraphPad Prism 7. Two-way ANOVA was used to compare the inhibitory effect of fluticasone on cytokine and chemokine production by TNFα alone with that by CSE alone and CSE co-treatment and pre-treatment with TNFα. 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. One-way ANOVA followed by a t-test was used to determine the significant difference between the control and the treated cells. The p values less than 0.05 were considered significant. To calculate IC_{50}, 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 (with TNFα or any CSE + TNFα treatment as 100%) was plotted on Y-axis. Non-linear regression analysis in the software was used to generate the curve and IC_{50} values.

Chapter 3: Results

3.1. Effect of CSE on Cell Viability

The MTT assay was used to evaluate cell cytotoxicity of CSE (1%, 2%, 3.5% and 5%) on HASMCs. The assay was preformed after the cells were incubated with CSE for 24h as described in Section 2.9. 5% CSE treatment, but not the other lower concentrations, significantly reduced the cell viability as shown in Figure 3.1 ($p<0.0001$). The results suggest that 5% CSE is toxic to the cells. Therefore, 3.5% CSE was used in further experiments.

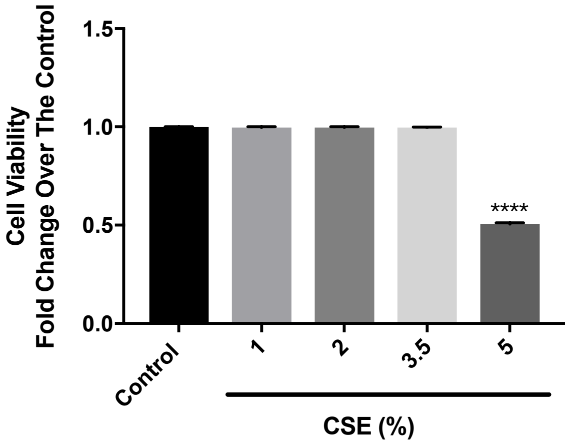

Figure 3.1. Effect of CSE on Cell Viability

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. ****$p<0.0001$ versus control.

3.2. Effect of fluticasone with TNFα or CSE on Cell Viability

To assess the cytotoxicity of TNFα (1 ng/ml) or CSE (3.5%) with and without fluticasone (10^{-11}-10^{-6} M) on HASMCs, MTT assay was used. Confluent and growth-arrested HASMCs were pre-treated with fluticasone (10^{-11}-10^{-6} M) for 1h. Then, the cells were treated with TNFα or CSE for 24h as described in Section 2.7. There was no significant difference in cell viability when cells were treated with TNFα or CSE with and without fluticasone compared with control. The result suggests that fluticasone with TNFα (1 ng/ml) or CSE (3.5%) is not toxic to the cells.

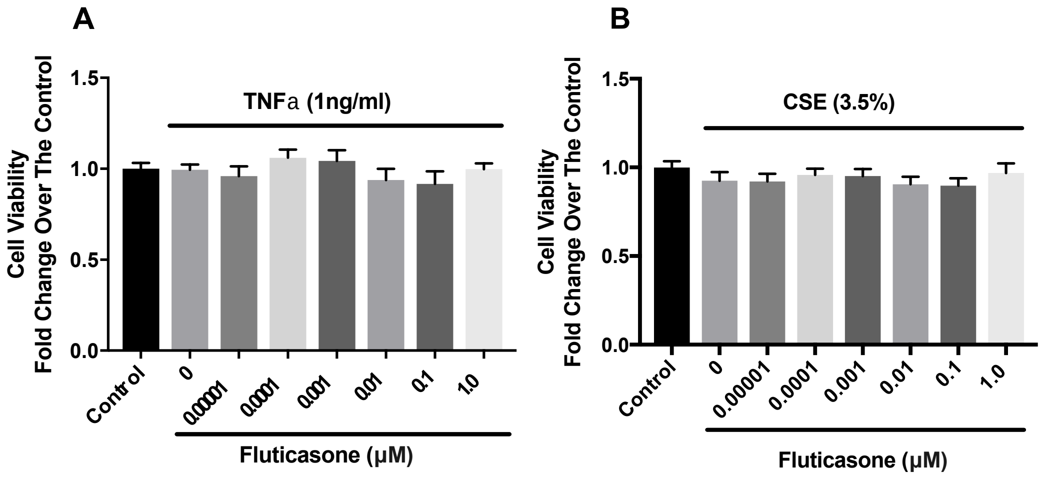

Figure 3.2 Effect of fluticasone with TNFα or CSE on Cell Viability

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

3.3. Effect of TNFα and CSE on CXCL8 and IL-6 production in HASMCs

CXCL8 and IL-6 are pro-inflammatory chemokines and cytokines that participate in the pathogenesis of asthma and it is known that fluticasone can inhibit their production. To assess the effect of TNFα-, CSE-, TNFα and CSE- and TNFα- with 24 and 48h CSE pre-treatment on CXCL8 and IL-6 production in HASMCs, confluent and growth-arrested HASMCs were pre-treated with or without CSE (3.5%) for 24 and 48h prior to stimulation with TNFα (1 ng/ml), CSE (3.5%) or TNFα + CSE for 24h. There was no significant difference among different treatments in the basal level production of either CXCL8 or IL-6. There was a significant increase in CXCL8 concentration when the cells were stimulated with TNFα or CSE compared with the controls (p<0.0001 and p<0.001). A significant increase was also observed on CXCL8 concentration when the cells were co-treated with TNFα and CSE or pre-treated with CSE for 24 and 48h prior to TNFα stimulation for 24h compared with the controls (p<0.0001) (Figure 3.3). A further increase on CXCL8 concentration was observed when the cells were co-treated with TNFα and CSE or pre-treated with CSE for 24 and 48h prior to TNFα compared with TNFα alone (3.6, 1.23 and 3.04 fold, respectively). A significant increase was seen in IL-6 concentration after stimulation with TNFα, CSE, co-treatment of TNFα and CSE and with CSE pre-treatment for 24 and 48h prior to TNFα treatment (p<0.01 to p<0.0001). However, co-treatment of CSE and TNFα and CSE pretreatment for 24 and 48h prior to TNFα stimulation did not further increase IL-6 production over TNFα alone (Figure 3.3).

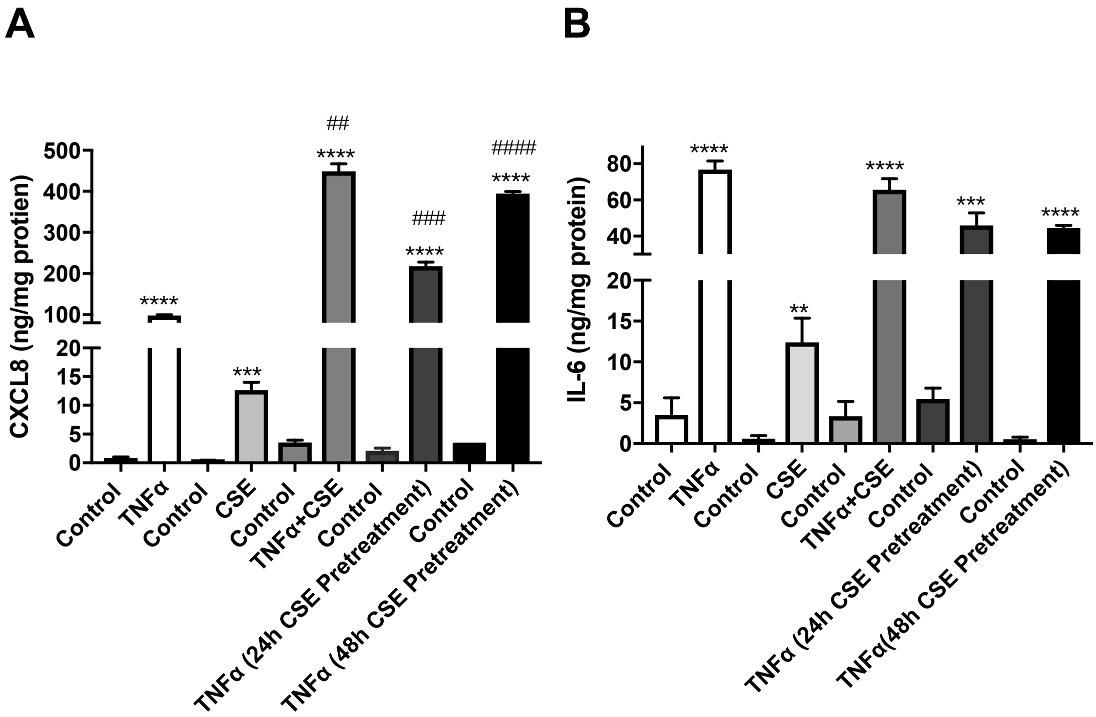

Figure 3.3. Effect of TNFα and CSE on CXCL8 and IL-6 production in HASMCs

Confluent and growth-arrested HASMCs were pre-treated with or without CSE (3.5%) for 24 and 48h prior to stimulation with TNFα (1 ng/ml), CSE (3.5%) or TNFα + CSE for 24h. The collected medium was used to measure CXCL8 and IL-6 concentrations by ELISA. The data was normalised to total protein. Each data point represents mean ± SEM of one experiment carried out in triplicates. ***p*<0.01, ****p*<0.001 and *****p*<0.0001 compared with control and ##*p*<0.01, ###*p*<0.001 and ####*p*<0.0001 compared with TNFα alone.

3.4. Effect of fluticasone on TNFα- and CSE-induced CXCL8 production

To compare the inhibitory effect of fluticasone on TNFα- or CSE-induced CXCL8, confluent and growth-arrested HASMCs were pre-treated with fluticasone for 1h in fresh DMEM- and then treated with either TNFα (1 ng/ml) or CSE (3.5%) for 24h. Medium was collected to measure CXCL8 by ELISA. The inhibitory effect of fluticasone on TNFα- and CSE-induced CXCL8 production was plotted together for comparison. As expected, fluticasone inhibited TNFα-induced CXCL8 production in HASMCs in a concentration-dependent manner with IC_{50}=0.2135nM. Fluticasone also inhibited CSE-induced CXCL8 production in a concentration-dependent manner with IC_{50}=0.5253nM. Complete inhibition of CXCL8 production by fluticasone was seen at 10^{-6} M in both TNFα- and CSE-stimulated cells (Figure 3.4). Two-way ANOVA analysis showed no significant difference on the inhibition of TNFα- and CSE-induced CXCL8 production by fluticasone. The results suggest that fluticasone has a similar inhibitory effect on TNFα- or CSE-induced CXCL8 production in HASMCs.

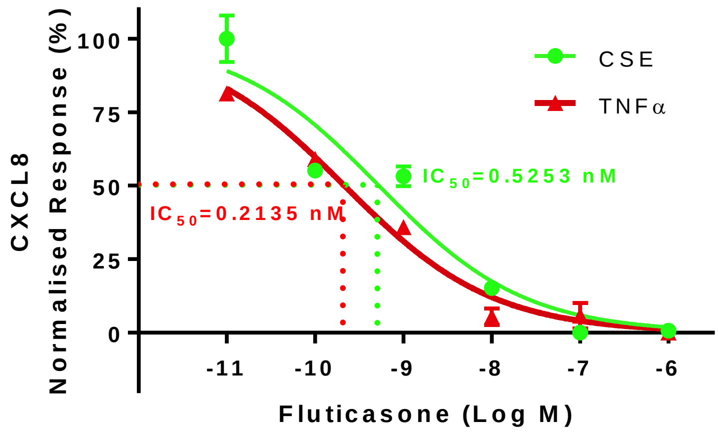

Figure 3.4. Effect of fluticasone on TNFα- and CSE-induced CXCL8 production. Confluent and growth-arrested HASMCs were pre-treated with fluticasone (10^{-11}-10^{-6} M) for 1h prior to incubation with either TNFα (1 ng/ml) or CSE (3.5%) for 24h. The collected supernatants were used to measure CXCL8 concentration by ELISA. Data were normalised to total protein and then expressed as percentage against either TNFα or CSE alone (100%). Each data point represents mean ± SEM of one experiment carried out in triplicates.

3.5. Effect of fluticasone on TNFα- and CSE-induced IL-6 production

To assess the inhibitory effect of fluticasone on TNFα or CSE-induced IL-6, confluent and growth-arrested HASMCs were pre-treated with fluticasone for 1h in fresh DMEM- prior to TNFα (1 ng/ml) or CSE (3.5%) treatment for 24h. Medium was collected to measure IL-6 by ELISA. The inhibitory effect of fluticasone on TNFα- and CSE-induced IL-6 production was plotted together to compare the inhibitory effect of fluticasone on both. Fluticasone inhibited TNFα- and CSE-induced IL-6 production in HASMCs in a concentration-dependent manner with IC_{50}=0.5646nM and IC_{50}=0.0209nM, respectively (Figure 3.5). At fluticasone concentrations of 10^{-7} M and 10^{-6} M complete inhibition of IL-6 production was achieved in TNFα- and CSE-stimulated cells, respectively. Two-way ANOVA analysis showed a significant difference of the inhibitory effect of fluticasone on TNFα-induced IL-6 production compared with CSE-induced IL-6 production (p<0.0001). At 10^{-11} M, fluticasone reduced TNFα-induced IL-6 production to 96.59% but reduced CSE-induced IL-6 production to 54.6% (p<0.05). No difference was observed at any other concentrations. The results suggest CSE-induced IL-6 production is more sensitive to inhibition by fluticasone than TNFα-induced IL-6 production in HSAMCs

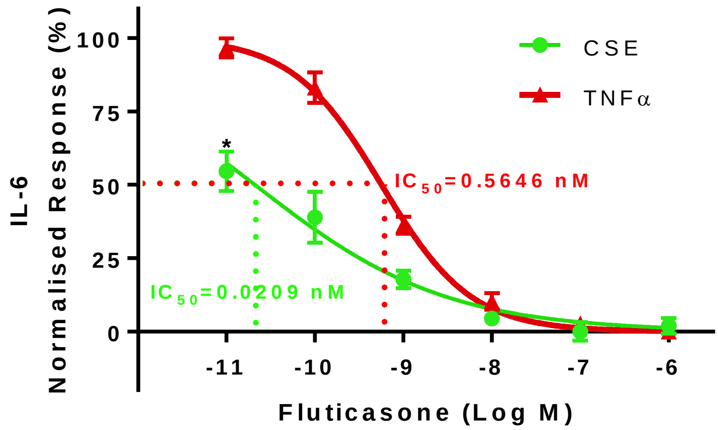

Figure 3.5. Effect of fluticasone on TNFα and CSE-induced IL-6 production

Confluent and growth-arrested HASMCs were pre-treated with fluticasone (10^{-11}-10^{-6} M) for 1h prior to TNFα (1 ng/ml) or CSE (3.5%) treatment for 24h. The supernatants were collected to measure IL-6 by ELISA. The data was normalised to total protein and then expressed as percentage against either TNFα or CSE alone (100%). Each data point represents mean ± SEM of one experiment carried out in triplicate. *p<0.05 versus corresponding TNFα alone.

3.6. Effect of fluticasone on TNFα + CSE-induced CXCL8 production

In order to explore if CSE can alter the inhibitory effect of fluticasone on TNFα-induced CXCL8 production when it is combined with TNFα in HASMCs, confluent and growth-arrested HASMCs were pre-treated with fluticasone (10^{-11}-10^{-6} M) for 1h in fresh DMEM- and then incubated with TNFα (1 ng/ml) + CSE (3.5%) for 24h. Medium was collected to measure CXCL8 by ELISA. The inhibitory effect of fluticasone on TNFα- and TNFα + CSE-induced CXCL8 production was plotted together for comparison. As shown in Figure 3.6, fluticasone inhibited TNFα-induced CXCL8 production and TNFα + CSE-induced CXCL8 production in HASMCs in a concentration-dependent manner with IC_{50}=0.2135nM and IC_{50}=2.914nM, respectively. IC_{50} of fluticasone was 13.6 times higher in TNFα + CSE-induced CXCL8 production compared with TNFα-induced CXCL8 production. Complete inhibition of CXCL8 production by fluticasone was not achieved in TNFα + CSE-induced CXCL8 production even at 10^{-6} M. Two-way ANOVA analysis showed a significant difference in the inhibition of TNFα + CSE-induced CXCL8 production compared with TNFα-induced CXCL8 production (p<0.0001). There was a significant reduction in the inhibitory effect of fluticasone at three concentrations (10^{-10}, 10^{-9} and 10^{-8} M) on TNFα + CSE-induced CXCL8 production compared with TNFα-induced CXCL8 production (p<0.05, normalised responses 83% vs 59.07%, 64.98% vs 35.87% and 36.93% vs 5.39%, respectively). The results suggest that CSE co-treatment with TNFα reduces the inhibitory effect of fluticasone on TNFα-induced CXCL8 production in HASMCs.

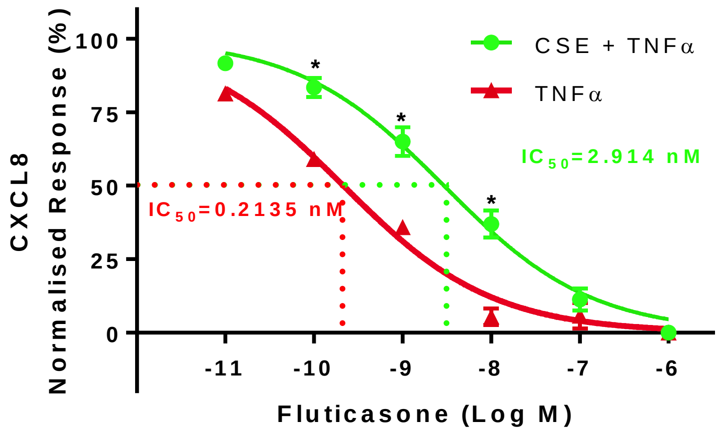

Figure 3.6. Effect of fluticasone on TNFα + CSE-induced CXCL8 production

Confluent and growth-arrested HASMCs were pre-treated with fluticasone (10^{-11}-10^{-6} M) for 1h prior to incubation with TNFα (1 ng/ml) + CSE (3.5%) or TNFα alone for 24h. The collected supernatants were used to measure CXCL8 concentration by ELISA. Data were normalised to total protein and then expressed as a percentage against either TNFα alone or TNFα + CSE alone (100%). Each data point represents mean ± SEM of one experiment carried out in triplicates. *p<0.05 versus corresponding TNFα alone.

3.7. Effect of fluticasone on TNFα + CSE-induced IL-6 production

To assess if CSE can reduce the inhibitory effect of fluticasone on TNFα-induced IL-6 production when it is combined with TNFα in HASMCs, confluent and growth-arrested HASMCs were pre-treated with fluticasone (10^{-11}-10^{-6} M) for 1h in fresh DMEM-. Then, cells were treated with TNFα (1 ng/ml) + CSE (3.5%) and incubated for 24h. Medium was collected to measure IL-6 by ELISA. The inhibitory effect of fluticasone on TNFα- and TNFα + CSE-induced IL-6 production was compared by plotting the two curves together. Fluticasone inhibited TNFα- and TNFα + CSE-induced IL-6 production in HASMCs in a concentration-dependent manner with IC_{50}=0.5646nM and IC_{50}=2.719nM, respectively as shown in Figure 3.7. Maximum inhibition of IL-6 production by fluticasone was seen at 10^{-6} in both TNFα alone and TNFα + CSE stimulated cells. Two-way ANOVA analysis showed no significant difference in the inhibition of TNFα + CSE-induced IL-6 production compared with TNFα-induced IL-6 production. The results suggest that CSE co-treatment with TNFα has no significant effect on the inhibitory effect of fluticasone on TNFα-induced IL-6 production in HASMCs.

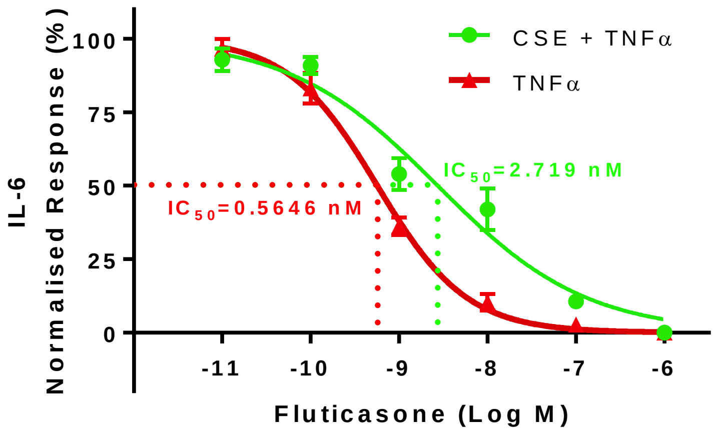

Figure 3.7. Effect of fluticasone on TNFα + CSE-induced IL-6 production

Confluent and growth-arrested HASMCs were pre-treated with fluticasone (10^{-11}-10^{-6} M) for 1h prior to incubation with TNFα (1 ng/ml) + CSE (3.5%) or TNFα alone for 24h. The collected supernatants were used to measure IL-6 concentration by ELISA. Data were normalised to total protein and then expressed as percentage against either TNFα alone or TNFα + CSE alone (100%). Each data point represents mean ± SEM of one experiment carried out in triplicates.

3.8. Effect of 24h CSE pre-treatment on the inhibition of TNFα-induced CXCL8 production by fluticasone

To assess if 24h CSE pre-treatment can reduce the inhibitory effect of fluticasone on TNFα-induced CXCL8 in HASMCs, confluent and growth-arrested HASMCs were pre-treated with CSE (3.5%) for 24h. Then, the plate was washed 3 times with PBS, and the cells were treated with fluticasone (10^{-11}-10^{-6} M) in fresh DMEM- for 1h prior to incubation with TNFα (1 ng/ml) for 24h. Medium was collected to measure CXCL8 by ELISA. The inhibitory effect of fluticasone on TNFα- and TNFα-(24h CSE pre-treatment)-induced CXCL8 production was compared by plotting two curves together. Fluticasone inhibited TNFα-induced CXCL8 and TNFα-(24h CSE pre-treatment)-induced CXCL8 production in HASMCs in a concentration-dependent manner with IC_{50}=0.2135nM and IC_{50}=3.438nM, respectively (Figure 3.8). When the cells were pre-treated with CSE for 24h prior to TNFα, IC_{50} of fluticasone on CXCL8 production was increased by 16.1 times compared with TNFα alone (IC_{50} 3.438nM vs 0.2135nM). There was a significant difference (p<0.0001) on the inhibitory effect of fluticasone between the two groups as analysed by two-way ANOVA. At concentrations of 10^{-10} and 10^{-9} M, the inhibitory effect of fluticasone was significantly reduced by CSE pre-treatment compared with TNFα alone (p<0.05, normalised responses 80.15% vs 59.07% and p<0.001, 75.14% vs 35.87%, respectively). The results suggest that 24h CSE pre-treatment reduces the inhibitory effect of fluticasone on TNFα-induced CXCL8 production in HASMCs.

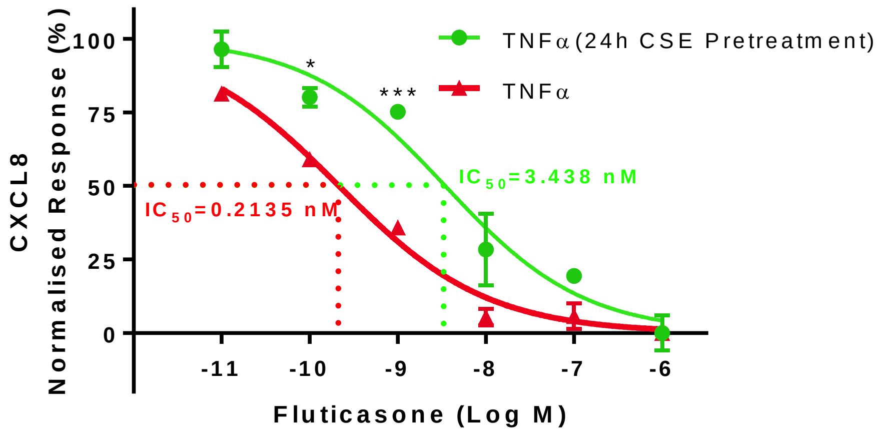

Figure 3.8. Effect of 24h CSE pre-treatment on the inhibition of TNFα-induced CXCL8 production by fluticasone

Confluent and growth-arrested HASMCs were pre-treated with or without CSE (3.5%) for 24h, then the cells were pre-treated with fluticasone (10^{-11}-10^{-6} M) for 1h prior to incubation with TNFα (1 ng/ml) for 24h. The collected supernatants were used to measure CXCL8 concentration by ELISA. Data were normalised to total protein and then expressed as percentage against either TNFα alone or TNFα-(24h CSE pre-treatment) alone (100%). Each data point represents mean ± SEM of one experiment carried out in triplicates. *$p<0.05$ and ***$p<0.001$ versus corresponding TNFα alone.

3.9. Effect of 24h CSE pre-treatment on the inhibition of TNFα-induced IL-6 production by fluticasone

To assess if 24h CSE pre-treatment can alter the inhibitory effect of fluticasone on TNFα-induced IL-6 production in HASMCs, confluent and growth-arrested HASMCs were pre-treated with CSE (3.5%) for 24h. After washing the plate with PBS 3 times, cells were treated with fluticasone (10^{-11}-10^{-6} M) in fresh DMEM- for 1h prior to incubation with TNFα (1 ng/ml) for 24h. Medium was collected to measure IL-6 by ELISA. Fluticasone inhibited TNFα-induced IL-6 production and TNFα-(24h CSE pre-treatment)-induced IL-6 production in HASMCs in a concentration-dependent manner with IC_{50}=0.5646nM and IC_{50}=0.7341nM, respectively. Two-way ANOVA analysis showed no significant difference in the inhibition TNFα-(24h CSE pre-treatment)-induced IL-6 by fluticasone compared with TNFα-induced IL-6 production. The results suggest that 24h CSE pre-treatment has no significant effect on the inhibitory effect of fluticasone on TNFα-induced IL-6 production in HASMCs.

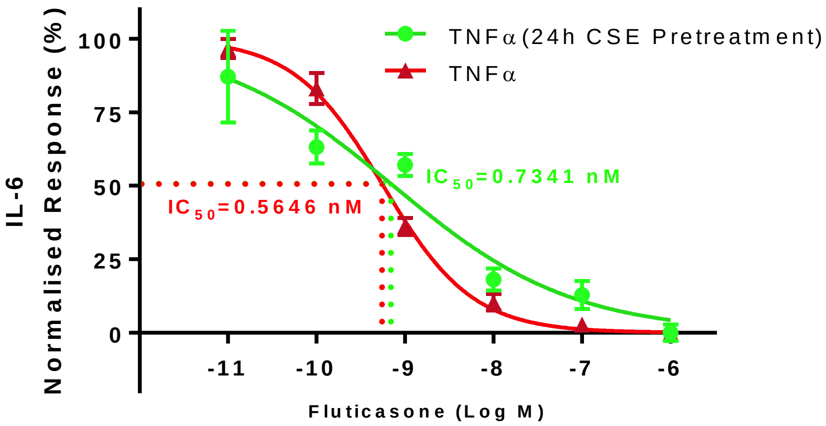

Figure 3.9. Effect of 24h CSE pre-treatment on the inhibition of TNFα-induced IL-6 production by fluticasone

Confluent and growth-arrested HASMCs were pre-treated with or without CSE (3.5%) for 24h, then the cells were treated with fluticasone (10^{-11}-10^{-6} M) for 1h prior to incubation with TNFα (1 ng/ml) for 24h. The collected supernatants were used to measure CXCL8 concentration by ELISA. Data were normalised to total protein and then expressed as percentage against either TNFα alone or TNFα-(24h CSE pre-treatment) alone (100%). Each data point represents mean ± SEM of one experiment carried out in triplicates.

3.10. Effect of 48h CSE pre-treatment on the inhibition of TNFα-induced CXCL8 production by fluticasone

To explore whether longer CSE pre-treatment for 48h can also reduce the inhibitory effect of fluticasone on TNFα-induced CXCL8 production in HASMCs, confluent and growth-arrested HASMCs were pre-treated with CSE (3.5%) for 48h. After that, the plate was washed 3 times with PBS prior to treating the cells with fluticasone (10^{-11}-10^{-6} M) in fresh DMEM- for 1h. Then, cells were incubated with TNFα (1 ng/ml) for 24h. Medium was collected to measure CXCL8 by ELISA. Fluticasone inhibited TNFα-induced CXCL8 production and TNFα-(48h CSE pre-treatment)-induced CXCL8 production in HASMCs in a concentration-dependent manner. With CSE pre-treatment for 48h, IC_{50} was 13.8 times higher in TNFα-(48h CSE pre-treatment)-induced CXCL8 compared with TNFα-induced CXCL8 production (IC_{50}=0.2135nM and IC_{50}=2.956nM, respectively). A significant difference was observed on the inhibitory effect of fluticasone on TNFα-(48h CSE pre-treatment)-induced CXCL8 production compared with TNFα-induced CXCL8 production using two-way ANOVA analysis ($p<0.0001$). There is a significant reduction in the inhibitory effect of fluticasone on TNFα-(48h CSE pre-treatment)-induced CXCL8 compared with TNFα-induced CXCL8 production at 10-8 M ($p<0.05$, normalised responses 28.35% vs 5.39%). The results suggest that 48h CSE pre-treatment reduces the inhibitory effect of fluticasone on TNFα-induced CXCL8 in HASMCs.

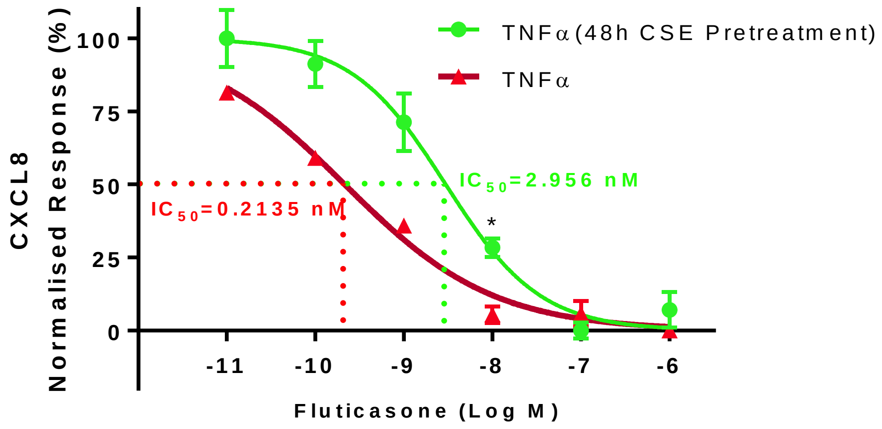

Figure 3.10. Effect of 48h CSE pre-treatment on the inhibition of TNFα-induced CXCL8 production by fluticasone

Confluent and growth-arrested HASMCs were pre-treated with or without CSE (3.5%) for 48h, then the cells were treated with fluticasone (10^{-11}-10^{-6} M) for 1h prior to incubation with TNFα (1 ng/ml) for 24h. The collected supernatants were used to measure CXCL8 concentration by ELISA. Data were normalised to total protein and then expressed as a percentage against either TNFα or TNFα-(48h CSE pre-treatment) alone (100%). Each data point represents mean ± SEM of one experiment carried out in triplicates. *$p<0.05$ versus corresponding TNFα alone.

3.11. Effect of 48h CSE pre-treatment on the inhibition of TNFα-induced IL-6 production by fluticasone

Experiment was conducted to investigate if 48h CSE pre-treatment can reduce the inhibitory effect of fluticasone on TNFα-induced IL-6 production in HASMCs. Confluent and growth-arrested HASMCs were pre-treated with CSE (3.5%) for 48h. Then, the plate washed 3 times with PBS, and the cells were then treated with fluticasone (10^{-11}-10^{-6} M) in fresh DMEM- for 1h prior incubation with TNFα (1 ng/ml) for 24h. Medium was collected to measure IL-6 by ELISA. Fluticasone inhibited TNFα-induced IL-6 production and TNFα-(48h CSE pre-treatment)-induced IL-6 production in HASMCs in a concentration-dependent manner with IC_{50}=0.5646nM and IC_{50}=0.7758nM, respectively. Two-way ANOVA analysis showed no significant difference in the inhibition of TNFα-(48h CSE pre-treatment)-induced IL-6 by fluticasone compared with TNFα-induced IL-6 production. The results suggest that even 48h CSE pre-treatment does not reduce the inhibitory effect of fluticasone on TNFα-induced IL-6 production in HASMCs.

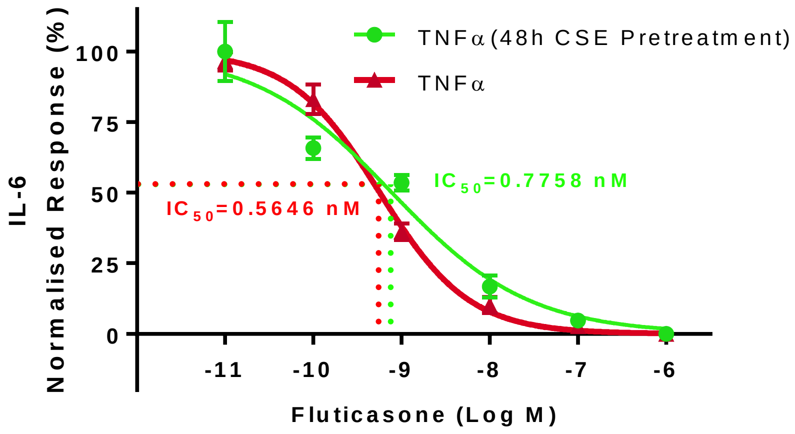

Figure 3.11. Effect of 48h CSE pre-treatment on the inhibition of TNFα-induced IL-6 production by fluticasone

Confluent and growth-arrested HASMCs were pre-treated with or without CSE (3.5%) for 48h, then the cells were treated with fluticasone (10^{-11}-10^{-6} M) for 1h prior to incubation with TNFα (1 ng/ml) for 24h. The collected supernatants were used to measure CXCL8 concentration by ELISA. Data were normalised to total protein and then expressed as percentage against either TNFα alone or TNFα-(48h CSE pre-treatment) alone (100%). Each data point represents mean ± SEM of one experiment carried out in triplicates.

3.12. Effect of fluticasone on TNFα-induced IL-5, IL-13 and VEGF production in HASMCs

As shown earlier, CSE can reduce the inhibitory effect of fluticasone on TNFα-induced CXCL8 but not IL-6 in HASMCs. In order to explore the effect of CSE on the inhibitory effect of fluticasone on TNFα-induced production of IL-5 and IL-13 (Th2 cytokines) and VEGF (a growth factor contributing to airway remodeling in asthma), confluent and growth-arrested HASMCs were pre-treated with fluticasone for 1h followed by TNFα stimulation for 24h. The medium was collected to measure the above listed cytokine and chemokine concentrations by ELISA. We found that IL-5, IL-13 and VEGF were undetectable in the medium from unstimulated cells and were still undetectable after TNFα treatment. Raw microplate spectrophotometer data of the standards and samples from ELISA are shown in Figure 3.12. The results suggest that TNFα has no effect on the production of IL-5, IL-13 and VEGF in HASMCs. Therefore, further experiments to explore the effect of CSE on the inhibition of TNFα-induced production of IL-5, IL-13 and VEGF by fluticasone were not carried out.

A

		1	2	3	4	5	6	7	8	9	10	11	12
Standards	A	305.771	158.531	73.588	42.653	20.101	9.765	5.888					
	B	294.072	142.91	71.738	37.556	17.603	6.973	3.226					
Samples	C	2.414	3.622	<< Y range	<< Y range	<< Y range	<< Y range	std range	std range	<< Y range			
	D	<< Y range	<< Y range	<< Y range	std range	<< Y range	3.622	4.011	std range	std range			
	E	4.011	<< Y range	<< Y range	std range	<< Y range	<< Y range	<< Y range	2.414	std range			
	F	std range	<< Y range	std range	<< Y range	<< Y range	<< Y range	3.622	std range	std range			
	G	std range	6.253	std range	<< Y range	<< Y range	<< Y range						
	H	<< Y range	<< Y range	std range	<< Y range	std range	<< Y range						

B

Samples: columns 1–9; Standards: columns 10–11

	1	2	3	4	5	6	7	8	9	10	11	12
A										6762.74	5298.268	
B										3345.15	2696.473	
C	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	1623.188	1247.84	
D	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	899.373	777.104	
E	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	265.913	218.041	
F	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	189.268	170.951	
G	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range				131.47	128.766	
H	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range	<< Y range						

C

		1	2	3	4	5	6	7	8	9	10	11	12
Standards	A	2369.868	1001.532	472.795	232.105	95.281	65.927	22.645					
	B	1857.579	671.438	588.914	237.236	139.413	25.267	22.01					
Samples	C	< std range	41.532	< std range	< std range	< std range	< std range	23.613	41.953	21.383			
	D	< std range	< std range	< std range	< std range	< std range	< std range	30.528	36.629	27.317			
	E	< std range	< std range	< std range	< std range	< std range	< std range	< std range	< std range	19.852			
	F	23.613	15.839	< std range	< std range	16.944	< std range	< std range	24.932	< std range			
	G	22.01	< std range	< std range	<< Y range	< std range	< std range						
	H	< std range	< std range	< std range	<< Y range	< std range	31.263						

Figure 3.12. Effect of fluticasone on TNFα induced IL-5, IL-13 and VEGF production in HASMCs

Confluent and growth-arrested HASMCs were pre-treated with fluticasone (10^{-11}-10^{-6} M) for 1h. Then, the cells were treated with TNFα (1 ng/ml) and incubated for 24h. The medium was collected to measure the IL-5, IL-13 and VEGF concentrations by ELISA. (A) IL-5, (B) IL-13 and (C) VEGF. Raw absorbance data for standards were highlighted in orange colour and samples were in green colour.

Chapter 4: Discussion

Glucocorticoids are commonly used in treating asthma to maintain lung function and prevent exacerbation. It has been shown clinically that cigarette smoke can reduce the efficiency of glucocorticoids such as fluticasone in asthmatic patients, which can lead to poor control of the disease. In the present study we sought to understand whether cigarette smoke extract (CSE) can reduce the inhibitory effect of fluticasone on TNFα-induced CXCL8 and IL-6 production in HASMCs using an *in vitro* cell experimental model. We found that co-treatment of CSE and TNFα, as well as CSE pre-treatment for 24h and 48h prior to TNFα treatment, reduced the inhibitory effect of fluticasone on TNFα-induced CXCL8 but not IL-6 in HASMCs.

4.1. Role of TNFα in airway inflammation

TNFα is a cytokine that plays an important role in the immune response, which prompts the host defence against foreign organisms [101]. TNFα contributes to pathological mechanisms of asthma [102]. For example, inhalation of recombinant TNFα causes airway hyperresponsiveness and neutrophil infiltration, two features of asthma [103]. In addition, patients with mild asthma shows an increase in airway hyperresponsiveness after administration of recombinant TNFα by inhalation as assessed by reduction in methacholine by measuring PC_{20} (the concentration of methacholine needed to produce a 20% fall in FEV_1 from baseline) [104]. It is well known that TNFα induces CXCL8 and IL-6 production in HASMCs and that this effect can be inhibited by the glucocorticoid fluticasone [71, 105]. Therefore, TNFα was used in this study to induce CXCL8 and IL-6, to mimic an asthma-like inflammatory scenario *in vitro*. Our findings are in line with the literature, and we have confirmed in this study that TNFα induced the production of CXCL8 and IL-6 in HASMCs significantly.

In addition to investigating the effect of TNFα on CXCL8 and Il-6 we sought to assess the effect of TNFα on other Th2 cytokines including IL-5 and IL-13 that play a role in eosinophilic inflammation. To our knowledge from the available literature, no study has investigated the effect of TNFα on IL-5 and IL-13 in HASMCs. Interestingly, our results showed that IL-5 and IL-13 were undetectable after stimulation with TNFα. These findings may suggest that TNFα is not involved in eosinophilic inflammation in asthma. However, TNFα can induce the production of IL-13 in other cell types such as endometrial epithelial (EEC) and stromal (ESC) cells [106]. It is, therefore, possible that TNFα might affect eosinophilic inflammation via IL-13 production in other cell types but not HASMCs. The effect of TNFα on the production of IL-5 has not been explored before on any cells from available literature.

We also aimed to assess the effect of TNFα on the production of vascular endothelial growth factor (VEGF), a cytokine that plays a key role in airway remodeling by increasing angiogenesis. TNFα has been reported to induce VEGF in HASMCs obtained from patients with bronchus carcinoma undergoing lung resection [107] but did not stimulate VEGF production in our study. The fact that HASMCs used in their study were from smokers with COPD along with the higher TNFα concentration, and longer incubation time compared with our study (20 ng/ml vs 1 ng/ml and 72h vs 24h, respectively) could explain this inconsistency with our finding.

4.2. Role of CSE in airway inflammation

Smoking in asthma induces neutrophilic inflammation as well as eosinophilic inflammation [94]. A study performed by our group has demonstrated that CSE can increase CXCL8, IL-6, IL-5, IL-13, VEGF and MCP-1 production in HASMCs (unpublished study). Supporting our data, Ling Chen and colleagues have previously shown that cigarette smoke increases the production of CXCL8 in HASMCs obtained from healthy smoker and COPD smokers [108]. Furthermore, others have shown that CSE can increase CXCL8 and VEGF in HASMCs [109], and IL-5, IL-6 and VEGF in mouse macrophage cells [110]. In this study, we confirmed that CSE can increase the pro-inflammatory cytokine and chemokine IL-6 and CXCL8 in HASMCs. Our results are consistent with findings from other studies and suggest that cigarette smoke could contribute to airway inflammation (eosinophilic and neutrophilic) by increasing the production of pro-inflammatory cytokines and chemokines, particularly IL-6 and CXCL8.

4.3. CSE and glucocorticoids insensitivity

Administration of glucocorticoids is the most common therapy used to treat inflammatory diseases such as asthma [111]. Glucocorticoids are not as effective in asthmatic smokers as in non-smokers in clinical settings [97-99]. However, the mechanisms driving this effect are still unclear and it is not known whether cigarette smoke can reduce the anti-inflammatory effect of steroids in cellular studies.

In this study, the inhibitory effect of the glucocorticoid fluticasone on TNFα-induced CXCL8 and IL-6 production in the presence of CSE and the inhibitory effect of fluticasone on CSE-induced production of CXCL8 and IL-6 in HASMCs was assessed. Oltmanns et al. have shown previously that fluticasone inhibits TNFα- and CSE-induced CXCL8 production in HASMCs, similar to our findings in this study [71]. Additionally, we have investigated the inhibitory effect of fluticasone on co-treatment of CSE and TNFα compared with TNFα alone. Our findings demonstrated that co-treatment of CSE and TNFα reduced the inhibitory effect of fluticasone on TNFα-

induced CXCL8 production significantly. We found that CXCL8 release was increased after co-treatment of CSE and TNFα together compared with TNFα alone, suggesting that the presence of CSE causes increased CXCL8 release in addition to TNFα-mediated effect. This effect has been observed previously by Oltmanns et al. who found that combined treatment of TNFα and CSE enhanced production of CXCL8 compared with either stimulus alone [71]. It is important to note that in our study CXCL8 was increased by 3.6 fold by TNFα and CSE co-treatment over TNFα alone, while the IC_{50} of fluticasone was increased by around 13.6 fold by the co-treatment over TNFα alone. This disproportion between the increase of CXCL8 production and the IC_{50} of fluticasone suggest that CSE reduces the inhibitory effect of fluticasone on TNFα-induced CXCL8 production (glucocorticoid insensitivity) and that although increased CXCL8 production may contribute, to some extent, to CSE-induced glucocorticoid insensitivity, it is most likely that other important factors may play a key role in CSE-induced glucocorticoid insensitivity.

In order to assess whether the effect of CSE on the response to fluticasone is long lasting, we used an experimental model where the cells were pre-treated with CSE for 24h or 48h prior to treatment with TNFα. In this study, our novel findings showed that the effect of CSE on reducing the inhibitory effect of fluticasone on TNFα-induced CXCL8 in HASMCs remained even when cells were pre-treated with CSE prior to the addition of TNFα. Interestingly, we found that CSE pre-treatment for either 24h or 48h increased CXCL8 release compared with TNFα alone. It is possible that CSE pre-treatment could stimulate the release of TNFα or other CXCL-8 stimulating mediators in HASMCs, which then contribute to the further increase in CXCL8 production when the cells incubated with TNFα alone for 24h. Like CSE and TNFα co-treatment, there was also a disproportion between the increase of CXCL8 in the CSE pre-treatment and the increase of the IC_{50} of fluticasone (1.23 and 16.1 fold, respectively), suggesting that other important factors, in addition to increased CXCL8 production, may contribute to CSE-induced glucocorticoid insensitivity. This warrants further study.

While our study is the first to report that CSE affects responses to a glucocorticoid in HASMCs, others have assessed the effect of CSE on the inhibitory effect of dexamethasone (another glucocorticoid drug) on TNFα-induced CXCL8 production in human bronchial epithelial cells (BEAS-2b) [72]. They found that CSE reduced the inhibitory effect of dexamethasone on TNFα-induced CXCL8 with CSE pre-treatment for 6h in BEAS-2b cells [72]. Unlike our study, they did not find a significant difference in CXCL8 production between the co-treatment and TNFα alone. This is different from our finding that CSE pre-treatment and co-treatment increased TNFα-induced CXCL8 production and further supports the concept that increased CXCL8 production is not an important factor for CSE-induced glucocorticoid insensitivity, although it may contribute to some extent, as the above study shows CSE still induces glucocorticoid insensitivity even without further increase of CXCL8 compared with TNFα alone. Additionally, the effect of CSE on the inhibitory effect of budesonide and dexamethasone on lipopolysaccharide (LPS)-induce CXCL8 production in alveolar macrophages has been assessed [73]. CSE reduces the inhibition of LPS-induced CXCL8 production by budesonide and dexamethasone compared with the inhibition by either glucocorticoid on LPS alone, this finding could suggest that CSE induce glucocorticoid insensitivity regardless of what the inflammatory stimulants were used [73]. Although in the previous two studies, different glucocorticoid drugs, different cell types, CSE preparation and pre-treatment durations were applied, their results support our ongoing hypothesis that CSE reduces the response of inflammation to glucocorticoids. Furthermore, this effect of CSE on glucocorticoid sensitivity is a general phenomenon not only in HASMCs but also in other cell types.

In this study, we showed that CSE reduced the inhibitory effect of fluticasone on TNFα-induced CXCL8 but not IL-6 production. This result may suggest that CSE reduces the inhibitory effect of fluticasone in neutrophilic more than eosinophilic inflammation. In agreement with this finding, one study shows that an increase in the neutrophil count by the increase of the neutrophilic chemoattractant CXCL8 in smokers with asthma might lead to a poor response to glucocorticoid therapy [112, 113]. In order to confirm this finding, we aimed to explore the effect of CSE on the inhibitory effect of fluticasone on TNFα-induced other Th2 cytokines that participate in eosinophilic airway inflammation, particularly IL-5 and IL-13, and VEGF [43, 51, 66]. But as mentioned earlier that TNFα has no effect on the production of IL-5, IL-13 and VEGF in HASMCs in our study. Therefore, further experiments to explore the effect of CSE on the inhibition of TNFα-induced production of these cytokines by fluticasone were not carried out.

4.4. Limitations and Conclusion

4.4.1 Limitations

In this study, some limitations need to be addressed. Firstly, we explored whether CSE can reduce the effect of fluticasone on TNFα-induced cytokines and chemokines using only one cell line of HASMCs from a healthy donor, and the effect could be limited to this cell line. Secondly, in our experimental setting, we showed that CSE can reduce the effect of fluticasone on TNFα-induced cytokine and chemokine in HASMCs, but whether CSE reduces the inhibitory effect of other glucocorticoid drugs such as budesonide has not been explored. Thirdly, we used healthy HASMCs in our experiments, and the response could be different in the asthmatic cells. Finally, cigarette smoke contains many harmful chemicals such as acetaldehyde and acrolein that which may not be extracted in CSE, and therefore their effects are not explored in the current study.

4.4.2 Conclusion

We conclude that CSE selectively reduces the anti-inflammatory effect of fluticasone on TNFα-induced production of the neutrophil chemoattractant CXCL8 in HASMCs. This *in vitro* model may be useful for further studies to explore the mechanisms of cigarette smoke-induced glucocorticoid insensitivity in asthma.

4.5. Future studies

There are a number of studies that will be carried out by our group in line with this particular study. First, to confirm that CSE-induced glucocorticoid insensitivity in normal and asthmatic HASMCs from different donors as well as human bronchial epithelial cells (HBECs) using fluticasone to confirm whether CSE can induce glucocorticoid insensitivity in other airway cells. Second, to assess if CSE can induced glucocorticoid insensitivity in the same cells by using other glucocorticoid drugs such as budesonide. Third, to investigate whether electronic cigarette vapor extract (ECVE) has a similar effect as CSE on glucocorticoids sensitivity in both HASMCs and HBECs. Fourth, to compare the effect of CSE and ECVE on the inhibitory effect of glucocorticoids in asthmatic HASMCs and HBECs with normal HASMCs and HBECs. Finally, to carry some mechanistic studies to investigate how CSE affects the response to glucocorticoids in both HASMCs and HBECs.

References:

1. Global Initiative for Asthma. *Global strategy for asthma management and prevention*. 2016[24.Feb.2019]; Available from: https://ginasthma.org/wp-content/uploads/2016/04/GINA-Appendix-2016-final.pdf.
2. Global Initiative for Asthma. *Pocket Guide for Asthma Management and Prevention*. 2018 [23.Feb.2019]; Available from: https://ginasthma.org/wp-content/uploads/2018/03/wms-GINA-main-pocket-guide_2018-v1.0.pdf.
3. British Thoracic Society. *A Practical Guide to Using Spirometry in Primary Care*. 2005 [22.Feb.2019]; Available from: https://www.brit-thoracic.org.uk/document-library/delivery-of-respiratory-care/spirometry/spirometry-in-practice-a-practical-guide-(2005)/.
4. Nunes, C., A.M. Pereira, and M. Morais-Almeida, *Asthma costs and social impact.* Asthma research and practice, 2017. **3**: p. 1-1.
5. Naghavi, M., et al., *Global, regional, and national age-sex specific mortality for 264 causes of death, 1980–2016: a systematic analysis for the Global Burden of Disease Study 2016.* The Lancet, 2017. **390**(10100): p. 1151-1210.
6. Masoli, M., et al., *The global burden of asthma: executive summary of the GINA Dissemination Committee report.* Allergy, 2004. **59**(5): p. 469-78.
7. Asthma UK. *Asthma UK Annual Report & Accounts*. 2017 [23.Feb.2019]; Available from: https://www.asthma.org.uk/about/annual-report-accounts/.
8. The World Health Organization. *Asthma, Key facts*. 2017 [25.Feb.2019]; Available from: https://www.who.int/news-room/fact-sheets/detail/asthma.
9. The Global Asthma Network. *The Global Asthma Report*. 2018 [15.Feb.2019]; Available from: http://www.globalasthmareport.org/Global%20Asthma%20Report%202018.pdf.
10. British Thoracic Society, *British guideline on the management of asthma*. 2016.
11. Kips, J.C. and R.A. Pauwels, *Long-acting Inhaled β2-Agonist Therapy in Asthma.* Am J Respir Crit Care Med, 2001. **164**(6): p. 923-932.
12. Ordonez, C.L., et al., *Increased neutrophil numbers and IL-8 levels in airway secretions in acute severe asthma: Clinical and biologic significance.* Am J Respir Crit Care Med, 2000. **161**(4 Pt 1): p. 1185-90.
13. Dijkstra, A., et al., *Lung function decline in asthma: association with inhaled corticosteroids, smoking and sex.* Thorax, 2006. **61**(2): p. 105-10.
14. Barnes, P.J., *Corticosteroid resistance in patients with asthma and chronic obstructive pulmonary disease.* J Allergy Clin Immunol, 2013. **131**(3): p. 636-45.
15. Barnes, P.J., *Inhaled Corticosteroids.* Pharmaceuticals (Basel), 2010. **3**(3): p. 514-540.
16. Fahy, J.V., *Eosinophilic and Neutrophilic Inflammation in Asthma.* Proceedings of the American Thoracic Society, 2009. **6**(3): p. 256-259.
17. Tillie-Leblond, I., et al., *Balance between proinflammatory cytokines and their inhibitors in bronchial lavage from patients with status asthmaticus.* Am J Respir Crit Care Med, 1999. **159**(2): p. 487-94.
18. Murphy, D.M. and P.M. O'Byrne, *Recent advances in the pathophysiology of asthma.* Chest, 2010. **137**(6): p. 1417-26.
19. Barnes, P.J., *Pathophysiology of asthma.* Br J Clin Pharmacol, 1996. **42**(1): p. 3-10.
20. Carr, T.F., A.A. Zeki, and M. Kraft, *Eosinophilic and Noneosinophilic Asthma.* Am J Respir Crit Care Med, 2018. **197**(1): p. 22-37.
21. Wenzel, S.E., et al., *Evidence that severe asthma can be divided pathologically into two inflammatory subtypes with distinct physiologic and clinical characteristics.* Am J Respir Crit Care Med, 1999. **160**(3): p. 1001-8.
22. Brusselle, G.G., T. Maes, and K.R. Bracke, *Eosinophils in the spotlight: Eosinophilic airway inflammation in nonallergic asthma.* Nat Med, 2013. **19**(8): p. 977-9.
23. Carr, T.F., et al., *Eosinophilic bioactivities in severe asthma.* World Allergy Organ J, 2016. **9**: p. 21.

24. Kaur, D., et al., *IL-33 drives airway hyper-responsiveness through IL-13-mediated mast cell: airway smooth muscle crosstalk.* Allergy, 2015. **70**(5): p. 556-67.
25. McBrien, C.N. and A. Menzies-Gow, *The Biology of Eosinophils and Their Role in Asthma.* Front Med (Lausanne), 2017. **4**: p. 93.
26. Gao, H., S. Ying, and Y. Dai, *Pathological Roles of Neutrophil-Mediated Inflammation in Asthma and Its Potential for Therapy as a Target.* J Immunol Res, 2017. **2017**: p. 3743048.
27. Green, B.J., et al., *Potentially pathogenic airway bacteria and neutrophilic inflammation in treatment resistant severe asthma.* PLoS One, 2014. **9**(6): p. e100645.
28. Fu, J.J., et al., *Systemic inflammation is associated with differential gene expression and airway neutrophilia in asthma.* Omics, 2013. **17**(4): p. 187-99.
29. Robinson, M.B., et al., *IL-6 trans-signaling increases expression of airways disease genes in airway smooth muscle.* Am J Physiol Lung Cell Mol Physiol, 2015. **309**(2): p. L129-38.
30. Robinson, D., et al., *Revisiting Type 2-high and Type 2-low airway inflammation in asthma: current knowledge and therapeutic implications.* Clin Exp Allergy, 2017. **47**(2): p. 161-175.
31. Chung, K.F. and P.J. Barnes, *Cytokines in asthma.* Thorax, 1999. **54**(9): p. 825-57.
32. Barnes, P.J., *The cytokine network in asthma and chronic obstructive pulmonary disease.* J Clin Invest, 2008. **118**(11): p. 3546-56.
33. Howarth, P.H., et al., *Synthetic responses in airway smooth muscle.* J Allergy Clin Immunol, 2004. **114**(2 Suppl): p. S32-50.
34. Lukacs, N.W., S.H. Oliveira, and C.M. Hogaboam, *Chemokines and asthma: redundancy of function or a coordinated effort?* J Clin Invest, 1999. **104**(8): p. 995-9.
35. John, A.E., et al., *Human airway smooth muscle cells from asthmatic individuals have CXCL8 hypersecretion due to increased NF-kappa B p65, C/EBP beta, and RNA polymerase II binding to the CXCL8 promoter.* Journal of immunology (Baltimore, Md. : 1950), 2009. **183**(7): p. 4682-4692.
36. Nocker, R.E., et al., *Interleukin-8 in airway inflammation in patients with asthma and chronic obstructive pulmonary disease.* Int Arch Allergy Immunol, 1996. **109**(2): p. 183-91.
37. Brightling, C.E., et al., *Differential expression of CCR3 and CXCR3 by human lung and bone marrow-derived mast cells: implications for tissue mast cell migration.* J Leukoc Biol, 2005. **77**(5): p. 759-66.
38. Strieter, R.M., et al., *CXC chemokines in angiogenesis.* Cytokine Growth Factor Rev, 2005. **16**(6): p. 593-609.
39. Hirano, T., *Interleukin 6 and its receptor: ten years later.* Int Rev Immunol, 1998. **16**(3-4): p. 249-84.
40. Ammit, A.J., et al., *Tumor necrosis factor-alpha-induced secretion of RANTES and interleukin-6 from human airway smooth muscle cells: modulation by glucocorticoids and beta-agonists.* Am J Respir Cell Mol Biol, 2002. **26**(4): p. 465-74.
41. Neveu, W.A., et al., *Elevation of IL-6 in the allergic asthmatic airway is independent of inflammation but associates with loss of central airway function.* Respir Res, 2010. **11**: p. 28.
42. Marini, M., et al., *Expression of the potent inflammatory cytokines, granulocyte-macrophage-colony-stimulating factor and interleukin-6 and interleukin-8, in bronchial epithelial cells of patients with asthma.* J Allergy Clin Immunol, 1992. **89**(5): p. 1001-9.
43. Hamelmann, E. and E.W. Gelfand, *IL-5-induced airway eosinophilia--the key to asthma?* Immunol Rev, 2001. **179**: p. 182-91.
44. Ohnishi, T., et al., *Eosinophil survival activity identified as interleukin-5 is associated with eosinophil recruitment and degranulation and lung injury twenty-four hours after segmental antigen lung challenge.* J Allergy Clin Immunol, 1993. **92**(4): p. 607-15.
45. Corrigan, C.J., et al., *CD4 T-lymphocyte activation in asthma is accompanied by increased serum concentrations of interleukin-5. Effect of glucocorticoid therapy.* Am Rev Respir Dis, 1993. **147**(3): p. 540-7.

46. Keatings, V.M., et al., *Late response to allergen is associated with increased concentrations of tumor necrosis factor-alpha and IL-5 in induced sputum.* J Allergy Clin Immunol, 1997. **99**(5): p. 693-8.
47. Hamid, Q., et al., *Expression of mRNA for interleukin-5 in mucosal bronchial biopsies from asthma.* J Clin Invest, 1991. **87**(5): p. 1541-6.
48. Schwarze, J., et al., *CD8 T cells are essential in the development of respiratory syncytial virus-induced lung eosinophilia and airway hyperresponsiveness.* J Immunol, 1999. **162**(7): p. 4207-11.
49. Walker, C., et al., *Activated T cells and eosinophilia in bronchoalveolar lavages from subjects with asthma correlated with disease severity.* J Allergy Clin Immunol, 1991. **88**(6): p. 935-42.
50. Farne, H.A., et al., *Anti-IL5 therapies for asthma.* Cochrane Database Syst Rev, 2017. **9**: p. Cd010834.
51. Wills-Karp, M., et al., *Interleukin-13: Central Mediator of Allergic Asthma.* Science, 1998. **282**(5397): p. 2258.
52. Berry, M.A., et al., *Sputum and bronchial submucosal IL-13 expression in asthma and eosinophilic bronchitis.* J Allergy Clin Immunol, 2004. **114**(5): p. 1106-9.
53. Brightling, C.E., et al., *Interleukin-4 and -13 expression is co-localized to mast cells within the airway smooth muscle in asthma.* Clin Exp Allergy, 2003. **33**(12): p. 1711-6.
54. Komai-Koma, M., et al., *Immuno-regulatory cytokines in asthma: IL-15 and IL-13 in induced sputum.* Clin Exp Allergy, 2001. **31**(9): p. 1441-8.
55. Park, S.W., et al., *Interleukin-13 and interleukin-5 in induced sputum of eosinophilic bronchitis: comparison with asthma.* Chest, 2005. **128**(4): p. 1921-7.
56. Huang, S.K., et al., *IL-13 expression at the sites of allergen challenge in patients with asthma.* J Immunol, 1995. **155**(5): p. 2688-94.
57. Humbert, M., et al., *Elevated expression of messenger ribonucleic acid encoding IL-13 in the bronchial mucosa of atopic and nonatopic subjects with asthma.* J Allergy Clin Immunol, 1997. **99**(5): p. 657-65.
58. Naseer, T., et al., *Expression of IL-12 and IL-13 mRNA in asthma and their modulation in response to steroid therapy.* Am J Respir Crit Care Med, 1997. **155**(3): p. 845-51.
59. Howard, T.D., et al., *Gene-gene interaction in asthma: IL4RA and IL13 in a Dutch population with asthma.* Am J Hum Genet, 2002. **70**(1): p. 230-6.
60. Corren, J., et al., *A randomized, controlled, phase 2 study of AMG 317, an IL-4Ralpha antagonist, in patients with asthma.* Am J Respir Crit Care Med, 2010. **181**(8): p. 788-96.
61. Corren, J., et al., *Lebrikizumab treatment in adults with asthma.* N Engl J Med, 2011. **365**(12): p. 1088-98.
62. Kasaian, M.T., et al., *IL-13 antibodies influence IL-13 clearance in humans by modulating scavenger activity of IL-13Ralpha2.* J Immunol, 2011. **187**(1): p. 561-9.
63. Piper, E., et al., *A phase II placebo-controlled study of tralokinumab in moderate-to-severe asthma.* Eur Respir J, 2013. **41**(2): p. 330-8.
64. Corren, J., *Role of interleukin-13 in asthma.* Curr Allergy Asthma Rep, 2013. **13**(5): p. 415-20.
65. Lee, Y.C. and H.K. Lee, *Vascular endothelial growth factor in patients with acute asthma.* J Allergy Clin Immunol, 2001. **107**(6): p. 1106.
66. Feltis, B.N., et al., *Increased vascular endothelial growth factor and receptors: relationship to angiogenesis in asthma.* Am J Respir Crit Care Med, 2006. **173**(11): p. 1201-7.
67. Hoshino, M., Y. Nakamura, and Q.A. Hamid, *Gene expression of vascular endothelial growth factor and its receptors and angiogenesis in bronchial asthma.* J Allergy Clin Immunol, 2001. **107**(6): p. 1034-8.
68. Lee, C.G., et al., *Studies of vascular endothelial growth factor in asthma and chronic obstructive pulmonary disease.* Proc Am Thorac Soc, 2011. **8**(6): p. 512-5.
69. van der Velden, V.H., *Glucocorticoids: mechanisms of action and anti-inflammatory potential in asthma.* Mediators of inflammation, 1998. **7**(4): p. 229-237.

70. Hirst, S.J. and T.H. Lee, *Airway smooth muscle as a target of glucocorticoid action in the treatment of asthma.* Am J Respir Crit Care Med, 1998. **158**(5 Pt 3): p. S201-6.
71. Oltmanns, U., et al., *Fluticasone, but not salmeterol, reduces cigarette smoke-induced production of interleukin-8 in human airway smooth muscle.* Pulm Pharmacol Ther, 2008. **21**(2): p. 292-7.
72. Luo, Q., et al., *The anti-malaria drug artesunate inhibits cigarette smoke and ovalbumin concurrent exposure-induced airway inflammation and might reverse glucocorticoid insensitivity.* Int Immunopharmacol, 2015. **29**(2): p. 235-245.
73. Milara, J., et al., *Oxidative stress-induced glucocorticoid resistance is prevented by dual PDE3/PDE4 inhibition in human alveolar macrophages.* Clin Exp Allergy, 2011. **41**(4): p. 535-46.
74. van der Velden, V.H., *Glucocorticoids: mechanisms of action and anti-inflammatory potential in asthma.* Mediators Inflamm, 1998. **7**(4): p. 229-37.
75. Fahy, J.V., *Type 2 inflammation in asthma--present in most, absent in many.* Nat Rev Immunol, 2015. **15**(1): p. 57-65.
76. Jang, A.S., *Steroid response in refractory asthmatics.* Korean J Intern Med, 2012. **27**(2): p. 143-8.
77. Berthon, B.S., et al., *A sputum gene expression signature predicts oral corticosteroid response in asthma.* Eur Respir J, 2017. **49**(6).
78. Moiseenko, A., et al., *Origin and characterization of alpha smooth muscle actin-positive cells during murine lung development.* STEM CELLS, 2017. **35**(6): p. 1566-1578.
79. Ozier, A., et al., *The pivotal role of airway smooth muscle in asthma pathophysiology.* J Allergy (Cairo), 2011. **2011**: p. 742710.
80. Zuyderduyn, S., et al., *Treating asthma means treating airway smooth muscle cells.* Eur Respir J, 2008. **32**(2): p. 265-74.
81. Lazaar, A.L. and R.A. Panettieri, *Airway smooth muscle: A modulator of airway remodeling in asthma.* Journal of Allergy and Clinical Immunology, 2005. **116**(3): p. 488-495.
82. Parameswaran, K., et al., *Extracellular matrix regulates human airway smooth muscle cell migration.* Eur Respir J, 2004. **24**(4): p. 545-51.
83. McDonald, D.M., *Angiogenesis and remodeling of airway vasculature in chronic inflammation.* Am J Respir Crit Care Med, 2001. **164**(10 Pt 2): p. S39-45.
84. Simcock, D.E., et al., *Induction of angiogenesis by airway smooth muscle from patients with asthma.* Am J Respir Crit Care Med, 2008. **178**(5): p. 460-8.
85. Kadowaki, M., et al., *Extracellular acidification-induced CXCL8 production through a proton-sensing receptor OGR1 in human airway smooth muscle cells: a response inhibited by dexamethasone.* J. Inflamm, 2019. **16**(1): p. 4.
86. Stapleton, M., et al., *Smoking and asthma.* J Am Board Fam Med, 2011. **24**(3): p. 313-22.
87. Lange, P., et al., *A 15-year follow-up study of ventilatory function in adults with asthma.* N Engl J Med, 1998. **339**(17): p. 1194-200.
88. Marquette, C.H., et al., *Long-term prognosis of near-fatal asthma. A 6-year follow-up study of 145 asthmatic patients who underwent mechanical ventilation for a near-fatal attack of asthma.* Am Rev Respir Dis, 1992. **146**(1): p. 76-81.
89. Boulet, L.P., et al., *Smoking and asthma: clinical and radiologic features, lung function, and airway inflammation.* Chest, 2006. **129**(3): p. 661-8.
90. Tamimi, A., D. Serdarevic, and N.A. Hanania, *The effects of cigarette smoke on airway inflammation in asthma and COPD: therapeutic implications.* Respir Med, 2012. **106**(3): p. 319-28.
91. Thomson, N.C., R. Chaudhuri, and E. Livingston, *Asthma and cigarette smoking.* European Respiratory Journal, 2004. **24**(5): p. 822.
92. Rasmussen, F., et al., *Impact of airway lability, atopy, and tobacco smoking on the development of asthma-like symptoms in asymptomatic teenagers.* Chest, 2000. **117**(5): p. 1330-5.

93. Raherison, C., et al., *Asthma phenotypes according to the timing of smoking onset in young adults.* Int J Tuberc Lung Dis, 2003. **7**(1): p. 84-92.
94. Shimoda, T., et al., *Influence of cigarette smoking on airway inflammation and inhaled corticosteroid treatment in patients with asthma.* Allergy Asthma Proc, 2016. **37**(4): p. 50-8.
95. Koo, J.B. and J.S. Han, *Cigarette smoke extract-induced interleukin-6 expression is regulated by phospholipase D1 in human bronchial epithelial cells.* J Toxicol Sci, 2016. **41**(1): p. 77-89.
96. Mio, T., et al., *Cigarette smoke induces interleukin-8 release from human bronchial epithelial cells.* Am J Respir Crit Care Med, 1997. **155**(5): p. 1770-6.
97. Pedersen, B., et al., *Eosinophil and neutrophil activity in asthma in a one-year trial with inhaled budesonide. The impact of smoking.* Am J Respir Crit Care Med, 1996. **153**(5): p. 1519-29.
98. Chalmers, G.W., et al., *Influence of cigarette smoking on inhaled corticosteroid treatment in mild asthma.* Thorax, 2002. **57**(3): p. 226-30.
99. Chaudhuri, R., et al., *Cigarette smoking impairs the therapeutic response to oral corticosteroids in chronic asthma.* Am J Respir Crit Care Med, 2003. **168**(11): p. 1308-11.
100. Jang, A.S., et al., *Factors influencing the responsiveness to inhaled glucocorticoids of patients with moderate-to-severe asthma.* Chest, 2005. **128**(3): p. 1140-5.
101. Berry, M., et al., *TNF-alpha in asthma.* Curr Opin Pharmacol, 2007. **7**(3): p. 279-82.
102. Brightling, C., M. Berry, and Y. Amrani, *Targeting TNF-alpha: a novel therapeutic approach for asthma.* J Allergy Clin Immunol, 2008. **121**(1): p. 5-10; quiz 11-2.
103. Thomas, P.S., D.H. Yates, and P.J. Barnes, *Tumor necrosis factor-alpha increases airway responsiveness and sputum neutrophilia in normal human subjects.* Am J Respir Crit Care Med, 1995. **152**(1): p. 76-80.
104. Thomas, P.S. and G. Heywood, *Effects of inhaled tumour necrosis factor alpha in subjects with mild asthma.* Thorax, 2002. **57**(9): p. 774-8.
105. McKay, S., et al., *Tumor necrosis factor-alpha enhances mRNA expression and secretion of interleukin-6 in cultured human airway smooth muscle cells.* Am J Respir Cell Mol Biol, 2000. **23**(1): p. 103-11.
106. Roberts, M., X. Luo, and N. Chegini, *Differential regulation of interleukins IL-13 and IL-15 by ovarian steroids, TNF-alpha and TGF-beta in human endometrial epithelial and stromal cells.* Mol Hum Reprod, 2005. **11**(10): p. 751-60.
107. Raidl, M., et al., *Impaired TNFalpha-induced VEGF expression in human airway smooth muscle cells from smokers with COPD: role of MAPkinases and histone acetylation--effect of dexamethasone.* Cell Biochem Biophys, 2007. **49**(2): p. 98-110.
108. Chen, L., et al., *Effects of cigarette smoke extract on human airway smooth muscle cells in COPD.* Eur Respir J, 2014. **44**(3): p. 634-46.
109. Volpi, G., et al., *Cigarette smoke and alpha,beta-unsaturated aldehydes elicit VEGF release through the p38 MAPK pathway in human airway smooth muscle cells and lung fibroblasts.* Br J Pharmacol, 2011. **163**(3): p. 649-61.
110. Zhao, J., et al., *Immunomodulatory effects of cigarette smoke condensate in mouse macrophage cell line.* Int J Immunopathol Pharmacol, 2017. **30**(3): p. 315-321.
111. Adcock, I.M. and S.J. Lane, *Corticosteroid-insensitive asthma: molecular mechanisms.* J Endocrinol, 2003. **178**(3): p. 347-55.
112. Thomson, N.C., R. Chaudhuri, and E. Livingston, *Asthma and cigarette smoking.* Eur Respir J, 2004. **24**(5): p. 822-33.
113. Ray, A. and J.K. Kolls, *Neutrophilic Inflammation in Asthma and Association with Disease Severity.* Trends in immunology, 2017. **38**(12): p. 942-954.