Modulating steroid insensitive pathways in airway smooth muscle cells

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ABSTRACT

Approximately 5 to 10% of patients with asthma have difficult-to-control or severe disease that is refractory or poorly responsive to glucocorticoids (GC). This group represents a significant unmet clinical need and is responsible for a disproportionate share of health care costs and morbidity associated with the disease.

This thesis investigated the underlying molecular mechanisms of steroid insensitivity in airway smooth muscle (ASM) in patients with severe asthma. We used a natural compound isolated from an African plant called Compound A (CpdA) in an *in vitro* model of steroid insensitivity of ASM cells treated with TNFα and IFNγ.

This is the first report to show that CpdA, independently of the GRα, differentially suppressed the expression of GC-insensitive chemokines in ASM cells. We elucidated one mechanism responsible for the inhibition of fluticasone-resistant CXCL10 by CpdA via up-regulation of the MAPK deactivator, MKP-1. This thesis described the modulation of fluticasone-resistant chemokines (CCL5, CX3CL1) via inhibition of the MAPK pathway (p38MAPK, JNK), raising the possibility that targeting these kinases in ASM could be beneficial in treating steroid insensitivity. The potential synergistic effect of using MAPK inhibitors (p38, ERK) in combination with fluticasone showed a beneficial effect in reducing the production of GC-resistant chemokines (CCL5 and CXCL10). Finally, this study identified another potential mechanism involved in CpdA action via inhibition of the transcription factor IRF-1 which we showed was important in driving cytokine-induced CCL5 and CX3CL1 expression. More importantly, IRF-1 was activated within the ASM in biopsies from severe asthmatics and its expression was unaffected by GC treatment. IRF-1 expression within the ASM from severe asthmatic biopsies correlated with serum IgE, sputum eosinophils and ENo levels, with some differences depending on treatment.

This thesis provided evidence for the effectiveness of CpdA in the suppression of GC-insensitive genes in our *in vitro* model. This work supports the involvement of IRF-1 in driving steroid insensitive pathways in ASM cells and suggests that targeting IRF-1 axis could offer an alternative therapy for asthma. Inhibition of MAPK kinases and up-regulation of endogenous MAPK deactivators could also be potential strategies for treatment.

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PUBLICATIONS

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ORAL PRESENTATIONS

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2015   European Respiratory Society Congress (Amsterdam, Netherlands)
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ABBREVIATIONS

α-actin	Alpha smooth muscle actin
ADAM	A Disintegrin and metalloproteinase
ANOVA	Analyses of variance
AHR	Airway hyper-responsiveness
APC	Antigen presenting cells
ATS	American Thoracic Society
ASM	Airway Smooth Muscle
AP-1	Activator protein-1
aENo	Alveolar exhaled nitric oxide
ARE	Adenylate-uridylate-rich elements
ALK	Activin receptor-like kinase
BTS	British Thoracic Society
BAL	Bronchoalveolar Lavage
BW	Bronchial wash
B cells	B lymphocytes
BCR	B cell receptor
BSA	Bovine serum albumin
bFGF	Basic fibroblast growth factor
CpdA	Compound A
Ca2+	Calcium
COX-2	Cyclooxygenase-2
Cys-LT	Cysteinyl leukotriene
CBP	CREB binding protein

CCL	Chemokine ligand
CXC	Chemokine (C-X-C motif) ligand
CCR	Receptor for CC-family chemokines
CSR	Corticosteroid resistance
cAMP	Cyclic adenosine monophosphate
COPD	Chronic obstructive pulmonary disease
DMEM	Dulbecco's modified eagles medium
DNA	Deoxyribonucleic acid
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
ENo	Exhaled nitric oxide
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal transition
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ERK	Extracellular-signal-regulated kinases
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FcεRI	The high-affinity IgE receptor
FEV1	Forced expiratory volume in 1 second
FP	Fluticasone propionate
FVC	Forced vital capacity
GC	Glucocorticoids
GILZ	Glucocorticoid-induced leucine zipper

GINA	The Global Initiative for Asthma
GM-CSF	Granulocyte/macrophage-colony stimulating factor
GR	Glucocorticoid receptor
GRE	Glucocorticoid-response element
GC	Glucocorticoid
H_2SO_4	Sulphuric acid
HBSS	Hank's balanced salt solution
HRP	Horseradish peroxidise
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
ICAM-1	Intercellular cell adhesion molecule 1
IFNγ	Interferon gamma
ITS	Insulin-Transferrin-Selenium
ICS	Inhaled corticosteroid
IgG	Immunoglobulin G
ISRE	Interferon Stimulated Response Element
iNOS	Inducible nitric oxide synthase
IL	Interleukin
JAK	Janus tyrosine kinases
JNK	c-Jun N-terminal kinase
LABA	Long-acting β2-agonist
LPS	Lipopolysaccharide
mRNA	messenger ribonucleic acid
MBP	Major basic protein
MAPK	Mitogen-activated protein kinase

MKP-1	MAP kinase phosphatase 1
miRNA	MicroRNA, small non-coding RNA
MCP-1	Monocyte chemoattractant protein 1
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MMP	Matrix metalloprotease
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
Na3VO4	Sodium orthovanadate
NAF	Sodium fluoride
Na+	Sodium
NF-κB	Nuclear factor-κB
NP-40	nonidet-40
PAF	Platelet activating factor
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cells
PDGF	Platelet derived growth factor
PGD2	Prostaglandin D2
PMSF	Phenylmethanesulfonyl fluoride
RIPA	Radioimmunoprecipitation Assay
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase polymerase chain reaction
SABA	Short-acting β2-agonist
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SLPI	Secretory leukocyte protease inhibitor

SEM	Standard error of the mean
STAT	Signal transducers and activators of transcription
TBST	Tris-buffered Saline containing 0.001% Tween®20
T cells	T lymphocytes
TGF-β	Transforming growth factor-beta
Th	T helper cells
TLR	Toll-like receptors
TNFα	Tumour necrosis factor-alpha
T_{reg}	Regulatory T cells
TF	Transcription factor
TSLP	Thymic stromal lymphopoietin
VEGF	Vascular endothelial growth factor
VCAM-l	Vascular cell adhesion molecule-I

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CHAPTER 1

General Introduction

1.1   Overview

Asthma is a very common disease, affecting nearly 300 million people in the world (1). There are 5.4 million people suffering from asthma in the UK, with one in five households affected, representing one of the highest prevalence rates of asthma symptoms in Europe. Furthermore, poor asthma management is associated with high morbidity and mortality and also with increased costs, causing a great burden on the health care system. It is estimated that better management of asthma symptoms could avoid as many as 75% of hospital admissions and approximately 90% of the deaths from asthma (2).

Currently, the most common treatment for asthma has been the administration of corticosteroids, used to manage the typical symptoms of cough, shortness of breath and wheeze. However 5 to 10% of patients suffer with severe asthma and do not respond to treatment. Although a small proportion, they account for more than 50% of the total asthma health care costs. Furthermore, they experience recurrent asthma attacks, have high morbidity and high mortality. These patients are also exposed to severe side effects due to the use of higher doses of corticosteroids, which greatly impacts on the quality of their life. There is an urgent need for finding better alternatives for asthma treatment in these patients.

1.2   Thesis statement

This thesis focuses on investigating the underlying molecular mechanisms contributing to reduced sensitivity to corticosteroid therapy in airway smooth muscle cells from patients with severe asthma. For this work, we used a natural compound isolated from an African plant called compound A (CpdA) in an *in vitro* model of steroid insensitivity of airway smooth muscle cells treated with cytokines. This thesis provided evidence for the effectiveness of CpdA in the suppression of genes that are not

inhibited by current glucocorticoids in our *in vitro* model of GC insensitivity and formulated a mechanism to explain its mode of action. This work contributes to our understanding of the mechanisms affecting the anti-inflammatory actions of corticosteroids in airway smooth muscle.

1.3   Asthma pathophysiology

Asthma is a chronic respiratory disease characterized by variable symptoms of wheeze, shortness of breath, expiratory airflow limitation, chest tightness and cough. These symptoms can be triggered by various stimuli (eg. allergen exposure, irritants, exercise, weather changes, and viral infections) and their intensity can vary over time (3). In some cases symptoms may resolve spontaneously or in response to treatment. Other times patients can experience exacerbations, which are episodic flare-ups of asthma symptoms that may be life threatening and that add to the high costs of asthma management (3).

Asthma symptoms are accompanied by the underlying pathophysiology of the disease, characterized by ***airway inflammation***, ***airway hyperresponsiveness***, and in more severe disease, persistent ***airflow obstruction*** and ***airway remodelling*** (4).

Airway inflammation is fundamental to asthma pathogenesis and is summarized in **Figure 1.1**. Firstly, the rapid phase is initiated by the allergen activation of IgE-specific cells (eg. mast cells, macrophages), which releases proinflammatory mediators (eg. histamine, eicosanoids, reactive oxygen species, cysteinyl leukotrienes (Cys-LTs) and prostaglandin D_2 (PGD_2)). These mediators cause airway smooth muscle (ASM) contraction, mucous secretion, and vasodilatation. Damaged epithelial cells release stem-cell factor (SCF) (5), which is responsible for the survival of mast cells within the airways. Epithelial cells also secrete thymic stromal lymphopoietin (TSLP) which

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contributes to the processing of allergens by dendritic cells (6), and IL-25 and IL-33 which can both activate innate lymphoid cells (ILC2) in an additive manner. The late-phase reaction occurs at 6 to 9 hours after allergen provocation and involves the recruitment and activation of inflammatory cells. Dendritic cells produce CCL17 and CCL22, which are Th2 chemoattractants via the CCR4 receptor (7). In turn, Th2 cells release a variety of mediators: IL‑4 and IL‑13 which stimulate IgE release by B cells; IL‑5, IL-3 and GM-SCF which recruit eosinophils; IL‑9, IL-3, IL-4 and IL-13 which activate mast cells and lead to their proliferation (8). Epithelial cells also release a plethora of cytokines including CCL11, CCL5 and CCL2, which act as eosinophil chemoattractants via CCR3 (9). Activated mast cells can in turn act on eosinophils and epithelial cells via the release of IL-4, IL-5 and IL-13 (10), further enhancing the cycle of cytokine-driven inflammation. Patients with asthma may have a defect in regulatory T (Treg) cells, as their reduced production may favour further Th2-cell proliferation (11). Since the involvement of T cells appears to occur later after allergen challenge, this supports their role in the more chronic phase of the response. The mechanisms for neutrophilic recruitment are less clear and Th1 factors are thought to contribute to some degree to the pathogenesis of asthma (12). More recently, Th17 immunity has been implicated in driving neutrophilia, primarily in murine models of asthma, with some supporting data from severe asthma (12), (13), (14), (15). Th17-related cytokines were elevated in the airway tissues in severe asthma. IL-17 was associated with the activation of epithelial cells *in vitro* and with the induction of IL-6 and IL-8 which had the downstream effect of recruiting and activating neutrophils (16). Furthermore, IL-17 was also reported to affect structural cells and to stimulate the production of pro-fibrotic cytokines and extracellular matrix proteins. A similar study highlighted that patients with severe asthma and neutrophilic inflammation had increased expression of chitinase

3-like protein 1 (YKL-40) and matrix metalloproteinase (MMP) 1, 3, 8, and 12 (17), further supporting the concept that features of inflammation may lead to remodelling.

Figure 1.1: Th2 and non-Th2 Immune mechanisms involved in the pathogenesis of asthma (Adapted from (11))

As a result of inflammation there is leakage of acute plasma proteins, which leads to thickening of the airway lumen, damage of the epithelial integrity and reduced mucus clearance. These contribute to increased *airway obstruction* (18) which is usually a reversible process initiated by the contraction of ASM. However, in some patients airway obstruction can be irreversible, due to structural changes in the airways known as *airway remodelling.* As a consequence, patients have poor lung function and are at increased risk of exacerbations, which results in sensitive airways and worsening of asthma symptoms (19).

The various structural changes leading to airway remodelling have been summarised in **Table 1.1**. One paradigm suggests that asthma pathogenesis begins with defects in the epithelial barrier function and repair mechanisms caused by predisposed genetic alterations coupled with environmental triggers. As shown in **Figure 1.1,** this leads to activation of dendritic cells, which initiates Th2-driven inflammation. Moreover, the damaged epithelium releases a variety of growth factors (Transforming Growth Factor β (TGFβ), Epidermal Growth Factor (EGF), and Vascular Endothelial Growth Factor (VEGF)) and cytokines which are responsible for driving fibrosis and the excessive deposition of sub-epithelial matrix proteins (collagen, tenascin, matrix proteins) (18). Myofibroblast activation alongside with fibroblast and smooth muscle hyperplasia also lead to increased thickness of smooth muscle. This process makes the airways stiffer and less distensible, leading to fixed airway lumen narrowing (20).

Table 1.1: Structural changes involved in airway remodelling in asthma

Abnormal changes	Proposed mechanisms	Reference
Abnormal epithelium	↑Th2 cytokines-induced goblet cell hyperplasia ↑Cell shedding ↑Ciliated cell loss	(21) (22) (23) (24)
Sub-basement membrane thickening (fibrosis)	↑Cytokines (IL-13, IL-21, TGF-β) ↑Chemokines (MCP-1, MIP-1β) ↑Growth factors (VEGF, PDGF) ↑Peroxisome proliferator-activated receptors (PPARs) ↑Acute phase proteins (SAP) ↑Caspases ↑Myofibroblast, fibroblast and ASM proliferation	(25) (26)
Matrix abnormalities	↑Collagen I, III, V, fibronectin, tenascin, hyaluronan, versican and laminin α2/β2 ↓Collagen IV and elastin ↑*De novo* ECM proteins synthesis	(27) (28) (29)

	↓Activity of ECM degrading enzymes (metalloproteinases, MMPs); ↑Expression of tissue-specific inhibitors of MMPs (TIMP).	
↑ Vascularisation and angiogenesis	↑VEGF ↑Profibrogenic cytokines, growth factors and MMPs ↑Proteolysis ↑Angiogenic cytokines (Fibroblast Growth Factor 2 (FGF-2), serine proteases tryptase and chymase, IL-8, TGF-β, TNFα and Nerve Growth Factor (NGF))	(30) Reviewed in (31)
↑ Mucus production	↑Mucin production and secretion (MUC5A, MUC5B) via IL-13/IL-4 receptor-α complex and EGFR (Epidermal Growth Factor Receptor) pathways	(32) (33), (34), Reviewed in (35)

1.4   The role of airway smooth muscle (ASM) in asthma

There is plenty of evidence to support the critical role of ASM cells in asthma. ASM cells **(1)** are part of the inflammatory process, **(2)** contribute to airway remodelling, and **(3)** have an altered contractile, proliferative and secretory function in asthmatic airways. Therefore pro-asthmatic responses in ASM cells may represent novel therapeutic targets to treat asthma.

This section aims to highlight the different mechanisms by which ASM contributes to asthma pathogenesis. It is important to note that these mechanisms may interact with each other so that the overall contribution of ASM is multi-fold and highly complex. For example, the ASM plays a role in airway hyperresponsiveness (AHR) via its increased response to various contractile stimuli and decreased response to relaxing stimuli such as β2-agonists. ASM contributes to inflammation via its secretion of multiple cytokines, chemokines and growth factors, potentiating the inflammatory responses within the airways. Finally, ASM plays a role in the remodelling of the airway wall via its increased mass (due to hyperplasia, hypertrophy, and decreased

apoptosis) and altered ECM deposition. Last but not least, these underlying features are interconnected. For example, ASM cells have the potential to attract other inflammatory cells such as mast cells to the ASM bundle of asthmatic patients to further potentiate the inflammatory cascade (36). Upon adhesion to ASM, mast cells release mediators (e.g. histamine, PGD_2 and Cys-LTs), which can modulate ASM contraction. This bidirectional interaction may be functionally important in driving AHR, as a negative correlation was found between the number of infiltrated mast cells within ASM and PC_{20}, (provocative concentration of methacholine that results in a 20% reduction in forced expiratory volume (FEV1) in one second, a marker of AHR).

1.4.1   Role of ASM in bronchial hyperresponsiveness (AHR)

The excessive narrowing of the airways, also known as AHR is thought to occur mainly due to the increased sensitivity of the ASM, the main tissue that regulates the degree of bronchoconstriction, that is further enhanced by the magnitude of swelling of airway wall compartments and mucus plugging (37), (38). Apart from its direct role in airway obstruction, ASM has a strong inflammatory potential, which will be discussed later in this section (39).

AHR can be triggered by various stimuli, including direct bronchoconstrictor stimuli (e.g. cholinergic agonists, histamine, prostaglandins, leukotrienes), indirect chemical stimuli (e.g. adenosine, non-steroidal anti-inflammatory drugs, bradykinin, endotoxin, allergens) and indirect physical stimuli (e.g. exercise, heat, water loss, increased ASM mass and vagal tone) (Reviewed in (39)). Direct mediators stimulate ASM cell membrane receptors while indirect mediators stimulate other cells (inflammatory cells present in the lungs), which in turn release contractile agonists that then induce ASM contraction (40).

It is known that AHR can be reversed using bronchodilators that directly relax ASM (41). In asthma, there appears to be a reduced production of broncho-relaxant mediators, which may indirectly contribute to AHR. These mediators can be inflammatory (eg. prostanoids) or neurotransmitter molecules (adrenaline, noradrenaline, vasoactive intestinal peptide) (Reviewed in (42)). For example, a reduced release of adrenaline was observed during asthma attacks (43). Also prostaglandin E_2 (PGE_2) production, a prostanoid known to relax ASM, was significantly lower in asthmatic cells compared to nonasthmatic ASM cells (44). A variety of mechanisms are thought to be responsible for the hyperresponsive properties of asthmatic airways as compared to healthy individuals (**Table 1.2**). Firstly, the asthmatic ASM is thought to generate more force and therefore contract to a greater extent (45) when compared to healthy airways. In sensitised animal models, it was shown that there was an increase in the contractility of ASM cells due to increased free calcium (Ca^{2+}) release (46) or increased expression of myosin light chains (47). Changes in Ca^{2+} signalling in ASM have been also associated with AHR (Reviewed by (48)) and calcium-sensing receptor antagonists were shown to abrogate AHR and inflammation in allergic asthma (49). In addition, the Rho kinase content and activity were also increased in guinea pigs (50). The altered composition of the extracellular matrix (ECM) or the thickening of the airway wall and the enhanced production of ECM proteins also moderate ASM shortening and stiffness in the airways (42).

Table 1.2: Mechanisms in ASM driving AHR

Mechanism	Reference
↑Release of contractile mediators (eg. histamine)	(51)
↑Mass	(52)
↑Stiffness	(53)
↑Cytokines → ↑ free calcium →↑Contractility	(46), (54), Reviewed by (48)
↑Myosin light chain expression →↑contractility	(47)
↑Maximum shortening velocity and capacity	(55)
↑Vagal tone and acetylcholine (Ach) release	(56), (57)
↑Activation of the Rho kinases	(58), (59)

1.4.2   Role of ASM in bronchial inflammation

As previously mentioned, ASM can secrete an array of inflammatory mediators which in turn regulate key asthmatic features of asthma such as airway inflammation (60), (61), (41). *In vivo* studies using immunohistochemistry have shown that many of these mediators are highly expressed in the ASM bundles of patients with asthma when compared to non-asthmatic subjects and their role in asthma is summarised in **Table 1.3**.

Table 1.3: Immuno-modulatory factors expressed *in vivo* by ASM in biopsies from asthmatic patients (Adapted from (62))

Mediators	Role in asthma	Reference
CCL5	Chemotaxis	(63)
CCL11	Chemotaxis	(64)
CCL19	Chemotaxis	(65)
CX3CL1	Chemotaxis	(66)
CCL15	Chemotaxis	(67)
Thymic stromal lymphopoietin (TSLP)	Th2 allergic response Corticosteroid insensitivity	(68)
TNF-related apoptosis-inducing ligand (TRAIL)	Eosinophil survival	(69)
IL-33	Chemotaxis Th2 allergic response Airway remodelling	(70)
Nerve Growth Factor (NGF)	ASM migration Mast cell activation and survival	(71)

Pentraxin 3 (PTX3)	Chemokine secretion ASM migration	(72)
ADAM Metallopeptidase domain 33 (ADAM 33)	Impaired lung function ASM contractile function	(73, 74)
Heparin-binding EGF	ASM remodeling	(75)

*Bold = mediators still expressed in biopsies from severe asthma patients

The synthetic function of ASM was first demonstrated *in vitro* using cultured ASM cells which showed its ability to express a wide variety of cell surface molecules (**Table 1.4**) and to produce various inflammatory molecules (**Table 1.5**), ECM proteins (**Table 1.6**) and enzymes (e.g. MMP- 9 and MMP-12) (76). As a consequence, ASM can play an active role through its autocrine and/or paracrine function in the asthmatic inflammatory process.

Table 1.4: Cell surface molecules expressed by ASM cells

ASM cell surface molecules	Cells activated	Functional relevance	Reference
Intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1)	T-cells	ASM DNA synthesis	(77)
CD44	T-cells	ASM DNA synthesis	(77)
CD40, CD80 and CD86	T-cells	ASM DNA synthesis	(78), (79), (80)
OX40 ligand	B-cells, dendritic cells	IL-6 increase	(81)
TLR2, TLR3 and TLR4	ASM cells	CXCL8 and CCL11 release	(82)
Toll-like receptors (TLR)	Mast cells	↑cytokine and chemokine production	(83)
Chemokine receptors (CCR3,7, CXCR1, 3, 4)	ASM cells	↑Pro-inflammatory responses	(84)
Receptors for IL-4, 6, 12, 13, 17, 22 and IFN-γ	ASM cells	↑Pro-inflammatory responses	(85), (86)
TNFR1 and TNFR2	ASM cells	↑TNFR1, important in AHR	(87, 88)

Table 1.5: Pro-inflammatory mediators produced by ASM cells

Chemokines, cytokines and growth factors	Reference
CCL2/5/7/8/11/17/19	Reviewed in (42); For CCL5 (89),
CXCL8/10	(90), (91)
CX3CL1	(66)
IL-2/5/6/11/12	Reviewed in (42)
IFNγ	(92)
Connective Tissue growth factor	(93)
Granulocyte macrophage-colony stimulating factors (GM-CSF)	(94)
Transforming growth factor-β1 (TGFβ)	(95)
Stem Cell Factor (SCF)	(96)
Vascular endothelial growth factor (VEGF)	(97)

Table 1.6: ECM molecules produced by ASM cells

ECM proteins	Reference
Collagen (I, III, IV, V)	(98)
Decorin, elastin, fibronectin, laminin, perlecan	(98)
Thrombospondin	(98)
Tissue inhibitor of MMPs- 1, 2	(99)

The receptor molecules on the surface of ASM cells play a crucial role in the interaction between ASM cells and other inflammatory cells. Experiments using human sensitised bronchi showed increased number of mast cells present in the smooth muscle as compared to nonsensitised bronchi (100). Brightling's group demonstrated that mast cells were infiltrated within the ASM bundle of asthmatic airways (36). This event was termed mast cell myositis (101), and appeared to be a specific feature of asthma. Interestingly, myositis did not take place in eosinophilic bronchitis and healthy patients, and was unchanged in treated and non-treated asthma and regardless of asthma severity (36), (102). *In vitro* studies demonstrated that mast cell-ASM cell interaction occurred via the cell adhesion molecule 1, CADM1 and SCF receptor (c-Kit) (103), (104). This direct cell-cell interaction was also demonstrated *in vitro* using electron microscopy (105) and was shown to be functionally important (106), with ASM cells promoting the survival, proliferation and constitutive activation of mast cells (104). In turn mast cells

released tryptase leading to cytokine release (107) and ASM proliferation (107), (108), (109). Tryptase also modulates ASM contractile responses as it induces the secretion of TGF-β1, which increases expression of ASM α-actin (110, 111). Chemoattractants such as CCL11 (112), CXCL10 (91) and CX3CL1 (66) can also help with mast cell recruitment to the ASM bundle. These different studies may explain why there is an increased mast cell number within the ASM bundles and why this infiltration positively correlates with the degree of AHR (36), (102) and with the intensity of α-actin expression (110).

T cells were also found to be infiltrated within the ASM layer of asthmatics (105), (113) and their numbers correlated with asthma severity (113). A few mediators produced by ASM cells act as chemotactic factors for T cells, such as CCL5 (89), however very little is known about the functional relevance of the interaction between these two cells types. One potential outcome of this interaction is that ASM could enhance T cell survival, thus possibly contributing to the perpetuation of bronchial inflammation (113). Also, through their ability to present superantigens via their MHC class II molecules to resting CD4+ T cells (114) ASM cells are able to activate these cells to release IL-13. IL-13 increases the contractile response to acetylcholine as shown in isolated rabbit ASM tissues (114).

1.4.3   Role of ASM in bronchial remodelling

Remodelling of the airways represents a key feature of asthmatics (115) and involves various structural alterations such as ASM hypertrophy or hyperplasia, epithelial changes, mucus hypersecretion, goblet cell hyperplasia and angiogenesis as described in detail in **Section 1.3**, **Table 1.1.** These alterations are shown in **Figure 1.2.** Furthermore there are no current medications for ASM remodelling (116).

The role of ASM in driving features of airway remodelling is unknown but most likely derives from various mechanisms including effects of inflammatory mediators, growth factors and aberrant deposition of ECM proteins (115). As evidenced in **Figure 1.2**, there is an increase in ASM in patients with severe asthma. Increased ASM mass was first observed in the airways of patients with fatal (117) and nonfatal asthma (113), (118), (119), (120). This increased ASM mass could be explained by the actions of various factors. Numerous mitogenic stimuli can promote ASM proliferation *in vitro* (e.g. cytokines, chemokines, enzymes, components of the ECM, reactive oxygen species (42)) with most factors acting via activation of receptor tyrosine kinase pathways or G protein coupled receptor pathways (121). Moreover, even without stimulation, asthmatic ASM cells were able to proliferate faster than nonasthmatic cells (122).
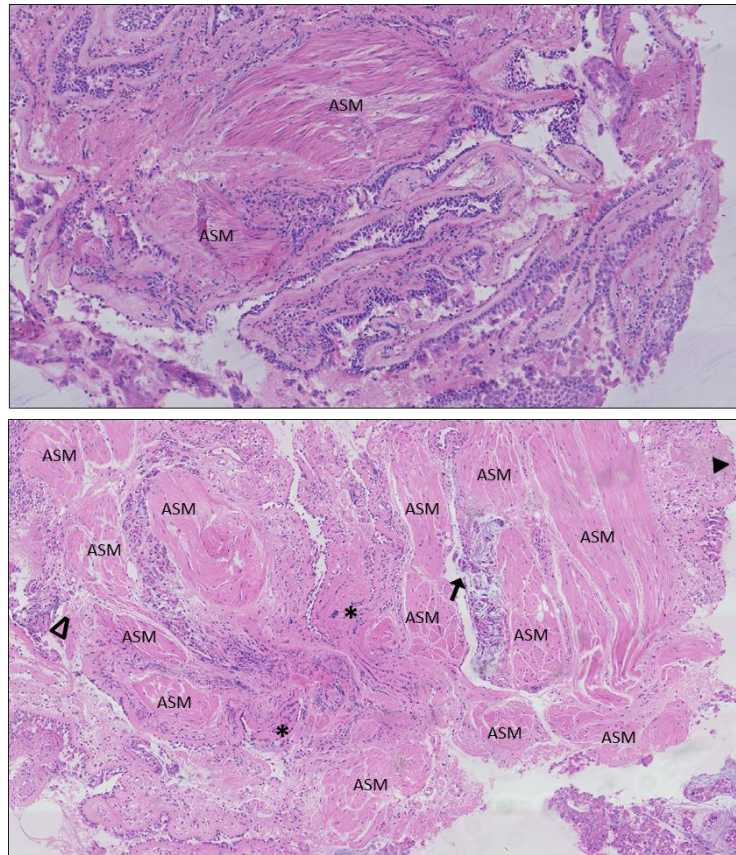
Figure 1.2: Haematoxylin and eosin (H&E) staining of 3μM thick airway tissue section from healthy (upper picture) and severe asthmatic (bottom picture). Specimens were taken from lung biopsies fixed in GMA. Airway smooth muscle (ASM); full arrowhead = denuded epithelial cells, empty arrowheads = angiogenesis; arrow = inflammatory cells; * = increased reticular membrane.

More recently, it was suggested that migration of ASM precursor cells towards the ASM bundles may participate in ASM hyperplasia (123). An increased accumulation of fibroblasts was found in the basement membrane in severe asthmatics when compared to healthy controls (119). Additionally, the myosin light chain kinase (MLCK) content in ASM from severe asthmatics was enhanced and correlated negatively with pre- and post-bronchodilator FEV1. More importantly, the increase in ASM area seemed to be the best marker for severity, and correlated negatively with FEV1 in severe asthmatics but not in the moderate asthmatics (124). Another possible explanation could be an imbalance between ASM proliferation and apoptosis however

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there is very little evidence so far in asthmatic patients to support this possibility (42). *Ex vivo* evidence showed increased expression of a cytokine that plays a role in apoptosis called TRAIL in asthmatic ASM following allergen challenge (69). Interestingly, there is contradicting evidence with regards to whether the increased ASM volume is due to an increase in cell number (hyperplasia) or cell size (hypertrophy). One study found an increase in cell number leading to increased ASM mass (125), while another showed that both hyperplasia and hypertrophy contributed to this process (117). In terms of its clinical relevance, the increase in ASM mass in the small airways of patients with severe asthma correlated with low FEV1 (below 50% predicted), recurrent and life-threatening exacerbations, and impaired quality of life in these patients (126).

Also contributing to remodelling is the production of an altered array of ECM proteins by asthmatic cells compared to nonasthmatics. Asthmatic cells produced increased perlecan and collagen I and less laminin-a1 and collagen IV (127). The changes in the ECM composition were shown to influence the rate of ASM proliferation (128). For example, fibronectin and collagen I increased the rate of ASM proliferation, while laminin had an inhibitory effect. Moreover, nonasthmatic cells grown on ECM produced by asthmatic cells were shown to proliferate faster. ECM was also shown to induce angiogenesis within the ASM bundle (129), stimulating its production of chemokines (e.g. eotaxin) (112) and maintaining its contractile phenotype (130).

In conclusion, characterising the pathophysiology of ASM could potentially improve our approaches for asthma treatment (131). In particular, the amount of ASM mass, mast cell infiltration within the ASM layer and the contractile versus synthetic-proliferative phenotype of ASM cells could be considered when classifying patients, as these features may correlate with severity and AHR. One very recent study looking at

biopsy features of cellular inflammation and remodelling in asthma identified three distinct clusters. Of these the early onset atopic eosinophilic group featured Th2 high inflammation, increased ASM mass, increased mast cells infiltration within the ASM and mixed granulocytic submucosal inflammation (132). In addition to this it would be advantageous to identify novel non-invasive markers of inflammation and remodelling. This is currently being investigated using cluster analysis in combination with RNA-Seq (high-throughput sequencing), microarray data and pathway analysis (**Table 1.7).**

Table 1.7: Recent studies looking at gene profiling in ASM cells

Stimuli	No. of genes	Potential candidates	Role	Reference
S1P	>80 (asthma vs healthy)	HBEGF TGFB3 TXNIP PLAUR SERPINE1 RGS4	**Steroid resistance** Airway remodelling Proliferation	(133)
Baseline	174 (asthma vs atopic) 108 (asthma vs non-atopic) 135 (atopic vs non-atopic)	RPTOR VANGL1 FAM129A LEPREL1	AHR	(134)
Prednisolone	15 (treatment vs baseline)	PPP2R1B SCUBE3 ADAM22 GCC2 ACTA2 KIAA0319 FAM129A KIF5C	Growth, Proliferation Development	(135)
Dexamethasone	316 (treatment vs baseline)	C7 CCDC69 CRISPLD2	**Steroid resistance**	(136)
Baseline	46 (asthma vs healthy)	STAU2 WARS BCL2 SLC26A4	AHR Inflammation Cellular functions	(137)
Dexamethasone	7500 (treatment vs baseline)	Kruppel-like factor 15 (Klf15)	ASM proliferation Contraction Inflammation	(138)

Baseline		miR-10a	ASM proliferation	(139)
Baseline	838 (fatal asthma vs. non-asthma)		Cellular functions	(140)
Vitamin D (VitD)	711 (VitD treatment vs baseline in fatal asthma) 867 (VitD treatment vs baseline in non-asthma)	CCL2 CCL13 CXCL12 IL8	Proliferation Inflammation	(140)

1.5   Targeting ASM for treatment of asthma

The evidence that ASM plays a major role in asthma pathogenesis has led to the suggestion that ASM could be the prime target for treatment, rather than targeting inflammation or treating symptoms, especially in patients with uncontrolled asthma. Supporting this, are results from studies using bronchial thermoplasty (BT) which showed very promising results in the treatment of severe asthmatics (141), (142). Through delivering radiofrequency energy, which heats up the airway wall, this technique obliterates ASM tissues from the wall and therefore reduces ASM mass. This technique was shown to positively impact asthma control and the quality of life of patients with uncontrolled asthma. The outcomes of these studies were summarised in **Table 1.8.** This experimental therapy requires further investigation, especially with regards to its long term effects on the outcomes on lung function, quality of life and symptoms control (41).

Other strategies to specifically target ASM could employ local delivery using a bronchoscope or administration via an aerosol. Although this type of targeting would avoid systemic involvement and reduce unwanted side effects, it is technically challenging since it is too invasive for the patients and also very costly. Current research

is focusing on developing ultrafine drugs that can reach the small airways. An overview of the potential targets within the ASM that could lead to the development of better strategies for treatment is summarized in **Figure 1.3**.

Table 1.8: Clinical studies using BT in severe asthma (Adapted from (143))

Effects	Adverse effects	References
↓ASM	No serious adverse effects	(144)
Improved PC_{20} ↑Symptom-free days Stable morning and evening peak flow	No serious adverse effects	(145)
↓Mild exacerbations ↑ACQ/AQLQ ↑Symptom-free days	Asthma worsening requiring hospitalization (7.6% BT vs 4.08% control group)	(141)
↑Pre-BT FEV_1 ↑ACQ/AQLQ ↓Requirement for OCS post-BT	↑Hospitalisation (23% in BT group versus 0% in control group)	(142)
↑AQLQ ↓Severe exacerbations ↓Hospitalisations	↑Hospitalisation (8.4% in the BT group vs 2% in the control group)	(146)
No clinical complications, stable FEV1 and FVC over 5 years	No long-term adverse events	(147)
↓Hospitalisations ↓ER visits FEV1 was maintained post-BT	No long-term adverse events	(148)
↓Severe exacerbation ↓ER visits ↓Hospitalisations	No long-term adverse events	(38)

*ACQ = Asthma Control Questionnaire; AQLQ = Asthma Quality of Life Questionaire; OCS = oral corticosteroids, FVC = Forced Vital Capacity; ER = Emergency Room
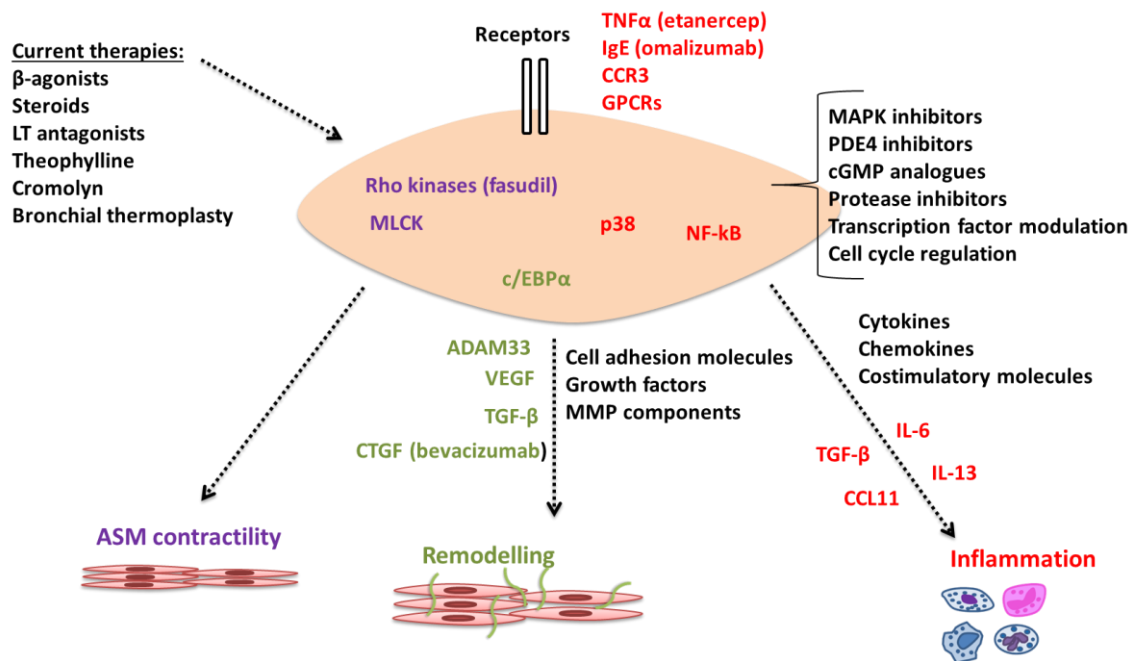
Figure 1.3: An overview of the potential targets in ASM for asthma treatment. ASM contractility could be reversed by targeting the myosin light chain (MLCK) and Rho kinases. A few proteins such as ADAM33, c/EBPα and growth factors (i.e. VEGF, CTGF) could be targeted for reversing airway remodelling. Finally, inflammation could be targeted by inhibiting the synthetic function of ASM using selective inhibitors of proinflammatory mediators (e.g. IgE), cytokines (e.g. IL-6), and chemokines (e.g. CCL11) as well as inhibitors for the MAPK kinases (i.e. p38), transcription factors (i.e. NF-κB), and for surface receptors (i.e. CCR3).

1.6   Asthma treatments

Despite an enormous increase in our understanding of the immune mechanisms involved in allergic diseases, very little progress has been made in terms of novel treatments. The mainstream treatment for asthma is still based on the use of inhaled corticosteroids (ICSs), short- and long-acting β2-adrenoceptor agonists (SABAs and LABAs) and oral corticosteroids (OCSs). The Global Initiative for Asthma (GINA) guidelines have designed steps (**1-5**) in the treatment of asthma, depending on the severity of the disease (3) (**Figure 1.4**).
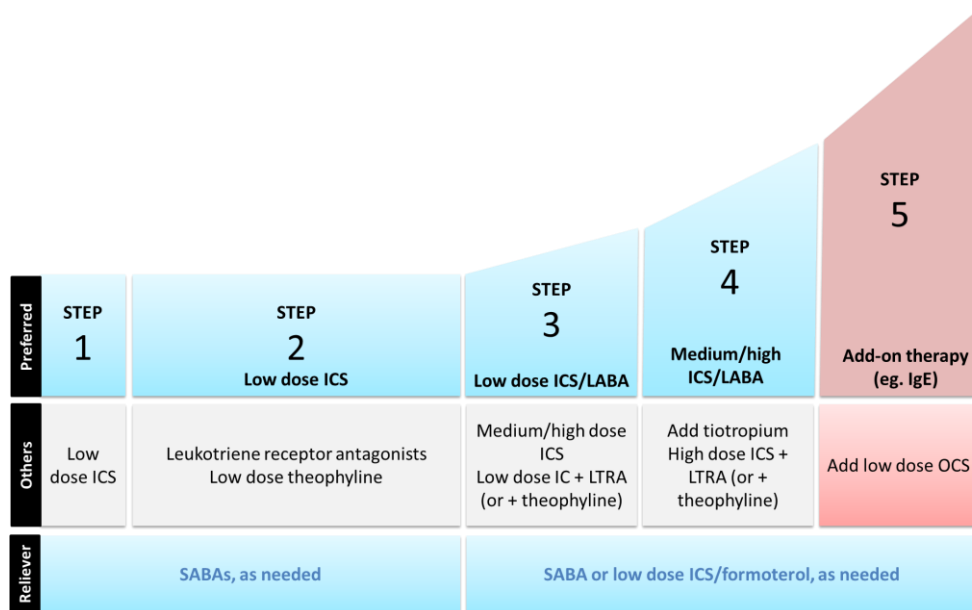
Figure 1.4: Stepwise GINA guidelines for asthma treatment (Adapted from (3)

When asthma symptoms are infrequent, short-lived, and mild (**steps 1-2**), administration of SABAs (salbutamol, terbutaline) is ideal as it reverses bronchospasm. One attractive supplementary therapy to ICSs has been the use of leukotriene-receptor antagonists (LTRA) (e.g. zileuton, montelukast, zafirlukast, pranlukast) (149). Of these, only zileuton has passed clinical trials and was shown to inhibit production of Cys-LTs by antagonizing the action of 5‑lipoxygenase (150). Also, in patients with moderate asthma, theophylline improved asthma control when added to ICS (151). Long-acting muscarinic antagonists have attracted attention as they were shown to reduce exacerbation frequency and improve lung function in COPD, however their potential use in asthma has not been much documented yet (152). As symptoms become more frequent or more severe, the emphasis changes to prevention of exacerbations, with patients requiring higher doses of ICSs (**Table 1.9**).

Table 1.9: High ICS thresholds recommended by GINA guidelines (μg) (Adapted from (3))

ICS	Age (6-12 years)	Age (>12 years)
Beclomethasone dipropionate (as chlorofluorocarbon or as dry powder inhaler)	≥800	≥2000
Budesonide	≥800	≥1600
Ciclesonide	≥160	≥320
Fluticasone propionate	≥500	≥1000
Mometasone furoate	≥500	≥800
Triamcinolone acetonide	≥1200	≥2000

When symptoms persist despite medication compliance and good inhalation technique (steps **4-5**), the use of LABAs (eg. formoterol, salmeterol) was shown to increase the efficacy of ICSs (153). More recently, tiotropium was shown to have a similar effect to salmeterol, and was beneficial in addition to ICS (154), (155). For patients with refractory asthma (step **5**), add-on therapies are being investigated especially for "Th2 high" patients such as the use of monoclonal antibodies and recombinant proteins to directly target various inflammatory mediators (**Table 1.10**). A few studies have looked at using soluble TNF-receptor fusion proteins such as etanercept, golimumab, infliximab and adalimumab (**Table 1.11**) however the results have been contradictory, unable to show a clear benefit without raising serious concerns regarding toxicity. A more successful approach has been the use of omalizumab which is now recommended as an add-on therapy in patients with severe uncontrolled asthma by the GINA guidelines (3). This monoclonal antibody directed against IgE (FcεR1) (high affinity receptor found on mast cells and basophils) was shown to be beneficial in inhibiting allergic reactions in the airways by reducing circulating IgE levels by 95% (156). The INNOVATE (Investigation of Omalizumab in severe asthma treatment) study specifically evaluated the efficacy and safety of this therapy. It revealed that

treatment with omalizumab significantly reduced the rate of severe asthma exacerbations and the rate of total emergency visits for asthma, with improvements in the AQLQ scores and better symptoms management (157). More recently, another IgE antibody was developed and tested in Phase I clinical trials (MEDI4212) and shown to have increased potency over omalizumab, however with modest duration of free IgE suppression. MEDI4212 was well tolerated by subjects and adverse events were of low severity (158).

Table 1.10: Clinical trials using monoclonal antibodies/recombinant proteins or kinase/cytokine inhibitors to target inflammatory mediators in asthma

Target	Antibodies/ Recombinant Proteins	Asthma severity	Effect	Reference
PDE-4	Roflumilast	Mild-moderate	Improved lung function but when used at high dose	(159)
IL-5	Mepolizumab	Severe	↓Sputum and blood eosinophils ↓Exacerbations ↑AQLQ scores No effect on FEV1, symptoms or AHR	(160), (161), (162)
	Benralizumab	Severe	↓Blood eosinophils ↓Sera eosinophil-derived neurotoxin (EDN) and eosinophil cationic protein (ECP) No effect on TNFα or IFN-γ ↑Serum IL-5, eotaxin, and eotaxin-2	(163)
	Resilizumab	Poorly controlled (↑ICSs)	↓Sputum eosinophils ↑ACQ score ↓Sputum eosinophils Improved FEV1	(164)
	SCH55700	Severe	↓Sputum eosinophils	(165)
IL-4, IL-13	Pitrakinra, dupilumab	Moderate-severe	↓Allergen-induced late asthmatic responses and the need for rescue medication ↓Side effects	(166), (167)
	AMG317	Moderate-severe	No clinical benefits	(168)

IL-13	Lebrikizumab,	Moderate-severe	Improved FEV1 No effect on exacerbations and asthma symptoms	(170)
IL-13 IL-2Ra	Tralokinumab	Moderate-severe	No effect on symptoms Improved FEV1	(171)
	GSK679586	Moderate-severe	No clinical efficacy No effect on asthma control, pulmonary function, or exacerbations	(172)
	Daclizumab	Moderate-severe	Improved FEV1 ↑Asthma control	(175)
CXCR2	SCH527123	Severe	↓Sputum neutrophilia ↓ Mild exacerbations No effect on asthma control	(176)
IL-17	Brodalumab	Moderate-severe	No clinical efficacy	(177)
CRTh2	BI671800	Moderate-severe	Small improvement in FEV1 in symptomatic naive asthma patients and in patients on ICSs	(178)
SCF PDGF	Masitinib (kinase inhibitor)	Severe GC-dependent	↑Asthma control ↓OCS doses No effect on lung function	(169)
IL-4, IL-5	Suplatast tosilate (Th2 cytokines inhibitor)	Mild atopic	Long-term treatment improved symptoms and inflammatory indices when used in combination with fluticasone	(173), (174)

Table 1.11: Anti-TNF therapy in asthma

Drug	Effect	Asthma severity	Reference
Etanercept	No clinical efficacy	Moderate-severe	(179)
Infliximab	Well tolerated ↓Number of patients with exacerbations in symptomatic moderate asthma	Moderate	(180)
Infliximab	↑Asthma control ↓Frequency of exacerbations and hospitalizations Some severe adverse effects (e.g. bacterial pneumonia, melanoma)	Severe GC refractory	(181)
Etanercept	Small improvement in asthma control and systemic inflammation	Severe GC refractory	(182)
Etanercept	Improvement in AQLQ, FEV1, AHR	Severe	(183)
Etanercept	Improvement in AQLQ, FEV1, AHR ↑Sputum histamine	Severe	(184)
Golimumab	Ineffective, with serious side-effects (infections in the treated group)	Severe	(185)

Also, as mentioned previously, Th17 cytokines have been identified as potential targets in the treatment of difficult to manage asthma, as shown by McKinley *et al* (13). IL-17 was increased in chronic sinusitis and its expression was steroid resistant (186). If so, targeting IL-17 cytokines may be of value in the therapy of severe asthma, in which steroid resistance, neutrophilic inflammation, and airway remodeling are substantial. Very interestingly, other preliminary studies identified the predominance of Th2/Th17 cells in bronchoalveolar lavage (BAL) of severe asthmatics. These cells had increased phosphorylation of p38 MAPK, MEK, ERK and IRF4, a transcription factor associated with chromatin reorganization and differentiation of Th2 and Th17 cells (187). Currently, there is a large body of experimental work ongoing, trying to identify small molecules that are important in the transcription of inflammatory genes, such as p38 MAPK and NF-κB (188), (189). Drugs that act on these pathways are in the early stages of development and not many have advanced to clinical trials as will be discussed later in this thesis.

1.7   Potential role of MAPK pathways

The MAPK pathway comprises of three members: c-Jun N-terminal kinases (JNK), p38MAPK (190), and extracellular signal regulated kinase (ERK) (191), (192). These are serine/threonine protein kinases that when activated play a role in the regulation of inflammatory gene expression at both transcriptional and post-transcriptional level. Their activation requires dual phosphorylation on threonine and tyrosine by upstream kinases and occurs in response to diverse stimuli, such as environmental stress (heat shock, UV irradiation), endotoxins, mitogenic stimuli, proinflammatory cytokines (IL-1β and TNFα), growth factors (epidermal growth factor and PDGF), or contractile agonists (histamine and thrombin) (193), (194). Once activated, they phosphorylate selected intracellular proteins including transcription

factors (88), RNA binding proteins (195), (196), (197), (198), (199), cytoskeletal proteins and other phosphoproteins (200). Since the MAPK can have effects on various cell types, this superfamily plays a crucial role in a wide variety of cellular functions ranging from proliferation, migration and synthesis of fibrotic and inflammatory proteins, pathways involved in asthma pathogenesis (201), (202) (**Figure 1.5**). **Chapter 4 of this thesis will investigate a potential role of these pathways in driving corticosteroid insensitivity in ASM cells**.
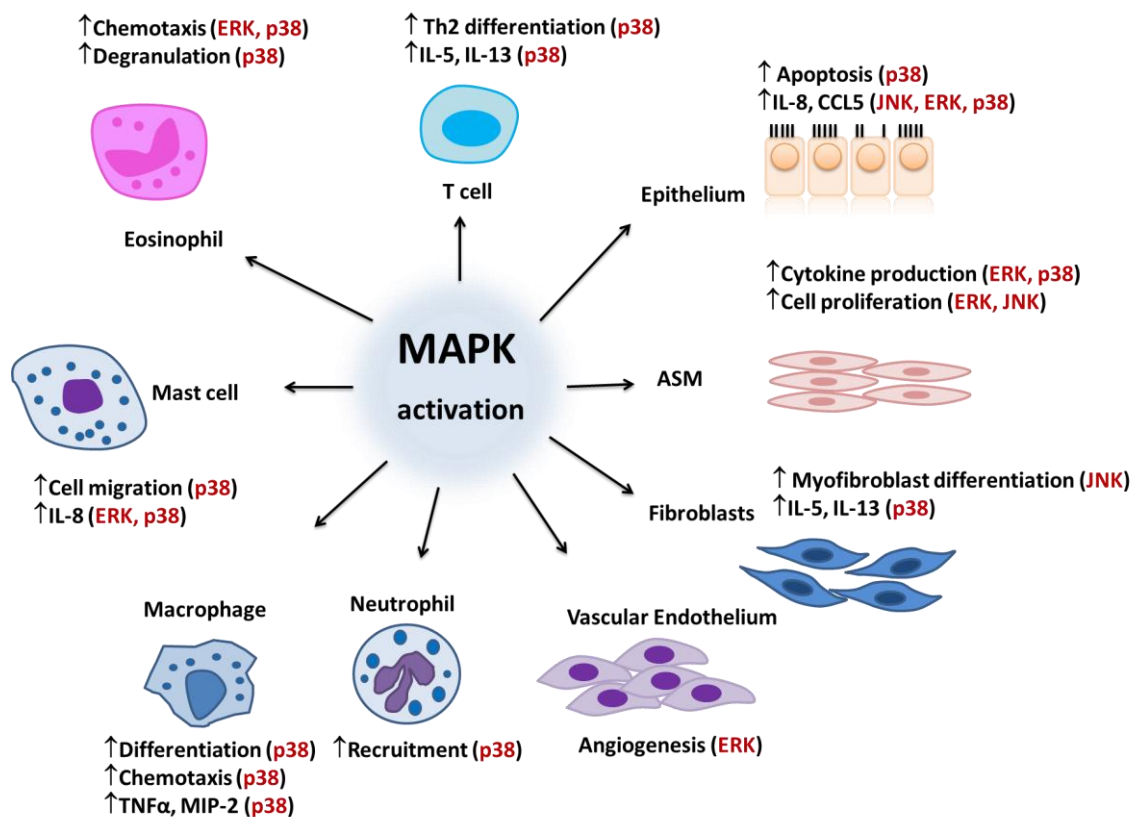
Figure 1.5: Effect of MAPK activation on structural and immune cells (Adapted from (201))

1.8   Severe asthma is an unmet clinical challenge

As previously mentioned, although most asthma patients can achieve good symptom control and minimal exacerbations with regular treatment, some patients will not achieve this even with optimal therapy (3, 203). These patients are at a high risk for adverse outcomes, mainly medication side effects, emergency visits, hospitalization, near-fatal events or even disability from persistent symptoms (1). Ultimately, recurrent asthma attacks and chronic symptoms of the disease in these patients lead to an increased risk of asthma death (204). 70-80% of asthma deaths occur in patients with poorly controlled severe disease (205).

1.8.1   Defining severe asthma

For the definition of severe asthma, criteria have been established by the collaboration of many research networks. The National Asthma Education and Prevention Program (NAEPP) and GINA were developed by the National Health Lung and Blood Institute to address the need for more structured recommendations for diagnosis and treatment. Their guidelines take into consideration various factors such as **(1)** use of SABAs, **(2)** frequency of exacerbations affecting the patient's daily activities, **(3)** nocturnal symptoms, and **(4)** the baseline pulmonary function measurements before treatment. In addition to this, in 2000 the ATS (American Thoracic Society) workshop led to a consensus for defining refractory asthma. This definition established two major criteria and seven minor criteria. The major criteria considered the use of high doses of ICSs and/or the additional requirement of OCSs. The minor aspects considered **(1)** the use of additional medications to control exacerbations, **(2)** presence of daily symptoms requiring an inhaler, **(3)** poor lung function, **(4)** emergency visits, **(5)** recurrent exacerbations requiring OCSs, **(6)** clinical deterioration without steroid treatment, and **(7)** history of near-fatal events. The Severe Asthma Research Program (SARP), used

these criteria to identify patients with persistent symptoms, asthma exacerbations or airway obstruction despite high medication use (206). The group TENOR (The Epidemiology and Natural History of Asthma: Outcomes and Treatment Regimens) (207) included patients with high use of the healthcare system or high medication in the past year. The latest GINA guidelines used four criteria for defining patients in the 'uncontrolled' group: **(1)** poor symptom control (i.e. ACQ >1.5 or Asthma Control Test (ACT) <20), **(2)** frequent severe exacerbations (>2 bursts of systemic GCs), **(3)** serious exacerbations (i.e. hospitalization) and **(4)** airflow limitation (i.e. FEV1<80% in the absence of SABAs and LABAs). All these studies attempting to define severe asthma emphasize the failure in some cases to manage asthma symptoms with current available treatments.

Finally, our current understanding of the asthma heterogeneity highlighted that the different asthma phenotypes, with their natural history and varying treatment responses may alter responsiveness to treatment and contribute to asthma refractoriness (208), (209). To aid relevant treatment decisions, phenotypic and pathophysiological markers are being considered for characterising patient populations. For example, treatment algorithms have been created using markers such as AHR (210), sputum eosinophils (211), (212) or exhaled nitric oxide (213), (214). Studies using airway biopsies and bronchoalveolar lavage (BAL) from the airways of severe asthmatics identified persistent airway tissue eosinophilia present in a large proportion of these patients, despite treatment with high-dose ICSs and OCSs (215). In addition, this 'high eosinophilic asthma' subtype of severe asthma was characterised by more active symptoms, reduced FEV1, recurrent exacerbations and more near-fatal events (216). Furthermore, this subgroup of severe asthmatics with high eosinophilia, responded well to anti-IL-5 therapy (160), which had reduced efficacy in the cohort of moderately

severe asthmatics (217) (**Section 1.6, Table 1.10**). It was also interesting to observe that in some cases where eosinophils were absent, this correlated with increased neutrophils (216), (218). The mechanisms and clinical implications of eosinophilic versus neutrophilic balance are unclear, pointing out to the need for better targeted and more personalised therapies for these different severe asthma subtypes. Finally, in 2009 the U-BIOPRED (Unbiased Biomarkers for the Prediction of Respiratory Disease Outcomes) project was set up. By using systems biology and combining state of the art transcriptomic, proteomic, lipidomic and metabolomic technologies, this study will help to identify multi-dimensional phenotypes of severe asthma and potential new targets for treatment. It is clear that there is a link between the concepts of asthma severity, control, and responsiveness and they all need to be considered when attempting to define severe asthma, to ultimately provide optimal treatment control.

1.8.2 Severe asthma represents a clinical and economic burden

As mentioned in the previous section, asthma control can often be achieved by increasing the dose of ICSs. At low-to-moderate doses, corticosteroids administered by inhalation are safe for long-term use. They get systemically absorbed from the gastrointestinal tract and undergo metabolic inactivation in the liver before reaching the systemic circulation (219). In addition, only small amounts of the administered doses are systemically absorbed across the respiratory tract mucosa as less than 20% of the delivered dose is deposited onto the airways (220). However, at high doses, the potential for long-term adverse effects becomes a concern (**Table 1.12**). Additionally, GCs negatively regulate ACTH, which is the key regulatory hormone in the hypothalamic pituitary adrenal (HPA) axis feedback loop. The HPA axis is a major component of the neuroendocrine system controlling many body functions (221). Due to their suppression of the HPA axis, GCs can affect immune responses, mood, and

emotions (221). The latest study by Sweeney and colleagues confirmed that morbidity rates for severe asthma were significantly higher for conditions associated with systemic steroid exposure such as type II diabetes, osteoporosis, cataracts, osteopenia and obstructive sleep apnoea, when compared to patients with mild/moderate disease (222). Furthermore, a systematic assessment of patients with difficult to treat asthma revealed a high prevalence of psychiatric illness in these patients (223). Psychosocial factors such as social isolation, alcoholism, anxiety and depression were also linked to poor asthma control (224). It is thought that these factors could further impact on patient compliance to treatment. Finally, as previously mentioned, severe asthma also places a large economic burden on the healthcare system and society, accounting for over 50% of total asthma care costs.

Table 1.12: Side effects associated with oral corticosteroid use

Side-effect	Reference
Skin bruising	(225)
Elevated intraocular pressure	(226)
Accelerated loss of bone mass increase	(227)
Growth retardation in children	(228)
Pharyngeal and laryngeal side effects (i.e. sore throat, coughing on inhalation of the medication, dysphonia, candidiasis)	(229)
Fracture and cataracts	(230), (231)
Weight gain	(232)
Diabetes/ Hyperglycemia	(233)
Osteoporosis	(234)
Cushing's syndrome	(235)

1.9   The anti-inflammatory actions of oral glucocorticoids

Glucocorticoids (GCs) have been used since the 1950s to treat various inflammatory diseases. Their efficacy can be attributed to their broad spectrum of action, resulting from the pleiotropic effects of the glucocorticoid receptor (GRα) on multiple signaling pathways. However, their clinical efficacy may be accompanied by acute and chronic adverse effects as described in the previous section.

1.9.1   Classical transcriptional regulation by GCs

GCs require binding to the glucocorticoid receptor (GRα), a member of the family of nuclear hormone receptors (236) in order to exert their anti-inflammatory actions either by increasing the transcription of anti-inflammatory genes (*transactivation*) or by inhibiting transcription of pro-inflammatory genes (*transrepression*). The number of genes per cell directly regulated by GCs is estimated to be between 10 and 100, but many are indirectly regulated through an interaction with other transcription factors and co-activators (237) and via post-transcriptional regulation (238).

GCs enter the cell via passive diffusion through the plasma membrane and bind to the cytosolic GRα. The inactive GRα resides primarily in the cytoplasm, where it is sequestered in a multimeric chaperone complex which prevents its degradation and subsequent activation and consists of heat shock proteins (hsp) (i.e. hsp70, hsp90, hsp90 binding protein p23) and immunophilins (FKBP51, Cyp44, PP5) (239). However, the inactive GRα is not rigidly compartmentalized and it was shown to shuttle continuously between the nucleus and the cytoplasm through the nuclear pore channels (240). Although constitutively expressed in virtually all cell types, GRα has a differential expression depending on the tissue, leading to tissue-specific outcomes in different diseases (241, 242). There are two isoforms of GR, GRα and GRβ, which have different transcriptional activities and display distinct cytoplasm-nucleus trafficking patterns: GRα is the isoform required for the anti-inflammatory effects of steroids, while GRβ plays a dominant negative effect on GRα through the formation of inhibitory heterodimers (243). Upon binding of the GC to GRα, the receptor undergoes conformational changes, it is released from the chaperone proteins and it translocates to the nucleus with the help of nuclear import proteins (eg. importin α).

In *transactivation,* the GRα homodimerizes and binds to DNA recognition sites known as glucocorticoid response elements (GREs) in the promoter region of steroid-responsive genes. These homodimers directly or indirectly bind to the transcriptional activator CBP (cAMP response element binding protein), which has HAT (Histone Acetyl Transferase) activity, causing the acetylation of core histones at lysine residues. The acetylation induces DNA relaxation, thus allowing activation of transcription of anti-inflammatory genes (237) (**Figure 1.6**). A list of these genes is provided in **Table 1.13**.

Table 1.13: Examples of anti-inflammatory genes induced by GCs

Anti-inflammatory gene	Effect	Reference
Lipocortin I	↓Arachidonic acid	(244)
p11/calpactin binding protein	↓Arachidonic acid	(245)
IL-1 type II receptor	↓IL-1-mediated inflammatory responses	(246)
Glucocorticoid-induced leucine zipper (GILZ)	↓Pro-inflammatory genes (e.g. NF-κB)	Reviewed in (247)
Mitogen-activated protein kinase phosphatase 1 (MKP-1)	↓Members of the MAPK family	(191), (248)
Annexin-1	Ca^{2+} signalling and membrane dynamics	(249)
Secretory leucocyte protease inhibitor (SLPI)	↓Proinflammatory genes	(250)
Inhibitor of NFκB α (I-κBα)	↓NF-κB	Reviewed in (247)
ß$_2$-adrenergic receptors	Sensitising target cells to additional anti-inflammatory stimuli	(238)
Tristetraprolin (TTP)	mRNA destabilisation	(251)
Downstream of tyrosine kinase (Dok-1)	↓signalling pathways critical to mast cell activation	(252)
Src-like adaptor protein (SLAP)	↓signalling pathways critical to mast cell activation	(253)
IL-10	↓Proinflammatory genes	Reviewed in (247)

Due to the activation of various enzymes involved in glucose and lipid metabolism (e.g. phosphoenol pyruvate carboxykinase, tyrosine aminotransferase, and glucose-6-phospate) it is believed that uncontrolled up-regulation of these genes could lead to the diabetogenic effects of GCs, resulting in the side-effects associated with their use (as discussed previously in **Section 1.8.2** (**Table 1.12)** (239)).

In *transrepression*, GRα acts as a monomer and binds to negative GREs (nGRE) to actively suppress transcription of inflammatory genes (e.g. IL-1β, IL-2). The monomers can bind directly to other transcriptional factors (e.g. NF-κB or AP-1) by altering their interaction with the transcriptional machinery, or indirectly via reversing the histone acetylation at sites of cytokine gene expression to suppress the activity of inflammatory genes. For example the degradation of IκBα by inflammatory stimuli (i.e. cytokines, interleukins) leads to the release of activated NF-κB as a dimer of p50 and p65 proteins. p50 increases DNA binding and p65 confers transcriptional regulation. The dimer translocates to the nucleus where it binds to the CBP (CREB Binding Protein), which by acetylation activates the expression of inflammatory genes. Suppression of the activated inflammatory genes occurs via the GC-GRα complex through the recruitment of HDAC2 (histone deacetylase 2), antagonizing the pro-inflammatory actions of AP-1 (activator protein-1) and of NF-kB (254). Finally, transrepression can also occur via competition for nuclear coactivators between the GC-GRα complex and other transcription factors (**Figure 1.6**). For example, our group showed that up-regulation of IRF-1 could compete with GRα for the transcriptional coactivator called glucocorticoid receptor interacting protein 1 (GRIP-1). GRIP-1 is part of the steroid receptor coactivators (SRC)/P160 family and through its interaction with the ligand-bound GRα, helps to recruit histone acetyltransferases (CBP/p300) and coactivator-associated arginine methyltransferase 1 (CARM1) that unpack the

condensed chromatin, allowing access of transcription factors to target genes. Therefore, the sequestration on GRIP-1 by IRF-1 leads to decreased GRα transcriptional activity, thus inhibiting GC anti-inflammatory activity (255).

1.9.2 Non-classical transcriptional regulation by GCs

Non-classical regulation of GC action can occur via interaction with negative-GREs, which recruit SMRT/NCoR co-repressors, leading to repression of transcription (256). Recently it was suggested that GCs may also function by non-genomic mechanisms, either via non-classical GRα signalling, or completely independent from GRα binding. One study showed that in human bronchial epithelial cells dexamethasone maintained its inhibitory effects in the presence of the GRα antagonist RU486 (257). Also, unlike the classical pathways, the non-genomic pathways do not involve GRα nuclear translocation. Non-genomic effects occur either at the cell membrane via the classic GRα (mGRα) or in the cytoplasm via non-classic GRα (cGRα) or protein/lipid components (258), (259) (**Figure 1.6**). For example, GCs were shown to induce the rapid phosphorylation, plasma membrane translocation and release of extracellular annexin-1 (260) which was important in resolving inflammation by its action on the formyl peptide receptors FPR1 and FPR2 (261). The mGRα has been shown to regulate three signalling pathways: p42 MAPK activation (262), ERK inhibition via MKP-1 (263), (264) and activation of proteins with SH3 domains (eg. Src, Ras) (265), (266) leading to apoptosis and cell lysis (267). Non-genomic effects can also involve regulation of Ca^{2+} signalling and changes in actin polymerisation (Reviewed in (268)).

Finally, post-translational events and mRNA destabilization are also central processes that contribute to the anti-inflammatory effects of GCs (269), (268). At translational level, GCs can affect the expression of translational initiation factors, as well as ribosomal gene expression levels (270). The effects of GCs on mRNA stability

are through the modulation of the levels of adenylate-uridylate-rich element (ARE)-binding proteins such as tristetrapolin (TTP), associated with mRNA decay, and Hu antigen R (HuR) (271). These proteins bind to the AREs in the 3' UTR of genes, regulating, promoting or disrupting RNA stabilization (272). One example is the inhibition of COX-2 and CCL11 by dexamethasone which reduces their stability via inhibition of p38 MAPK activity (273). Genomic and non-genomic pathways have been summarized in **Figure 1.6.**
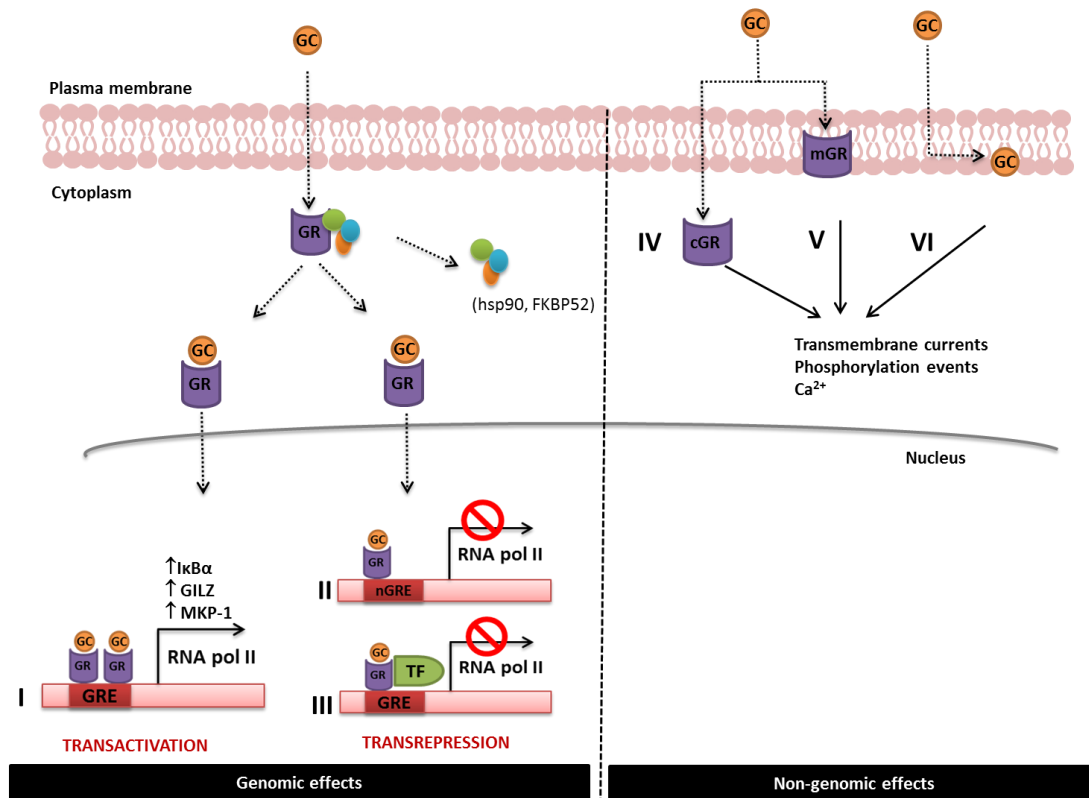
Figure 1.6: Genomic and non-genomic immunoregulation by glucocorticoids. GCs passively diffuse through the plasma membrane and bind to the GRα. Upon conformational change of the GRα, the GC-GR complex translocates to the nucleus where it can act via two major mechanisms, transactivation and transrepression. In transactivation, the GC-GRα homodimers can bind to GREs of anti-inflammatory genes (i.e. IκB, annexin-1, MKP-1) to enhance their transcription (**I**); In transrepression, the GC-GRα monomers can bind to negative GREs (nGRE) to actively suppress transcription of inflammatory genes (i.e. IL-1β, IL-2) (**II**) or they can directly bind and inhibit pro-inflammatory transcription factors (i.e. AP-1, NFAT, NF-κB and STATs) (**III**). In addition to the genomic actions, GCs induce rapid non-genomic effects via cytosolic GRα (**IV**), membrane-bound GRα (**V**), or via non-specific interactions with the cell membrane (**VI**) leading to changes in transmembrane currents, signal transduction and intracellular Ca^{2+} levels (Adapted from (274).

1.10   Separating transactivation from transrepression

Dissociated GC agonists have been designed with the notion that by favouring the ***transrepression*** (beneficial action) over the ***transactivation*** (linked to side effects) properties of GC, these compounds would have fewer side effects while retaining their anti-inflammatory effects (275). The construction of a GRα dimerization-deficient mutant mouse ($GR^{dim/dim}$) (with one point mutation at A458T) provided the first evidence for the separation between the two pathways of GC action (276). In contrast to this view, recent convincing evidence showed that the transactivation activities of GCs may also contribute to their anti-inflammatory effects (277), (278). For example the activation of certain genes (MKP-1, annexin 1) may have important roles in some of the anti-inflammatory properties of GCs (279). Studies in these GRα knockout mice showed that some genes were still activated, only not as strong as the wild type counterpart (280). Furthermore, GRα dimerization and subsequent GRE-dependent transcription proved to be indispensable for the suppression of certain inflammatory disorders such as TNF-induced inflammation, antigen- and G6PI-induced arthritis (281), and contact hypersensitivity (282). There is however very little evidence for the use of these compounds in the treatment of asthma. Several agents have been synthesized, but few have entered clinical development: BI-54903, GW870086X and AZD5423. The development of BI-54903 was discontinued due to recent evidence that the model of selective transrepression over transactivation is an oversimplification of GRα activity and the acknowledgement that it is difficult to uncouple the therapeutic and harmful effects mediated by GRα. Only one trial performed in mild to moderate asthmatics used a novel dissociated compound (GW870086), but this disappointingly showed no effect on lung function or rescue medication use (283). One possible explanation provided was the difficulty of maintaining full agonism in patients, despite the pre-clinical data

supporting its strong efficacy. They also acknowledged the small size of their study (only 36 patients), the possible inherent variability in patient's response to ICSs and the limitation of not assessing other efficacy endpoints for asthma such as symptom scores, exacerbations and quality of life (283). These contradictions create great controversy around the beneficial effects of dissociated steroids and urge for more research to be done to elucidate their mechanism of action and potential value for treating inflammatory conditions.

1.11   Glucocorticoid resistance is a defining feature of severe asthma

Glucocorticoid resistance in asthma patients was defined as the persistence of airflow obstruction associated with an increase of less than 15% in the forced expiratory volume in 1 s (FEV1) following 2 weeks of treatment with twice daily prednisolone (20mg) (284).

The requirement for high doses of medication and the need for combination therapies suggest that patients have developed a degree of resistance or insensitivity to treatment with ICSs or OCSs (285), (286). This insensitivity is not an absolute phenomenon but varies from patient to patient. Some patients in this category may also be labelled as corticosteroid dependent because asthma control may deteriorate when the maintenance dose of ICSs or OCSs is reduced. Furthermore, it is now recognized that many other factors may contribute to decreased corticosteroid responsiveness such as comorbidities, poor adherence, persistent environmental exposures, or psychosocial factors (**Table 1.14**). For a patient to fall into the 'steroid refractive' category, all reasonable efforts must have been made to eliminate all other non-asthma diagnoses.

Table 1.14: Possible factors contributing to GC insensitivity

Factors	Reference
Obesity, tobacco smoke	(287)
Comorbidities (eg. rhinosinusitis)	(288)
Poor adherence (poor inhaler technique)	(289)
Symptoms perceptions of individual patients	(290)
Allergen sensitization, viral infections, occupational agents, air pollutants	(288)

1.11.1 Molecular mechanisms of GC resistance

A variety of mechanisms have been described to drive GC resistance (**Figure 1.7, Table 1.15**). Most studies were performed in immune cells such as alveolar macrophages and PBMCs by comparing between healthy or non-severe and severe patients (238). Cells from bronchoalveolar lavage (BAL) fluid from GC resistant patients after 1 week of treatment with prednisolone displayed a reduction in the suppression of cytokines and chemokines (e.g. IL-4, IL-5 mRNA) on top of the reduced changes in the clinical symptoms of these patients (291). Since cytokines are known to recruit, activate, and promote the survival of inflammatory cells in the respiratory tract, and to mediate distinct effects on structural cells, their underlying profile of expression may alter the responsiveness to GCs in these patients. One of the mechanisms thought to lead to GC insensitivity involves defects in GRα activation and phosphorylation. This was shown in cells from severe patients and occurred via (**1**) reduced receptor expression, (**2**) altered affinity of the GC ligand for GRα, (**3**) reduced GRα binding to DNA, (**4**) decreased GRα nuclear translocation and (**5**) increased expression of inflammatory transcription factors (eg. AP-1) (285). Impaired GRα translocation was observed in PBMCs taken from patients with GC resistant asthma (292). In addition, there are studies to show that GRβ up-regulation contributes to GC resistance (293). GRβ was proposed to prevent GRα from binding to GRE, either by acting as a dominant negative inhibitor (294) or by forming heterodimers with GRα (295). In addition, a

GRβ polymorphism (GR-9β) was found to be associated with reduced transcriptional response to GCs, but there is no evidence yet of its involvement in GC resistance in asthma (238).
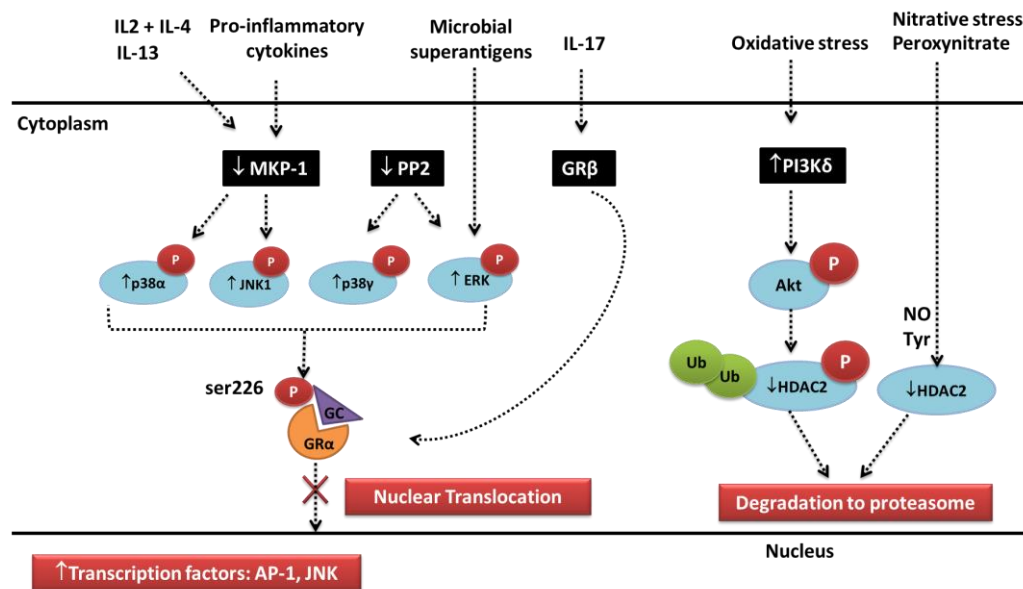
Figure 1.7: Proposed molecular mechanisms of GC insensitivity in asthma. IL-2 and IL-4 or IL-13 are overexpressed in the airways of patients with CSR asthma and via p38 activation reduce GRα nuclear translocation. Cytokines such as TNFα can activate members of the MAPK family and these kinases can affect GRα phosphorylation. For example JNK was shown to directly phosphorylate GRα at ser226, inhibiting its binding to GRE which is necessary for transcriptional activity. Microbial superantigens (i.e. staphylococcal enterotoxin B) can contribute to GC resistance through activation of ERK pathways, also via GRα phosphorylation. The serine/threonine phosphatase protein phosphatase 2A (PP2A) impairs GRα nuclear translocation via ERK and p38γ. Various pro-inflammatory cytokines (i.e. IL-17) can induce GRβ up-regulation which competes for the binding of GRα to GREs, thus acting as a dominant negative inhibitor. The excessive activation of transcription factors such as AP-1 was also shown to reduce GRα transcriptional activity since these factors physically interact with the receptor and block its binding to GRE or to other transcription factors. The PI3Kδ pathway is activated by oxidative stress and leads to the phosphorylation of downstream kinases (i.e. Akt), and ultimately to the inactivation of HDAC2. Oxidative and nitrative stress also inhibit the activity of HDAC2. This is possible via nitration at its tyrosine residues (Tyr) in response to peroxynitrite production. The modifications of HDAC2 result in its ubiquitination (Ub), which target the enzyme for degradation by the proteasome and lead to its reduced expression. Ultimately, all these different mechanisms contribute to GC resistance.

Table 1.15: Additional mechanisms of GC resistance in structural and inflammatory cells (Adapted from (296))

Cell type	Mediator	Intermediary mechanism	CSR mechanism
Structural cells	TGFβ	Activation of ALK5 (TGFβ receptor), via non-canonical pathways	↓GRα transactivation
	Denatured collagen	Activation of ECM-integrin signalling	↓GRα transactivation ↓cyclin D1
Immune cells	P-glycoprotein	↓MDR1 expression	↓GC concentration
	IL-2	p38 MAPK-induced STAT5 activation	↓GRα translocation
	IFNγ or IL-27/IFNγ	MyD88 activation by TLR	↓GRα translocation ↓GRα transactivation
	NFκB	Binding to GRα	↓GC-GRα binding ↓GRα transactivation
	AP-1	Binding to GRα	↓GC-GRα binding ↓GRα transactivation

As mentioned briefly in the description from **Figure 1.7**, one proposed mechanism involves defective histone acetylation, particularly through the inactivation of HDAC2. Low expression levels of HDAC2 have been shown in PBMCs and alveolar macrophages from patients with refractory asthma (297) (292). Cysteine oxidation was also shown to mediate GRα nuclear translocation via post-translational modification of GRα (298). Gene array studies in PBMCs revealed that 11 genes were differentially regulated between the GC resistant asthmatics and controls (299). Among these, the bone morphogenetic protein receptor type II was found to enhance steroid responsiveness when transfected into cells (300). Also oxidative stress was found to modulate pro-asthmatic responses in ASM. One recent study showed the presence of a defective innate immune defence against pathogens in severe asthmatics (301). In this study, low levels of BAL Surfactant protein D (SP-D) and altered bacterial presence with airway neutrophilia were found in patients with severe asthma.

More recently, studies using peripheral blood mononuclear cells (PBMCs) and lung macrophages from patients with severe asthma showed that, in these patients, GCs had a reduced inhibitory effect on the production of cytokines induced by LPS and this relative insensitivity to treatment correlated with increased activation of p38 MAPK (286, 302). An important regulator of p38MAPK is MKP-1, a well-known GC inducible gene that acts as a crucial anti-inflammatory mechanism to limit its activation (303). Due to its anti-inflammatory properties, MKP-1 has been shown to play a role in GC resistance. Studies using animal models showed that macrophages derived from MKP-1 knockout mice (MKP-1$^{-/-}$) displayed partial glucocorticoid-insensitivity (248). In these mice dexamethasone was unable to inhibit IL-1α mRNA expression, compared to wild type mice (MKP-1$^{+/+}$). It was interesting to observe that the effect of GCs varied between different genes. For example, many pro-inflammatory genes such as COX-2, TNFα and IL-1β showed intermediate responses, with dexamethasone having an impaired but not completely ablated inhibitory effect in MKP-1$^{-/-}$ mice (304). Also, in a simple mouse model of acute inflammation, GCs were unable to inhibit inflammation in the MKP-1$^{-/-}$ mice, as compared to the wild type (304). An abnormality of MKP-1 was also linked to the GC insensitivity found in obese asthmatic patients (305). **Chapter 4 in this thesis will address the potential involvement of the MAPK pathway in driving GC insensitivity in ASM cells.**

Current research is investigating the emerging role of vitamin D as a novel therapeutic option in the treatment of GC insensitivity in severe asthma patients. Results from various clinical trials reported the presence of low levels of vitamin D in patients who required high doses of GCs (306). Moreover, low levels of vitamin D also correlated with impaired response to GCs (307). More recently, a study by Nanser et al. found that calcitriol therapy significantly improved clinical response to prednisolone in

GC resistant asthmatics (308). Very interestingly, vitamin D was able to restore the ability of GCs to increase the expression of the anti-inflammatory cytokine IL-10 in the PBMCs of these patients (309). In addition, vitamin D enhanced GRα transcriptional activity (310). This was confirmed in studies by other groups reporting that vitamin D restored the ability of GCs to induce GR nuclear translocation, which was impaired in the PBMCs of severe asthmatics. It would be interesting to investigate a potential use for vitamin D in reversing steroid insensitivity in ASM cells from severe asthmatics. It is important to note that some proposed mechanisms may be cell-type specific, whilst others may be more broadly relevant. Also, these mechanisms may differ between patients.

1.12 Impaired sensitivity of ASM to glucocorticoids in severe asthma

Targeting ASM using bronchial thermoplasty (BT) was shown to improve clinical outcomes in patients with uncontrolled severe asthma who were refractory to GC therapy as indicated in **Table 1.8**. This suggests that directly targeting the ASM and its immunomodulatory responses could be beneficial for the treatment of GC resistance, a notion that this thesis is aiming to support. Also, as mentioned earlier in this section, RNA-seq studies in ASM cells have allowed the identification of numerous potential targets that could be involved in GC resistance such as CRISPLD2 (133), HBEGF, TGFB3, SERPINE1, and RGS4 (136) (shown in **bold** in **Table 1.7**, **Section 1.4.3**).

Immunohistochemistry experiments confirmed that the ASM from severe patients expresses a variety of pro-asthmatic mediators despite treatment with high doses of ICSs or OCSs (shown in **bold** from **Table 1.3**), clearly pointing to their resistance to therapy (62)). For example, IL-33 expression was observed in patients with severe GC resistant asthma and this correlated with features of airway remodelling (311). Another inflammatory protein, PTX3 was significantly elevated in ASM bundles

of severe asthmatics (72). Although its role in asthma has not been clearly defined, it was shown to induce eotaxin production. Also ADAM33 was reported to be increased in ASM tissues in severe asthmatics (73), (74) and shown to modulate ASM contractile properties (312) (Reviewed in (62)).

Finally, the mechanisms driving GC resistance within the ASM are complex, with the degree of refractoriness varying depending to the gene type studied (313), (314). For example, dexamethasone inhibited TNFα-induced CXCL8 and CCL11 production, but had no effect on cytokine-induced CX3CL1 (313). This suggests that different anti-inflammatory pathways are activated by GC in ASM cells.

1.13   Our *in vitro* model of steroid insensitivity

Our unique model of cytokine-induced GC insensitivity in ASM cells has uncovered potential molecular players involved in blunting GC function. Changes in receptor phosphorylation are one of the main events leading to dysfunctional GC signalling. As previously mentioned, there is a reduction in the suppression of inflammatory chemokines by GC therapy in ASM cells from patients with severe asthma. Moreover, the expression of some of these inflammatory mediators is completely refractory to GC treatment (313), (314), (315). For example, the TNFα-induced production of CCL11 and CXCL8 was not affected by dexamethasone in cells from severe asthmatics, compared to cells from healthy subjects. The underlying mechanism responsible for this effect was an impaired GRα translocation in response to GC treatment in the severe asthmatics. The following section will discuss some of the known molecular mechanisms susceptible of blunting GC responsiveness in ASM cells, with a focus on the evidence reported from our cellular model of GC insensitivity in ASM cells stimulated with TNFα and IFNγ.

1.13.1 Role of TNFα

TNFα is a cytokine produced mainly by macrophages, but also by other immune cells (i.e. monocytes, dendritic cells, B cells, CD41 cells, neutrophils, mast cells and eosinophils) and structural cells (i.e. fibroblasts, epithelial cells) (316), (317). It is produced in response to Toll-like receptors, which detect common bacterial cell-surface products (e.g. LPS), driving the immediate host defence against invading microorganisms before activation of the adaptive immune system (318). The role of TNFα in asthma has been well documented. Initial studies showed that TNFα was increased in the asthmatic airways both at mRNA (319) and protein (320) levels. In addition to this, the administration of inhaled recombinant TNFα to normal subjects led to the development of AHR and airway neutrophilia (321), (322), suggesting an involvement of this cytokine in the pathogenesis of asthma. Later studies confirmed an effect of TNFα on a variety of immune and structural cells (**Figure 1.8**). High levels of TNFα were found in the airways during allergic airway inflammation (323) and in BAL fluid and bronchial biopsies from asthmatic patients (324), (320). Moreover, as presented in **Figure 1.8**, TNFα has a direct effect on ASM, promoting its hyper-contractile phenotype and leading to AHR. Studies using CD38 knock out mice showed that TNFα increased ASM contractile properties in tracheal rings (325). Other mechanisms thought to account for a role of TNFα in driving AHR involve: an altered expression or affinity of the TNF receptor to various stimuli (e.g. bradikinin), and altered signal transduction or changes in Ca^{2+} sensitivity mediated by effects on RhoA/MLCK expression (Reviewed in (317)). As shown previously in **Table 1.11**, the beneficial effects of antagonising TNFα on markers of asthma control support the view that this cytokine plays an important role in disease pathogenesis. TNFα also has several properties that are relevant to severe refractory asthma. Experiments using PBMCs

from patients with refractory asthma showed increased expression of membrane-bound TNFα, TNFα receptor 1, and TNFα–converting enzyme, as compared to patients with mild-to-moderate asthma and controls. Moreover, treatment with etanercept in these patients significantly reduced the expression of membrane-bound TNFα and improved the PC_{20}, asthma-related quality of life, FEV1, and symptom scores, when compared to placebo.
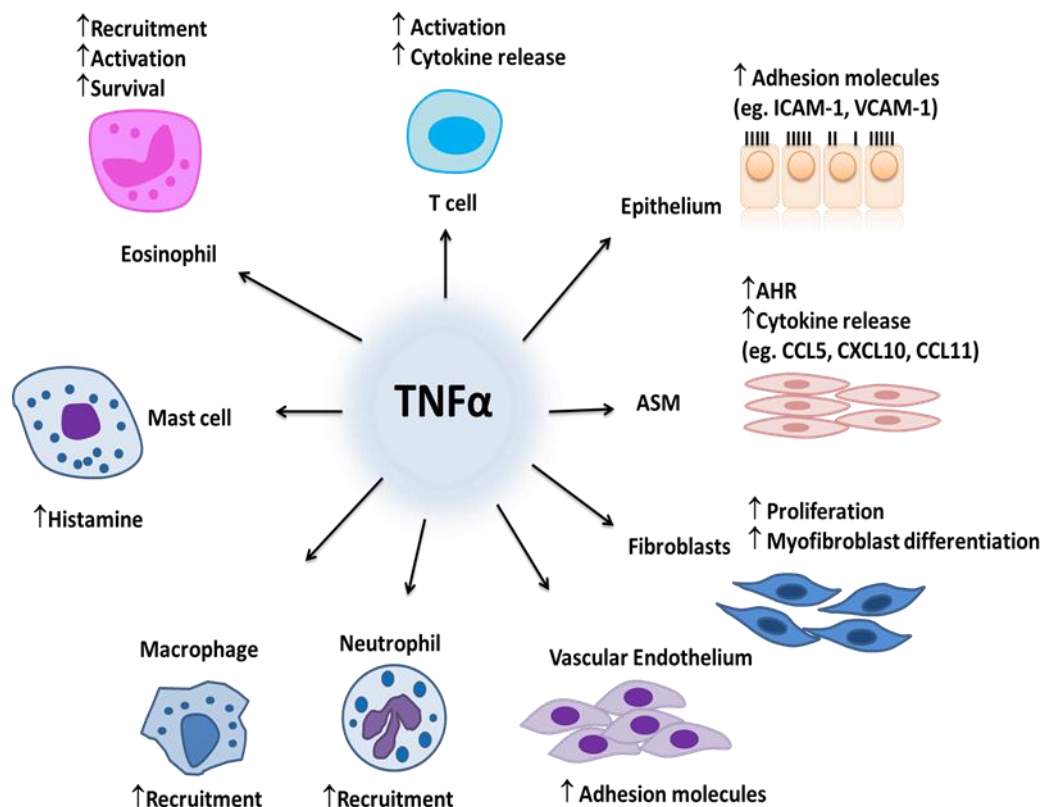
Figure 1.8: Role of TNFα in the pathogenesis of asthma. TNFα plays a central role in many of the features of the asthma paradigm by exerting important effects on both inflammatory and structural cells (Adapted from (317))

1.13.2 Role of IFNγ

IFNγ is a Th1 cytokine that is produced primarily by T-lymphocytes and natural killer cells in response to a variety of stimuli (e.g. IL-12, IL-18, IFNα, and IFNβ). In a very recent study, IFNγ was shown to be elevated in patients with severe asthma.

Counts from BAL fluid showed an increased percentage of IFNγ+CD4+ T cells and IL-17+CD4+ T cells in individuals with severe asthma compared to those with mild to moderate asthma (326). Studies performed in bronchial epithelial cells reported that high IFNγ levels in the airways promoted AHR through the suppression of Secretory leukocyte peptidase inhibitor 1 (SLP1) and the IFNγ-mediated immune response differentiated severe asthma from mild–moderate asthma in humans and mice (326). Earlier studies also support a role of IFNγ in driving features of asthma pathogenesis. For example, experiments using an ovalbumin challenged mice showed that treatment with antibodies to IFNγ selectively reduced AHR. In the same study they found that the median levels of serum IFNγ were significantly higher in the asthmatic group compared to the healthy control group and these elevated serum levels correlated with increased responses to methacholine (327). Its involvement in AHR was also shown to be via attraction of neutrophils to the lungs (328). Other studies found that production of IFNγ was significantly increased by T cells in the peripheral blood (329), (330). Reports in children with asthma showed that increased IFNγ levels correlated with disease severity (331). Finally, it has been recognised that IFNγ plays a role in increasing the cytotoxic function of lung macrophages and their secretion of an array of cytokines and chemokines such as CXCL10, CXCL11, and CXCL9 (328).

1.13.3 Role of TNFα and IFNγ combination in driving glucocorticoid resistance

A variety of triggers, including viral or bacterial proteins, may lead to increased expression of TNFα/IFNγ in severe asthma. This cytokine combination has a functional role in ASM cells. It stimulates ASM to produce various chemokines and cytokines which contribute to the inflammatory process. Secondly, via the activation of multiple transcription factors it leads to the up-regulation of numerous cell surface adhesion molecules found on ASM cells (**Figure 1.9**). More importantly, the synergistic

induction of some of these proteins is insensitive to different GC such as dexamethasone and fluticasone (**Table 1.16**). One possible mechanism was thought to be the failure of GCs to inhibit the transcription factor NF-κB, as shown with the fluticasone-resistant ectoenzyme CD38 (243). Another possible mechanism involves the GRβ, a dominant negative isoform of GRα which via the formation of GRα/GRβ heterodimers reduces the transactivation properties of GRα (238). Moreover, when cells were constitutively transfected with active GRβ, fluticasone lost its ability to suppress TNFα-induced CD38 expression and to induce GRE-dependent gene transcription (243). At shorter incubation times with TNFα/IFNγ (<6 hours), the GRα transactivation activity was still blunted (255) which occurred via the activation of IRF-1 and its competition for GRIP-1 (as was previously described). These studies demonstrate that IRF-1 may participate in the pathogenesis of asthma by affecting GC signalling, and ultimately by its ability to inhibit GRα function. **Chapter 5 of this thesis will explore the potential involvement of IRF-1 in driving GC insensitive genes in ASM cells.**
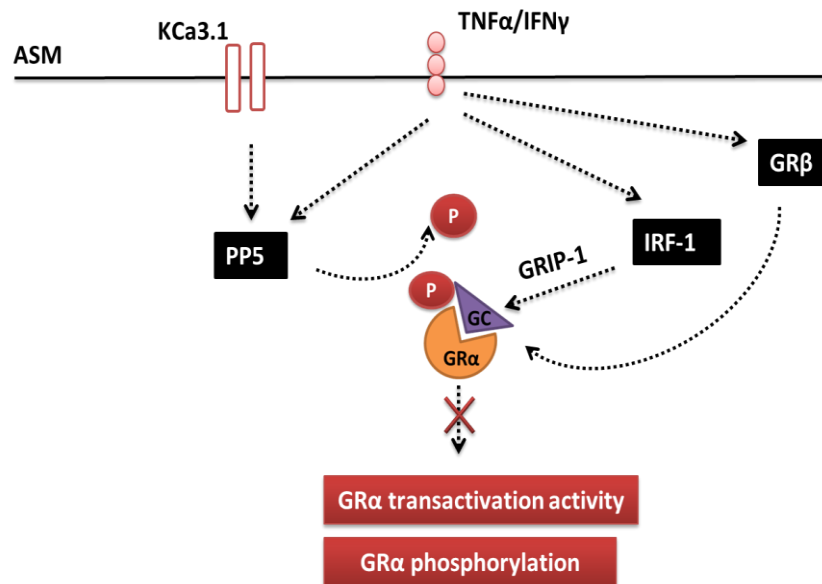
Figure 1.9: Mechanisms driving GC resistance induced by TNFα and IFNγ in ASM cells. As part of the rapid inhibitory mechanisms (>6 hours), the TNFα/IFNγ-induced IRF-1 competes with GRα for its transcriptional coactivator GRIP-1. Alternatively, the TNFα/IFNγ combination upregulates the $KCa_{3.1}$ channel which activates the serine protein phosphatase PP5. PP5 can in turn dephosphorylate GRα at ser211, blunting its transcriptional activity. The delayed inhibitory mechanisms (<12 hours following TNFα/IFNγ) involve the up-regulation of the GRβ isoform following stimulation with TNFα/IFNγ (Adapted from (62)).

Table 1.16: GC resistant proteins induced by TNFα/IFNγ in ASM cells and their role in asthma (Adapted from (62))

Mediators	Role in asthma	Reference
CXCL10	Chemotaxis	(97), (332), (333)
CCL5	Chemotaxis	(332), (333)
CX3CL1	Chemotaxis	(334), (332), (333)
IL-33	Chemotaxis Th2 allergic responses Airway remodelling	(70)
CCL15	Chemotaxis	(67)
IRF-1	Transcription of inflammatory genes Inhibition of GRα transactivation via GRIP-1 depletion	(255), (333)
CCL11	Chemotaxis	(332)
CD38	Airway inflammation AHR	(243)
GRβ	Inhibition of GRα transactivation via dominant negative action	(243)
PP5	Inhibition of GRα transactivation via dephosphorylation at ser211	(335), (332)

To conclude, our model represents a unique opportunity to test a range of drugs that could be used to either restore GC function or inhibit the expression of GC resistant genes. In this regard, this PhD thesis has provided novel observations about the effect of a natural product called Compound A.

1.14 Using Compound A to better understand the mechanisms underlying GC resistance.

Over the last decade, interesting observations have been made using Compound A (CpdA, 2-(4-acetoxyphenyl)-2-chloro-Nmethyl-ethylammonium chloride), a stable analog of the hydroxyphenyl aziridine precursor found in the Namibian shrub. The structure of CpdA is totally different from a normal steroidal ligand (**Figure 1.10**) however its biological effects are thought to be mediated by GRα. CpdA was reported to activate GRα-dependent repression of inflammatory mediators without triggering GRα related side effects (336).
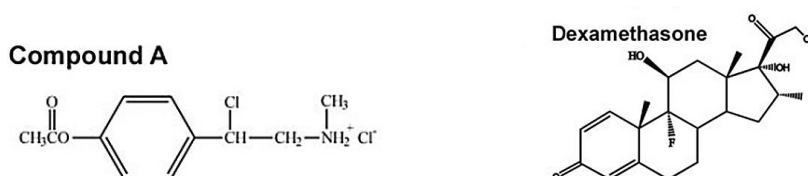
Figure 1.10: Chemical structures of Compound A and Dexamethasone

The first study by De Bosscher looked at the interaction of CpdA with the GRα using independent competitive whole-cell binding assays performed in the murine fibrosarcoma L929sA cells. Both CpdA and dexamethasone competed for GRα binding and CpdA displayed a higher affinity, with an IC50 approximately 4-fold lower than that of Dexamethasone (6.4nM compared to 25.9nM). The same study revealed that CpdA interacted with the ligand-binding domain of GRα. However, in comparison with dexamethasone, this compound induced a different conformational change and phosphorylation pattern of the GRα. Unlike dexamethasone, CpdA was unable to

phosphorylate the GRα at ser211, the site that is important for transactivation activities. This effect supported the lack of transactivation by CpdA on GRE-driven promoters (336). Earlier reports looking at the molecular properties of this compound revealed that it can also inhibit the enzyme responsible for the final step in the synthesis of GCs (337) by binding and displacing GCs from rat and sheep corticosteroid-binding globulin (CBG) (338). Moreover, CpdA did not elicit ligand induced receptor dimerization and this was thought to account for its dissociative behaviour (339). Finally, CpdA showed potent anti-inflammatory actions in various inflammatory conditions in mouse models (**Table 1.17**) including a Th2-driven asthma model. In addition to these studies, *in vitro* evidence revealed that it was also effective in inhibiting inflammatory responses in a variety of cells as shown in **Table 1.18**.

Table 1.17: *In vivo* anti-inflammatory properties of CpdA (adapted from (340))

Mouse model	Therapeutic effect	Molecular mechanisms	Reference
Zymosan-induced inflamed paw	Similar effects to dexamethasone	↓NF-κB activity ↓IL-8, IL-6, and E-selectin	(336)
Collagen-induced arthritis	↓Disease severity ↓Cytokine production ↑Anti-inflammatory action	↓TNFα-induced production of IL-1β, IL-6	(341-343)
Experimental autoimmune encephalomyelitis	↓Leukocyte infiltration ↓Neuronal damage ↓Clinical symptoms	↓NF-κB activity ↓IL-1β, TNFα, IL-23, IL-17	(344, 345)
Experimental autoimmune neuritis	↓Recruitment of macrophages and lymphocytes ↓Side effects ↑Treg response	↓IL-1β, IL-17, IL12p35, IFNγ, TNF-α, iNOS ↑Th2 and Foxp3 expression	(346)
Acute trinitobenzene sulfonic acid-induced colitis	↓Clinical symptoms ↓Inflammatory cells infiltration into colon wall	↓TNFα, IL-1β, and COX-2	(347)
Type 1 diabetes	Protection against diabetes ↑Treg response	↓ IL-1β, TNFα, IL-6 ↑IL-4 and IL-10	(348)

Th-2 driven asthma	↓Eosinophils, neutrophils, dendritic cells, B cells, T cells, macrophages, and mast cells in the lungs ↓AHR, lung inflammation, mucus production ↓Cytokine production ↓IgE production ↓Hyperglycemia ↓Hyperinsulinemia ↓HPA axis suppression	↓NF-κB and STAT6 nuclear translocation ↓IL-4, IL-5, IL-13	(349)
Duchene muscular dystrophy	↓Muscle inflammation ↑Limbs strength and function	↓NF-κB signalling ↓IL-6, CCL2, IFN-γ, TNF-α, IL-12p70	(350)
Contact dermatitis	↓Skin atrophy ↓Skin inflammation	↓REDD1	(351)

Table 1.18: *In vitro* anti-inflammatory properties of CpdA (adapted from (339))

Cell type	Molecular mechanisms	References
Synovial fibroblasts	↓NF-κB activity and IKK phosphorylation ↑IκB-α ↓IL-1β	(341, 352)
HepG2 hepatocyte cells infected with Dengue virus	↓CXCL10 and TNFα ↓Leukocyte migration	(353)
Murine T-lymphocytes	↓T-bet ↓GRα-dependent transrepression	(354)
Primary microglial cells	↓NF-κB activation ↓TNFα and IL-1β	(344)
Murine macrophage cell line RAW 264.7	↓TNFα, iNOS, and IL-1β ↑ IL-10 ↑Macrophage differentiation towards M2 anti-inflammatory phenotype	(346)
Bone marrow stem cells with osteoclasts	↓IL-1β, TNFα, IL-6 expression	(343)
Saos-2 osteosarcoma cells	↓IL-8 by TNFα	(355)

These studies prompted us to investigate whether CpdA had any effects on the production of steroid-resistant chemokines in our cellular model of GC insensitivity using human ASM cells treated with TNFα/IFNγ and to uncover the potential mechanisms involved.

1.15 HYPOTHESIS

There are still many questions regarding the exact molecular mechanisms driving steroid insensitive pathways in ASM. Here we tested whether the plant derivative CpdA can be used in the modulation of GC resistance in these cells. We hypothesised that CpdA-sensitive pathways could uncover novel potential targets for reversing GC resistance. Based on current literature and on our own preliminary data supporting their involvement in GC resistance, we focused on two potential avenues: modulation of the MAPK signalling and of the activation of the transcription factor IRF-1.

The specific aims of this PhD are:

- To examine the inhibitory profile of CpdA in our cellular model of corticosteroid insensitivity in ASM treated with both TNFα/IFN-γ

- To examine the exact mechanisms of CpdA action (GRα-dependent or -independent)

- To examine whether MAPK pathways modulate the expression of chemokine expression in ASM cells under steroid-resistant conditions

- To examine whether known transcription factors (i.e. IRF-1) play any role in TNFα/IFNγ-induced steroid resistance in ASM cells and their potential relevance *in vivo*

CHAPTER 2

Materials and Methods

A.   MATERIALS

The materials and reagents that have been used for each technique are listed below, with details of the catalogue numbers and company provided. Detailed methodology is described in the subsequent section.

2.1 Cell culture reagents

Table 2.1: Reagents used for cell culture of airway smooth muscle (ASM) cells

Reagent	Catalogue number	Company
DMEM + GlutaMAX-1[+4.5g/L glucose, pyruvate]	61965	GIBCO
Fetal Bovine Serum (FBS)	F9665/10500-064	Sigma/GIBCO
Non-essential amino acid (NEAA)	11140-035	GIBCO
Antibiotic/Antimycotic solution (AA)	15240-062	GIBCO
Sodium pyruvate (SP)	S8636	Sigma Aldrich
HANK's balanced salt solution (HBSS) 1x	9394	Sigma Aldrich
HANK's balanced salt solution (HBSS) 1x	14175-053	GIBCO
Trypan Blue	T8154-100ml	GIBCO
Dimethyl sulfoxide (DMSO)	D2650	Sigma Aldrich
0.25% Trypsin/Ethylendiaminetetracetic acid (EDTA)	25200-072	GIBCO
Stem Pro Accutase Dissociation Reagent	A11105-01	GIBCO
Insulin-Transferrin-Selenium-G Supplement	41400-045	Invitrogen
PBS Phosphate-Buffered Saline (1x) liquid	11540546	Fisher Scientific
Dulbecco's phosphate buffered saline (DPBS) (10x)	D1283	Sigma Aldrich

2.2   Reagents used for experimental design

ASM cells were serum deprived prior to experiments and stimulated with various chemicals, depending on the assay design. These reagents are shown in **Table 2.2** below.

Table 2.2: Reagents used for experimental design

Chemical	Catalogue number	Company
Glucocorticoid Receptor Modulator (Compound A)	346110-25mg	Calbiochem
Fluticasone propionate	F9428	Sigma Aldrich
Mifepristone (RU486)	M8046	Sigma Aldrich
Recombinant Human TNFα	210-TA-010 (10μl) 210-TA-020 (20μl)	R&D Systems
Recombinant human IFNγ	285-IF-100	R&D Systems
Actinomycin D	A9415	Sigma Aldrich
Dymethyl sulfoxide, anhydrous (DMSO)	27.685-5	Sigma Aldrich
Ethanol	200-578-6	Fisher Scientific
U0126 (ERK inhibitor)	9903	Cell Signalling
SP600125 (JNK inhibitor)	129-56-6	Santa Cruz Biotechnology
SB203580 (p38 inhibitor)	559389-1MG	Merck Chemicals Ltd
SR11302 (AP-1 inhibitor)	2476/10	R&D Systems

2.3 Reagents used for ELISA experiments

Table 2.3: Reagents used ELISA experiments

Reagent	Catalogue number	Company
10x Phosphate buffered saline (PBS)	P5493	Sigma-Aldrich
10x DPBS	14200-067	GIBCO
Albumin from bovine serum	A7030-100G	Sigma-Aldrich
ELISA Human CCL5/RANTES kit	DY278	Duoset (R & D)
ELISA Human CCL11/Eotaxin kit	DY320	Duoset (R & D)
ELISA Human CXCL10/IP-10 kit	DY266	Duoset (R & D)
ELISA Human CX3CL1/Fractalkine kit	DY365	Duoset (R & D)
Tween 20	P1379	Sigma-Aldrich
1x PBS	11540546	Fisher Scientific
3,3',5,5' Tetramethylbenzidine (TMB) Liquid Substrate System Super Sensitive Form for ELISA	T4444	Sigma Aldrich
Stop solution 2N H_2SO_4	7664-93-9	Sigma Aldrich

2.4 Reverse transcription and RT-PCR reagents

Table 2.4: Reagents used for RT-PCR

Reagent	Catalogue number	Company
100bp DNA ladder	G2101	Promega
CCL5 Forward Primer	H11781 4-3190-9/12	Eurofins MWG Operon
CCL5 Reverse primer	H11781 4-3190-10/12	Eurofins MWG Operon
CXCL10 Forward Primer	H424 20-2307-1/2	Eurofins MWG Operon
CXCL10 Reverse primer	H424 20-2307-2/2	Eurofins MWG Operon
CX3CL1 Forward Primer	105 39-4287-3/8	Eurofins MWG Operon
CX3CL1 Reverse primer	105 39-4287-4/8	Eurofins MWG Operon
β-actin Forward Primer	H925 20-3187-3/4	Eurofins MWG Operon
β-actin Reverse Primer	H018 20-3187-4/4	Eurofins MWG Operon
GAPDH Forward Primer	K1621	Fermentas - UK
GAPDH Reverse Primer	K1621	Fermentas - UK
Fast SYBR® Green Master Mix	4472908	Fisher Scientific
RevertAid First strand cDNA synthesis Kit	K1612	Fermentas - UK
PureLink® RNA Mini Kit	12183018A	Life technologies

2.5 Western Blot reagents

2.5.1 Protein extraction

Table 2.5.1: Reagents used for RIPA Protein Lysis extraction (Protocol 1)

Reagent	Product number	Company
RIPA buffer	492016 (100ml) D6750-100G L3771	NP-40:Calbiochem Sodium deocycholate: Sigma SDS: Sigma
Phenylmethanesulphonylfluoride (PMSF)	P7626	Sigma Aldrich
Sodium Fluoride (NaF)	S7920	Sigma Aldrich
Sodium orthovanadate (NA$_3$VO$_4$)	S6508	Sigma Aldrich
Aprotinin, from Bovine Lung (APOP)	A6279-10ml	Sigma Aldrich
Leupeptine (LP)	L8511	Sigma Aldrich
Ethylenediaminetetraacetic acid disodium (EDTA) salt solution 0.5M	E7889-100ml	Sigma Aldrich

Table 2.5.2: Reagents used for RIPA Protein Lysis extraction (Protocol 2)

Reagent	Product number	Company
RIPA Lysis buffer system	sc-24948A	Santa Cruz Biotechnology

2.5.2 Gel preparation

Table 2.5.3: Reagents used for gel preparation

Reagent	Product number	Company
Ultrapure protogel 30% (w/v) Acrylamide: 0.8% (w/v) Bis-Acrylamide Stock solution (37.5:1)	A2-0072 (1L)	Geneflow
4X Protogel Resolving Buffer (1.5M Tris-HCl, 0.4%SDS pH 8.8)	B9-0010 (1L)	Geneflow
Ammonium Persulfate (APS)	BP179-25 (25g)	Fisher Scientific
TEMED (N,N,N',N'-tetramethylethylenediamine)	1610801	BioRad
Protogel Stacking Buffer (0.5M Tris-HCl 0.4%SDS pH 6.8)	B9-0014 (200mls)	Geneflow
Isopropanol	W292907	Sigma Aldrich

2.5.3 Sample preparation

Table 2.5.4: Reagents used for sample preparation, electrophoresis and protein transfer

Reagent	Product number	Company
Laemmli buffer (5X)	N/A	N/A
Protein Ladder: Spectra Multicolor Broad Range Protein Ladder	26634	Thermo Scientific
Running Buffer Ultrapure 10X TRIS/Glycine/SDS (0.25M Tris-1.92M Glycine-1%SDS)	B9-0032 (4L)	Geneflow
Blotting Buffer Ultrapure 10X TRIS/Glycine (0.25M Tris-1.92M Glycine)	B9-0056 (4L)	Geneflow
Protran Nitrocellulose Membrane BA 83, 0.2μm roll Size 20cm x 3m	B3-0055	Geneflow
Spacer Plates	1653311	BioRad
Short Plates	1653308	BioRad
Washing Buffer IHC Select 20X Rinse Buffer	20845	Millipore

Pierce ECL	32106	Thermo Scientific
Pierce ECL 2	1188-4584	Fisher Scientific
Tween 20	P1379-100ML	Sigma Aldrich
Bovine Serum Albumin	A7906-100G	Sigma Aldrich
Milk Powder	N/A	Marvel
2-mercaptoethanol	M/P200/05	Fisher Scientific
Glycerol	#G/0650/08 (500ml)	Fisher Scientific
TRIS Base	#BP152-1 (1kg)	Fisher Scientific
HCl	#H1758	Sigma Aldrich

2.5.4 Antibodies for Western Blotting

Table 2.5.5: Antibodies used for Western Blotting

Antibody	Source	Dilution	Product number	Company
β-actin	mouse	1:1000	(C4): sc-47778	Santa Cruz Biotechnology
IRF-1	rabbit	1:1000	(C-20): sc-497	Santa Cruz Biotechnology
Phospho-p38 MAP Kinase (Thr180/Tyr182)	rabbit	1:1000	9910; 9211	Cell Signaling
p38 MAP Kinase Antibody	rabbit	1:1000	9926; 9212	Cell Signaling
Phosphor-SAPK/JNK (Thr183/Tyr185)	rabbit	1:500	4688	Cell Signaling
SAPK/JNK (56G8)	rabbit	1:500	9258	Cell Signaling
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	rabbit	1:1000	4695	Cell Signaling
p44/42 MAPK (Erk1/2)	rabbit	1:1000	9102	Cell Signaling
MKP-1 (C-19)	rabbit	1:500	sc-370	Santa Cruz
MKP-1 Peptide (C-19)	rabbit	1:5	sc-370	Santa Cruz
Anti-rabbit IgG, HRP-linked Antibody	goat	1:5000	7074	Cell Signaling
Donkey anti-rabbit IgG-HRP	rabbit	1:5000	sc-2313	Santa Cruz
Goat anti-mouse IgG-HRP	mouse	1:5000	sc-2005	Santa Cruz

2.6 Flow cytometry reagents – Annexin V

Table 2.6: Reagents used for Annexin V experiment

Reagent	Product number	Company
DPBS 1X	11540546	Fisher Scientific
NaCl	7647-14-5	Fisher Scientific
KCl	7447-40-7	Fisher Scientific
MgSO4.7H2O	231-229-8	Fisher Scientific
Hepes	H3375-100G	Sigma Aldrich
Annexin V FITC Apoptosis Detection Kit	APOAF-20TST	Sigma Aldrich
Annexin V FITC Conjugate	A9210	Sigma Aldrich
Propidium Iodide Solution	P2667	Sigma Aldrich
10x Binding Buffer	B9796	Sigma Aldrich
Staurosporine	APOAF-20TST	Sigma Aldrich

2.7 Immunofluorescence reagents

Table 2.7.1 Reagents used for Annexin V experiment (Protocol 1)

Reagent	Product number	Company
Phosphate Buffered Saline 1X	11540546	Fisher Scientific
Methanol	M/4056/17	Fisher Scientific
Bovine Serum Albumin	A7906	Sigma Aldrich
Tween20	10485733	Fisher Scientific
Lab Tek II 8 well chamber slides	13063043	Fisher Scientific
Coverslips 22 x 50mm	631-0137	VWR International
Vectashield mounting medium with DAPI	H-1200	Vector Laboratories

Table 2.7.2: Reagents used for Annexin V experiment (Protocol 2)

Reagent	Product number	Company
Methanol	M/4056/17	Fisher Scientific
Bovine Serum Albumin	A7906	Sigma
10% Neutral Buffered Formalin	PRC/R/132	Pioneer
Alexa-488-labelled secondary antibody	A-11001	Invitrogen
Lab Tek II 8 well chamber slides	13063043	Fisher Scientific
Coverslips 22 x 50mm	631-0137	VWR International
Vectashield mounting medium with DAPI	H-1200	Vector Laboratories

2.8   Cytotoxicity assays (MTT)

Table 2.8: Reagents used for MTT assay

Reagent	Product number	Company
Thiazolyl Blue Tetrazolium Bromide (MTT)	M2128	Sigma Aldrich
Nondet P-40(NP40)	74385	Sigma Aldrich
Isopropanol	W292907	Sigma Aldrich

2.9   Transfection experiments

Table 2.9: Reagents used for transfection with small interference RNA (siRNA)

Reagent/Kit	Product number	Company
Basic smooth muscle cells Nucleofector Kit	VPI-1004	Lonza
IRF-1 siRNA	AM16708 (106718)	Life Technologies
Silencer Negative Control No. 1 siRNA	AM4611	Life Technologies
Amaxa Nucleofector II		Amaxa Biosystems
TransIT-TKO Transfection Reagent	2154	Mirus
TransIT-siQUEST Transfection Reagent	2114	Mirus
ON-TARGETplus Human DUSP1 siRNA SMARTpool, 5nmol	L-003484-02	Dharmacon
MKP-1 scrambled	U-15	Santa Cruz

2.10   Immunohistochemistry

Table 2.10.1: Immunohistochemistry reagents

Reagent	Product number	Company
EnVision FLEX+, Mouse, High pH (Link)	K8002	Dako
Microscope slides Colourfrost PLUS	SD999102	Fisher Scientific
Coverslips 22x50mm	631-0137	VWR International
Gill's 2 haematoxylin	PRC/13/1	Pioneer research chemicals
DPX mountant	44581	Sigma
Xylene	X/0200/17	Fisher Scientific
Antibody diluent with background reducing components	S3022	Dako
Tween 20	2013-09	Dako
Immedge pen	H-4000	Vector Labs

Table 2.10.2: Antibodies used for immunohistochemistry experiments

Antibody	Source	Product number	Company
IRF-1 (H-205)	Rabbit	sc-13041	Santa Cruz
Rantes (CCL5)	Mouse	AHC1052	Invitrogen
α-actin	Rabbit	04-1094	Millipore
Nuclear Factor NF-1	Rabbit	LS-C164846	LSBio
Rabbit IgG	Rabbit	RBPLPU-01MG	Immunostep
Mouse IgG	Mouse	sc-2025	Santa Cruz

2.11 Other consumables

Table 2.11: List of other consumables

Item	Product number	Company
96 Well Plates for ELISA (Half Plates)	#675061	Greiner Bio-one
96 well polypropylene plates qPCR Plates (25 plates/box)	#410088	Stratagene Products/Agilent Technologies
Autoclave tape	#12986976	Fisher Scientific
Plate, multi-well, round, 6 wells	#734-0991	Nunclon

B)   METHODS

2.12   General Cell Culture

2.12.1 The *in vitro* model of corticosteroid resistance in cultured human airway smooth muscle (ASM) cells

All experiments were performed in human ASM cells obtained from endobronchial biopsy samples from normal and asthmatic subjects. ASM cells were exposed to a combination of two pro-asthmatic cytokines, TNFα (10ng/ml) and IFNγ (25ng/ml). These two cytokines were shown to work synergistically to model the GC-resistant state *in vitro*.

2.12.2 Human ASM cell culturing

ASM cells were grown from biopsy samples and maintained in DMEM+GlutaMAX-1 (+4.5g/L glucose, pyruvate) culture medium supplemented with 10% foetal bovine serum (FBS) (v/v), 1% L-glutamine (2mM) (v/v), 1% Antibiotic/Antimycotic solution (penicillin-streptomycin) (v/v), 1% sodium pyruvate (v/v) and 1% non-essential amino acids (v/v) and kept at 37°C in a humidified atmosphere containing 5% CO_2. Patient characteristics for the samples used are summarised in **Table 2.12.1**. For each experiment, confluent cells, as shown in **Figure 2.12.1**, were removed from culture flasks using trypsin. Following 3 minutes incubation at 37°C, the trypsin was neutralised with either HBSS or growth medium and the cell suspension was centrifuged at 1200RPM for 7 minutes at room temperature. The pellet was then re-suspended in 1ml of medium and the number of cells was counted using a haemocytometer. The appropriate number of cells was plated depending on the experiment (**Table 2.12.1**) and cells were incubated at 37°C until confluent. Prior to each experiment the growth media was replaced with Insulin Transferrin Selenium (ITS) media for 24 hours.
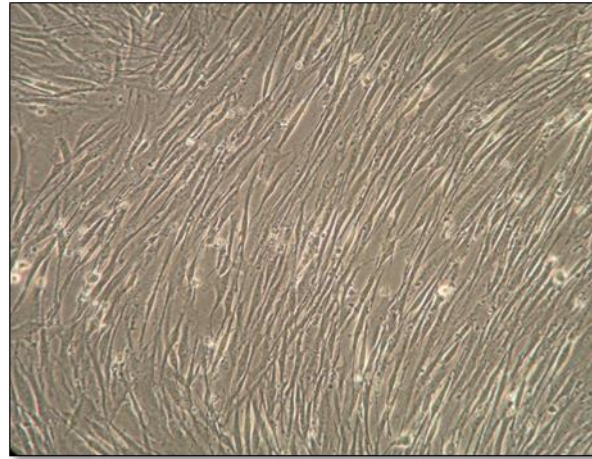
Figure 2.12.1: Confluent ASM cells under light microscopy

Table 2.12.1: Patient demographics for *in vitro* studies

	Healthy	**Asthma (steps 1-5)**	**p value**
Number	19	32	N/A
Age	44.47±4.08	51.25±1.92	0.0968
Gender (Male/Female/unknown)	9/10	16/14/2	N/A
FEV1 current	3.24±0.2	2.57±0.15	**0.0266**
FEV1 (% predicted)	85.59±7.04	82.33±4.21	0.397
FEV1 / FVC %	84.4±6.45	68.2±2.44	**0.0076**
PC_{20}	12.01±1.43	4.7±1.38	**0.0183**
Atopy (number)	4	23	N/A
ICS mg/day (BDP)	0	1009±116.4	**< 0.0001**
OCS (number)	0	6	N/A
Age of onset	N/A	27.05±3.43	N/A
Smoking	5	6	N/A

Data are expressed as means±SEM. Values in bold indicate significance when compared to healthy controls. The statistical tests used were unpaired t-tests for comparing between 2 groups. P<0.05; FEV1=Forced expiratory volume in 1 second; FVC=Forced vital capacity; PC_{20}= provocative concentration causing a 20% fall in FEV1; Atopy=positive response to skin prick test; ICS= inhaled corticosteroids; BDP=Budesonide; OCS=oral corticosteroids.

Table 2.12.2: Seeding densities for HASM cells

Container	Seeding density
96 well-plate	5.000-10.000
24 well-plate	20.000-40.000
6 well-plate	100.000-200.000
T75 flask	170.000
T25 flask	150.000
8 well-chamber slide	8.000-10.000

2.12.3 Counting human ASM cells

Cells were counted using a haemocytometer. 10μl of cell suspension were added to 10μl of Trypan Blue and mixed by pipetting up and down to ensure that the cells were not clumped together. 10μl of this mixture were added to the haemocytometer and cells were counted in the square area highlighted below:
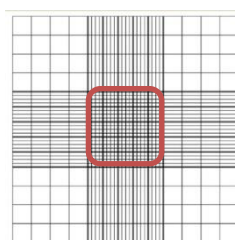
Following the count, the calculation below was used to determine the number of cells/ml: **number of cells x 2 (dilution factor) x 10.000 = number of cells/ml**

2.13 Basic analytical techniques

2.13.1 Supernatant collection

Cells were seeded on 24 well-plates and serum deprived for 24 hours prior to stimulation. Cells were treated with the various compounds followed by 24 hours stimulation with TNFα and IFNγ. DMSO (<0.1%) was used as vehicle. Following

stimulation, cell supernatants from confluent monolayers of cells (1ml) were transferred to clean tubes and stored at -80°C until subsequent assays and analyses.

2.13.2 Enzyme-linked immunosorbent assay (ELISA) for RANTES (CCL5), IP-10 (CXCL10) and Fractalkine (CX3CL1)

The concentrations of CX3CL1, CXCL10 and CCL5 in the supernatants were measured by commercially available Quantikine ELISA kits (R&D Systems). All ELISAs were performed according to the manufacturer's instructions. Briefly, capture antibodies were diluted in PBS to the recommended concentrations and added to 96 well half plates (50μl/well) and left overnight. Solutions were expelled forcefully and plates were washed three times with wash buffer (PBS containing 0.1% (v/v) Tween-20) prior to the addition of 100μl/well of blocking solution (PBS containing 1% BSA) for 1 hour to stop non-specific binding. Plates were washed again 3 times with wash buffer before the addition of the samples and standards (50μl/well) and incubation at room temperature for two hours. Samples for CCL5 and CXCL10 were diluted at 1:50 in blocking buffer, while samples for CX3CL1 were left undiluted. At the end of the incubation, plates were washed again 3 times with wash buffer and 50μl of diluted detection antibodies were added for another 2 hours at room temperature. Following another wash, 50μl of the recommended concentration of Streptavidin conjugated horseradish peroxidase was added to all wells and incubated for 20 minutes at room temperature. After 3 final washes with wash buffer, 50μl of TMB substrate solution (equal parts of A and B) was added to each well until significant signal had developed (5-30 minutes). The reaction was stopped by addition of 25μl 2N sulphuric acid (H_2SO_4) and the absorbance was determined using a Perkin Elmer plate reader at 450nm. The absorbance of the cytokine standards was used to determine the concentration of cytokine in the samples.

2.13.3 MTT Cytotoxicity Assay

MTT was used to assess the viability of cells. The mitochondria in living cells reduce yellow MTT to purple formazan and the absorbance can be quantified using a spectrophotometer. Cells were seeded at a density of 10^4/well on 96 well-plates and serum deprived for 24 hours prior to stimulation. 20µl of 5mg/ml MTT was added to each well and the cells were incubated for 3.5 hours at 37°C in culture hood. The media was removed and 150µl MTT solvent was added. After covering in tinfoil and agitating cells on orbital shaker for 15 minutes, the absorbance was read at 590 nm.

2.13.4 Flow cytometry for Annexin V-FITC and Propidium Iodide

The AnnexinV-FITC Apoptosis Detection kit was used to assess the cytotoxic effect of CpdA at different concentrations. Annexin V-FITC is a fluorescent probe which binds to phosphatidylserine expressed in the membrane of cells which are beginning the apoptotic process. Annexin V requires the presence of calcium. Propidium Iodide is a marker of late apoptosis as it binds to the cellular DNA in cells where the cells membrane has been totally compromised.

Human ASM cells were grown in T25 flasks until confluency and serum deprived 24 hours prior to stimulation. After stripping the cells using accutase, they were resuspended in 400µl PBS and distributed in 4 FACS tubes per condition as follows: Hepes without calcium with Annexin V (negative control for Annexin V), Annexin V Binding Buffer (ABB) with Annexin V (negative control for PE), ABB with Propidium Iodide (negative control for Annexin V) and ABB with both Annexin V and Propidium Iodide. After another spin, Hepes/Annexin V Binding buffer and 1µl Annexin V were added to the respective tubes and incubated for 15 minutes. 2µl Propidium Iodide were added just before running the samples on FACS machine. Results were recorded using a FACS machine and CellFlow.

2.13.5 Immunofluorescence

Human ASM cells were plated on sterile 8 well chamber slides. Cells were next washed in PBS (200µl/well) and fixed with methanol (200µl/well) for 20 minutes on ice. The methanol was removed and the slides were left to air dry at room temperature for 10 minutes. The cells were kept in blocking buffer (PBS-3%BSA) for 30 minutes at room temperature. The anti-GRα antibody was diluted at 1:200 in PBS-1%BSA and left to incubate overnight at room temperature. On the following day, three washing steps in PBS Tween (PBS-0.05% Tween 20) were performed and the secondary antibody diluted at 1:10 in PBS-1%BSA was applied for 90 minutes at room temperature (protected from light). Cells were rinsed 3 times in PBS Tween, 3 times in PBS followed by counterstaining with DAPI (4', 6-Diamidino-2-phenylindole at 1:1000 in PBS) for 40 seconds. After another 6 washes in PBS the slides were mounted with several drops of fluorescence media and analysed under the fluorescence microscope.

2.13.6 Isolation of total RNA and cDNA synthesis

Following stimulations, ASM were placed on ice to stop the reaction. After removing the media, cells were washed in PBS. For isolation of RNA, cells were purified using the PureLink RNA Mini Kit from Invitrogen according to the manufacturer's instructions. In short, after washing the cells with PBS, lysis buffer was added and the cells were scraped using a cell scraper and vortexed. The lysates were homogenised by pipetting up and down and then mixed and vortexed with one volume of 70% ethanol. The homogenates were spun down at 12000xg for 15 seconds at room temperature using the spin cartridges provided in the kit. For washing the membranes, 700µl of Wash Buffer I was added to the spin cartridge and spun at 12000xg for 15 seconds, followed by 2 washes with 500µl of Wash Buffer II. The membrane with bound RNA was dried by spinning at 12000xg for 2 minutes. 30-100µl of RNase-free

water were added to the centre of the spin cartridge and incubated for 1 minute at room temperature. RNA was isolated by spinning the cartridge at 12000xg for 2 minutes and the eluted RNA was collected into recovery tubes and either used for cDNA synthesis or stored at -80°C for further experiments. 200ng-2μg of total RNA were reverse transcribed using the RevertAid First Strand cDNA Synthesis Kit from Thermo Scientific. Briefly, nuclease free water, 1μl Random Hexamer and 2μg sample of RNA were mixed to a total volume of 12μl. To this, 4μl of 5x reaction buffer, 1μl of RiboLock RNase Inhibitor, 2μl of dNTP mix and 1μl of RevertAid M-MuLVRT were added to reach a final volume of 20μl. Microtubes were then incubated for 5 min at 25°C followed by 60 min at 42°C and the reaction was terminated by heating at 70°C for 5min. cDNA was used immediately for qPCR or stored at -80°C.

2.13.7 qPCR (Real-time PCR)

Real-time PCR was performed using the Fast SYBR Green Master Mix kit from Applied Biosystems using the manufacturer's instructions and the following thermal cycle program.

Thermal cycle program for CCL5, CX3CL1, CXCL10, MKP-1, GILZ and β-actin

	Incubation at 95°C for 10'		
Thermal cycle program	Denaturation	95°C for 15″	
	Annealing	60°C for 60″	40 cycles
	Extension	72°C for 30″	

Thermal cycle program for IRF-1 and β-actin

	Incubation at 95°C for 10'		
Thermal cycle program	Denaturation	95°C for 15″	
	Annealing	59°C for 60″	40 cycles
	Extension	72°C for 30″	

Primers for CCL5, CX3CL1, CXCL10, IRF-1 and MKP-1 were designed by Eurofins MWG Operon (**Table 2.13.1**). Human β-actin and GAPDH were used to normalize the results. Primers were reconstituted in molecular grade water. For the

qPCR, 10µl Fast SYBR Green Master Mix were mixed with 250-400nM of both sense and antisense primers, 2µl cDNA and water to a final volume of 20µl. Reactions were pipetted into a 96 well plate in duplicate, alongside with negative controls (Reverse Transcriptase Negative and No Template Control). Microseal sealer film was used to cover the plates and PCR was performed using a thermocycler to monitor sample fluorescence. Integration of the fluorescent SYBR Green into the PCR product was recorded after each annealing step. To confirm the amplification of one specific product a melt curve analysis was performed, where a single melting peak eliminated the possibility of primer-dimer association. All qPCR samples were run using Stratagene Mx3000P QPCR system. The cycle threshold (Ct), the point at which each PCR product is detectable above a fixed threshold, was determined for both the target gene and the housekeeping gene (β-actin or GAPDH). The relative mRNA expression of each gene was calculated with the comparative ΔCt method using the formula $2^{-\Delta\Delta Ct}$. The internal reference dye ROX was used.

Table 2.13.1: Primer sequences

Gene Name	Sequence	Product size (bp)	Gene ID NCBI
Human CCL5	F 5'-TCTGCGCTCCTGCATCTG-3' R 5'-GGGCAATGTAGGCAAAGCA-3'	69	NM_00298 5.2
Human CXCL10	F 5'- GGATGGACCACACAGAGGCTGC -3' R 5'- GCCCCTTGGGAGGATGGCAGT -3'	417	NM_00156 5.3
Human CX3CL1	F 5'-GCTGAGGAACCCATCCAT-3' R 5'-GAGGCTCTGGTAGGTGAACA-3'	165	NM_00299 6.3
Human GILZ	F 5′-CACTCCCCTTCTCACTCTGC-3′ R 5′-GAACTTTATAAGCAGTCATCCC-3′	294	NM_00408 9.3
Human MKP-1	F 5'-GACGCTCCTCTCTCAGTCCAA-3' R 5'- GGCGCTTTTCGAGGAAAAG-3'	82	NM_00441 7.3
Human β-actin	F 5'-CCCAAGGCCAACCGCGAGAAGAT-3' R 5'-GTCCCGGCCAGCCAGGTCCAG-3'	559	NM_00110 1.3
Human LAD-1	F 5'- AAG CGC CAC CTC TTT GAG AA -3' R 5'- TTC TGT CCC CAC TGA GTC CT-3'	203	NM_00555 8.3
Human IRF-1	F 5'- GCTGACCCCAGTCCGGTTGC-3' R 5'- GCCCCTCAGCCAAAGCAGGG -3'	624	NM_00219 8.2
Human GAPDH	F 5'- TGCACCACCAACTGCTTAGC-3' R 5'- GGCATGGACTGTGGTCATGAG -3'	87	NM_00204 6.5

2.13.8 Western Blotting

Western blot procedures allow for protein expression to be investigated based on the size of the protein that is being interrogated. For isolating total cell protein, ASM cells were washed with PBS and complete RIPA lysis was added before cells were scraped using a scraper. The complete RIPA lysis buffer contained 200μM PMSF, 10mM NAF, 2mM NA_3VO_4, 10μg/ml APOP, 10μg/ml LP and 2mM EDTE. To prepare this, in 5mls of RIPA lysis the following volumes were added: 10μl of NA_3VO_4, 250μl NAF, 50μl APOP, 5μl LP, 1μl EDTE and 5μl PMSF. Cells were kept on ice for 20 minutes, vortexed and spun at 12000 RPM for 20 minutes at 4°C. Following this supernatants containing total proteins were collected for further experiments. The protein concentration of each sample was detected by Bradford method using the Bio-Rad protein assay kit. Bovine Serum Albumin concentrations for the standards ranged from 0mg/ml to 3mg/ml. To 5μl of cell extracts or standards, 25μl reagent A' (made of 20μl of reagent S and 1ml of reagent A) and 200μl reagent B were added and left for 15 minutes at room temperature. The protein concentrations were calculated using the spectrophotometer where the absorbance was measured at 750 nm. The standard protein curve was plotted using Excel. The amount of total protein used was between 30-100μg per sample.

i)   *Preparation of polyacrylamide gels*

Gels were prepared at either 8% or 10% concentration depending on the molecular weights of the proteins to be separated, using the following recipes:

Resolving gel (De-gas)	8% (30-120 KDa)	10% (20-75 KDa)
Proto-gel	4 ml	5 ml
4 x Resolving buffer	3.75 ml	3.75 ml
dH20	7.08 ml	6.09 ml
AP (ammonium persulfate)	150 μl	150 μl
TEMED	15 μl	15 μl

Stacking gel	8% (30-120 KDa)	10% (20-75 KDa)
Proto-gel	1.3 ml	1.3 ml
Proto-stacking buffer	2.5 ml	2.5 ml
dH20	6.1 ml	6.1 ml
AP (ammonium persulfate)	50 µl	50 µl
TEMED	10 µl	10 µl

The resolving gel was added first and left to polymerise for approximately 20 minutes, followed by the staking gel in which the combs were inserted. Gels were used straight away or kept at 4°C for no longer than a week. The molecular weight of the proteins under investigation is shown in **Table 2.13.2**.

Table 2.13.2: Molecular weights of target proteins

Target Protein	Molecular Weight (kDa)
Phospho-p38	43
Total p38 MAP Kinase	43
MKP-1	40
IRF-1	48
β-actin	45
Phosphor-SAPK/JNK (Thr183/Tyr185)	46, 54
Total SAPK/JNK (56G8)	46, 54
Phosphor-p44/42 MAPK (Erk 1/2) (Thr 202/Tyr204)	44, 42
P44/42 MAPK (Erk1/2) (137F5)	42, 44

ii) *Sample Preparation for SDS-PAGE (Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis)*

30-100µg of whole cell protein from each sample was mixed with 4x or 5x Laemmli loading buffer to add up to a total volume of 30µl of mix/well for 1.0mm gels and 40µl of mix/well for the 1.5mm gels. Samples were vortexed, warmed at 100°C for 5mins, centrifuged and added to wells using a micro-syringe. For reference, 7µl of marker (Spectra Multicolour Broad Range Protein Ladder) was added to the first well of each gel.

iii) *SDS-PAGE electrophoresis and Western Blotting*

After the marker and samples were added, the cassettes were filled with running buffer (Running Buffer Ultrapure 10X with 0.25M Tris-1.92M Glycine-1%SDS), and ran at 160V for 1 hour in order for the proteins to separate according to their molecular weight.

iv) *Transfer of proteins to nitrocellulose membrane*

The separated proteins were electrophoretically transferred onto a nitrocellulose membrane. On the transfer device, cellulose and nitrocellulose membranes were soaked in transfer buffer (prepared as 25mls per membrane using 17.5mls distilled water, 2.5mls transfer buffer (Blotting Buffer Ultrapure 10X with 0.25M Tris-1.92M Glycine) and 5mls methanol) and set as follows: three cellulose membranes, one nitrocellulose membrane, the gel and three other cellulose membranes on top. The transfer was set to run for 20mins at 250A, 20 mins at 270A and 20 mins at 310A. Negatively charged proteins move up towards the positive cathode and onto the membrane. Immediately after the transfer, the nitrocellulose membranes were blocked in 5% Milk/TBS/Tween for an hour on the bench rocker, at low speed. After three 10 minutes washes in TBS/1% Tween, the membranes were incubated overnight with the respective diluted antibodies in either 5% Milk/TBS/Tween or 5% BSA/TBS/Tween. On the following day the membranes were washed three times for 10 minutes each in TBS/Tween and then incubated with the respective secondary antibodies of either mouse or rabbit origin diluted at 1:5000 in 5% dried Milk/TBS/Tween. After another three washes, the membranes were incubated for 5 minutes with the Pierce ECL cheluminescence reagent and visualised using a developer. The membranes were washed 3 times for 5 minutes each time and then stripped using the stripping buffer (25mls required per membrane and prepared as 5mls SDS 10%, 1.56mls TRIS pH 6.8, 26µl 2-mercaptoethanol and

18.5mls distilled water) for 15 minutes at $70^{o}C$ in the water bath. After another three 5 minutes washes, the membranes were incubated with primary antibodies at 1:5000 dilutions in 5% dried Milk/TBS/Tween. On the following day, membranes were washed again three times for 5 minutes and incubated with the respective secondary antibodies and then visualised by autoradiography. All X-ray films were scanned for further analysis using the ImageJ software.

2.14   Gene knockdown using small interfering RNA

ASM cells were transfected using the Basic Nucleofector Kit for Primary Smooth Muscle Cells, with the manufacturer's optimised protocols D-033 for IRF-1 and P-024 for MKP-1. Briefly, ASM cells ($2x10^6$ cells) were transiently transfected with either IRF-1 or MKP-1 siRNA at the specified concentrations. Briefly, the cells were harvested by trypsinisation and after counting, the cell pellet was resuspended in 100μl of room temperature Nucleofection Solution (82μl Nucleofector Solution plus 18μl of supplement, as provided by the supplier). The cell suspension was combined with the required concentration of siRNA and transferred to a cuvette. siRNA for IRF-1 and MKP-1 were used and a pool of scrambled sequences not complementary to the two genes was transfected as negative control. The cuvette was then placed in the Amaxa machine and the programme for each transfection was selected. Once the program was finished, the cuvettes were taken out, approximately 500μl of pre-warmed culture media was added and the samples were gently transferred into Eppendorf tubes using the supplied pipettes. Finally the samples were added to 6 well plates with pre-warmed culture media for 16 hours, before being growth-arrested for a further 24 hours. Cells were then stimulated according to the specific experiments. At the end of each experiment, RNA and protein were isolated.

siRNA	Concentration
IRF-1 Silencer Pre-Designed siRNA, Ambion (#AM16708)	300nM
IRF-1 siRNA Negative Control, Ambion (#AM4611)	300nM
MKP-1 siRNA: on-Target Plus SMART Pool, Dharmacon (#L-003484-02)	1µg
MKP-1 scrambled, Santa Cruz (U-15)	1µg

2.15 Actinomycin D mRNA chase experiments

To measure mRNA stability, growth-arrested ASM cells were stimulated with TNFα and IFNγ for 2 hours with or without 2 hours pre-incubation with CpdA. Cells were then washed and incubated with actinomycin D (5µg/ml) to inhibit further transcription. Total RNA was extracted after 0, 0.5, 1, 2, 4, and 6 hours of incubation with actinomycin D. mRNA expression was quantified by real-time RT-PCR as described in **Section 2.3.7.** Results are presented as % mRNA remaining (compared with steady-state levels of mRNA expression after TNFα and IFNγ cytokine treatment).

2.16 Immunohistochemistry

Bronchial biopsies from healthy subjects and subjects with severe asthma following corticosteroid treatment were obtained through bronchoscopy and were fixed in acetone and embedded in glycomethacrylate (GMA). Antibody titrations were performed to determine the optimal antibody dilution for the sections (1:50, 1:100, 1:200, 1:400 and 1:500). Sequential 3µm sections were cut from GMA embedded sections and were left to float on 0.2% ammonia water. Sections were collected onto positively charged slides and left to dry overnight at room temperature in a humid container. The staining was performed using EnVision™ FLEX Mini Kit (DAKO) as follows: the slides were first microwaved at 850W for 4 minutes, washed 3 times for 5

minutes each with EnVision™ FLEX Wash Buffer and then incubated with Peroxidase Block for 10 minutes at room temperature in a humid chamber before being washed again. The slides were next incubated with the right concentrations of diluted antibodies for IRF-1, CCL5, NF-1 and α-smooth muscle actin and the isotype controls used at the corresponding concentrations. All the antibody and isotype control dilutions were prepared in EnVision™ FLEX Antibody Diluent buffer. The slides were incubated for 1 hour at room temperature, washed 3 times for 5 minutes in EnVision™ FLEX Wash Buffer, incubated with EnVision™ FLEX/HRP for 30 minutes at RT, washed 3 x 5 minutes. The developing was performed using the EnVision™ FLEX DAB + Chromogen and substrate (1drop in 1ml of substrate) for 2 minutes. The slides were then washed with water and counterstained using Mayer`s Haematoxylin for 5 minutes, washed under running tap water and dried before finally being mounted using DPX mounting solution.

2.17   Statistical analysis

Results from all readings were initially analyzed using Excel. Data from all donors was transported to Prism 5 and Prism 6 and significant differences among groups were assessed, with values of $P < 0.05$ ($P < 0.05 = *$, $P < 0.01 = **$, $P < 0.0001 = ***$) sufficient to reject the null hypothesis for all analyses. Each set of experiments was performed with human ASM from at least three different donors in both normal and asthmatic subjects. Analysis was performed in Prism 6 using ANOVA, followed by post hoc tests (Bonferonni), and paired and unpaired student's t tests.

CHAPTER 3

Compound A modulates glucocorticoid-insensitive pathways in ASM cells

3.1   INTRODUCTION

As already mentioned in the **Introduction,** chronic treatment with GCs can lead to multiple severe side effects affecting various tissues and organs (i.e. diabetes, glaucoma, osteoporosis (356, 357) as summarised in **Section 1.8.2, Table 1.12**). These side effects have been linked to the transactivation property of GRα which is responsible for the induction of genes that encode gluconeogenetic enzymes. On the other hand, transrepression was thought to account for the anti-inflammatory properties of GCs. Dissociated GC agonists or Selective Nonsteroidal Glucocorticoid Receptor Agonists (SEGRAs) have been developed with the notion that favouring the transrepression (beneficial action) over the transactivation (linked to side effects) properties of GC would lead to fewer side effects, while retaining the anti-inflammatory effects (275). The use of SEGRAs in lung diseases has not been investigated and there is no information on whether these compounds have any advantage in steroid resistant states both *in vitro* and *in vivo.*

Over the last decade, interesting observations have been made using Compound A, a stable analogue of the hydroxyphenyl aziridine precursor found in the Namibian shrub, which was reported to selectively act via GRα-dependent transrepression, without triggering transactivation (336). Moreover, numerous studies highlighted the potential benefit of using this compound in inflammatory conditions. Unlike GCs, CpdA was unable to induce the expression of key liver gluconeogenic enzymes. Possibly because of this, CpdA was not associated with hyperglycemia, hyperinsulinemia and HPA axis inhibition following systemic treatment (342, 345). In mice with experimental inflammatory diabetes resembling type I diabetes, CpdA had a preventing and protective effect (348). In contrast to GCs, treatment with CpdA did not induce muscle waste in mice mimicking Duchenne Muscular Dystrophy (mdx mice)

(350), and had a significantly weaker effect on bone metabolism compared to GCs (343, 350). Also topically applied CpdA treatment did not induce skin atrophy in rats and mice (358). Finally, there was a lack of CpdA effect on levels of corticosterone and serum ACTH (344, 345). The most interesting observation was the strong anti-inflammatory profile of this compound. In most studies it showed similar or increased potency compared to regular GCs. Very important for our study is the evidence from the Th2 driven asthma model. In this mouse model, CpdA reduced inflammation and AHR. It also had a strong inhibitory effect on a plethora of Th1/Th17 cytokines (**Section 1.14**, **Table 1.17**), and was able to switch their differentiation towards a more anti-inflammatory profile, an important mechanism for resolving inflammation (346, 348). Moreover, CpdA was also able to induce the expression of anti-inflammatory cytokines such as IL-10 (346). Although the side effects associated with chronic *in vivo* treatment with CpdA have not been comprehensively analysed, most studies showed that CpdA was able to decrease both the severity of localized clinical symptoms and the systemic signs of inflammation. This indicates its potential safer therapeutic profile when compared to GCs.

There is very little known about the precise underlying mechanisms of CpdA anti-inflammatory action, with *in vitro* studies suggesting that its beneficial actions are highly dependent on multiple factors such as the cell type, the pro-inflammatory insult, the targeted inflammatory genes, and the inflammatory environment (339). Quite a few studies have looked at the interaction between CpdA and GRα and more importantly, at the effects of CpdA on GRα phosphorylation, dimerization, nuclear translocation and binding to DNA. CpdA was shown to bind the GRα with high affinity (336, 339, 359), however its effect on GRα nuclear translocation was very modest (336, 359, 360) and unlike GCs, CpdA was unable to elicit ligand induced GRα dimerization (336, 342, 359,

360). The first study published in 2005 from De Bosscher and colleagues showed that CpdA did not induce GRα phosphorylation at ser211, known to be critical for GR transactivational activity (336, 339, 359, 360). The ability of CpdA to supress the activity of a plethora of transcription factors (NF-κB, AP-1, Ets-1, Elk-1, SRF, NFATc, T-bet and STAT6) was also described. However, in contrast to GCs, CpdA was shown to decrease the nuclear import of these transcription factors and sequester them in the cytoplasm (as shown for NF-κB and AP-1 proteins (359, 361)). Using cells lacking GRα, CpdA inhibitory effects were shown to depend on the receptor (336, 360). Most of these studies confirmed the "dissociated" nature of this ligand and showed that it was unable to activate GRα inducible genes (i.e. MKP1, GILZ, and FKBP51 (Reviewed in (340)).

These studies prompted us to investigate whether CpdA had any effects on the production of steroid-resistant chemokines in our cellular model of GC insensitivity consisting of human ASM cells treated with TNFα/IFNγ (243, 255, 362, 363). The combination of TNFα/IFNγ is known to induce the expression of a variety of chemokines, transcription factors and ectoenzymes with pro-asthmatic functions. Moreover, some of them are totally resistant to fluticasone when compared to responses induced by TNFα alone (364). We investigated the effect of CpdA on the production of three chemokines (CXCL10, CCL5 and CX3CL1) based on their relevance in asthma pathogenesis and their potential role in driving in GC resistance in ASM cells. Firstly, CXCL10 was shown to be elevated in asthmatic subjects within the ASM in bronchial biopsies (91), as well as in the BAL fluid and bronchial mucosa (365). Moreover, CXCL10 was produced in response to viral infections and it is known that steroids are relatively ineffective in virus-induced acute asthma exacerbations (366). Additionally, CXCL10 was shown to be involved in the recruitment of mast cells to the asthmatic

ASM layer (91). Therefore inhibiting this chemokine could have a potential therapeutic value. Considering that the up-regulation of CXCL10 by exposure to TNFα/IFNγ was insensitive to fluticasone *in vitro*, this provides a possible pathway for its up-regulation in the asthmatics *in vivo* (97). In addition, CCL5 was shown to have important chemoattractant role for various inflammatory cells (i.e. monocytes, eosinophils and T cells) (367) suggesting that inhibiting this chemokine could limit airway inflammation. Also, CCL5 was found to contribute to the recruitment of fibrocytes in severe asthma (368) and increased BAL levels of CCL5 were indicative of severe disease in asthmatic children (369). Moreover, a reduction of CCL5 expression was one of the benefits for using anti-IgE therapy in patients with severe persistent allergic asthma (370). Currently an anti-CCR3, a receptor that recognises CCL5, is being investigated in Phase II clinical trials for moderate to severe asthma (i.e. Met-RANTES) (371). Finally, CX3CL1 was shown to mediate mast cell recruitment to the asthmatic ASM cells (66) and its expression was increased in ASM upon allergen challenge in asthmatic patients (66).

Therefore targeting the production of these chemokines could be beneficial in treating steroid insensitive conditions. To conclude, since it has become increasingly accepted that a substantial part of the efficacy of GC treatments may derive from their suppressive action on 'structural' cells such as smooth muscle, fibroblasts and epithelia, we argued that structural and inflammatory cell types may exhibit overlapping mechanisms that impair patients' sensitivity to GC therapy, and that novel therapeutic targets will emerge from an understanding of the mechanisms underlying GC resistance that occurs in lung structural cells.

3.2   AIMS

This chapter aims to test whether CpdA could affect the suppression of genes that are not inhibited by current GCs and to formulate a mechanism to explain its mode of action by examining the role of GRα-dependent or GRα-independent mechanisms.

The anti-inflammatory potential of CpdA was tested on the production of pro-asthmatic mediators (CCL5, CX3CL1, and CXCL10) in our experimental model of cytokine-induced steroid insensitivity. We have specifically addressed the following questions:

(1) Does CpdA inhibit the production of steroid-resistant chemokines induced by TNFα/IFNγ?

(2) Is CpdA effect at transcriptional/translational levels?

(3) Is CpdA effect non-cytotoxic?

(4) Is CpdA effect GRα-dependent?

❖ Does CpdA induce GRα-dependent genes?

❖ Does RU486 antagonise CpdA inhibitory effects?

❖ Does CpdA regulate GRα-inducible genes (GILZ, MKP-1)?

3.3   RESULTS

3.3.1   TNFα and IFNγ work synergistically to induce chemokine production by ASM cells

Experiments were performed in ASM cells stimulated with TNFα and with a combination of TNFα and IFNγ. Using ELISA assays, the production of the three chemokines CCL5, CXCL10 and CX3CL1 was measured in the supernatants following 24 hours stimulation. ASM cells stimulated with TNFα produced increased levels of CCL5 (3294 ± 933.6 pg/ml) and CXCL10 (5134 ± 338 pg/ml) as compared to unstimulated cells (445.9 ± 260.6 pg/ml and respectively 487.1 ± 138.5 pg/ml). On the other hand, CX3CL1 production was not induced by TNFα alone (3329 ± 727.7 ng/ml compared to 2341 ± 238.6 ng/ml). Subsequently, the combination of TNFα and IFNγ was shown to have a synergistic effect on the production of all three chemokines. This cytokine combination significantly enhanced the production of CCL5 (9234 ± 1632 pg/ml), CXCL10 (26131 ± 4265 pg/ml) and CX3CL1 (7741 ± 1388 ng/ml) when compared to ASM cells stimulated with TNFα alone and with unstimulated controls (**Figure 3.1**).
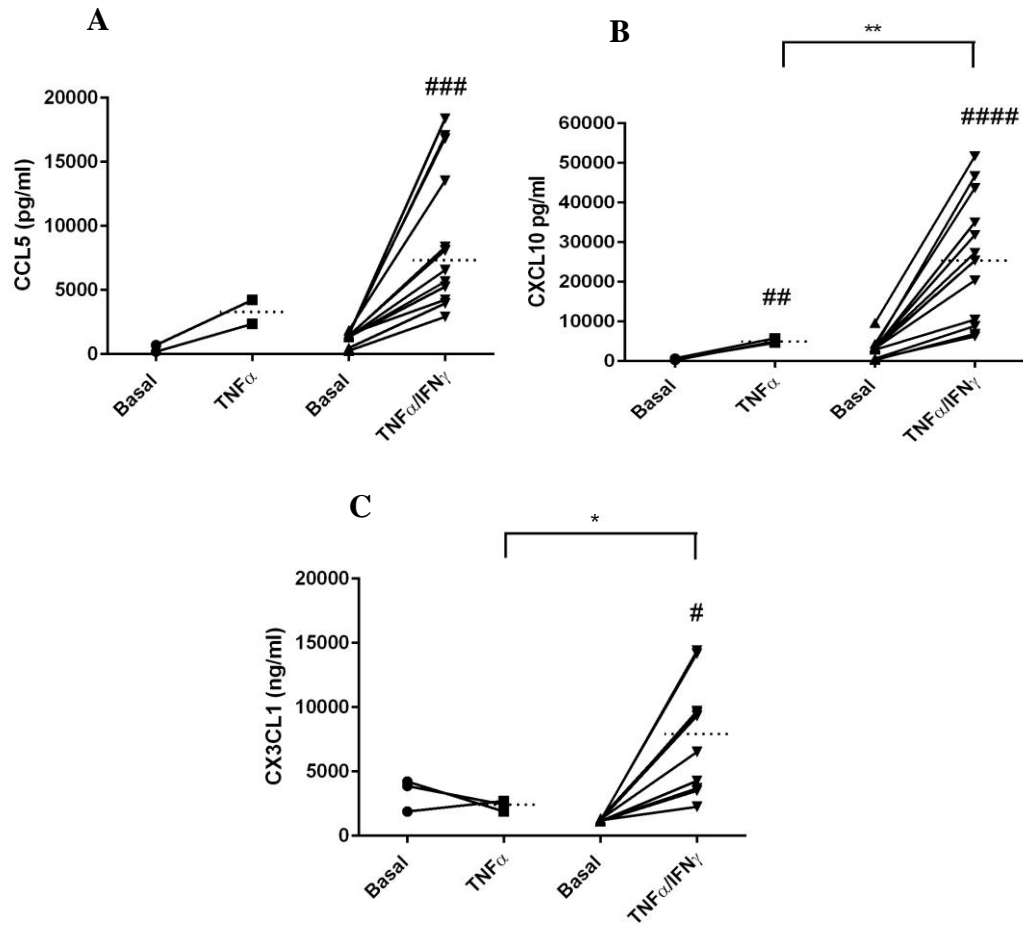
Figure 3.1: TNFα/IFNγ-induced production of CCL5, CXCL10 and CX3CL1 in ASM cells. ASM cells were growth arrested for 24 hours and stimulated with TNFα (10 ng/ml) alone or in combination with IFNγ (25 ng/ml) for 24 hours. CCL5 **(A)** CXCL10 **(B)** and CX3CL1 **(C)** levels released in the supernatants were measured by ELISA. Results are presented as means ± SEM in cells from n = 2-13 independent donors (10 healthy and 3 asthmatics), each point being performed in triplicate. Statistical analysis was performed using paired t test to compare between unstimulated cells and cells stimulated with TNFα or TNFα/IFNγ or Student's unpaired t test to compare between TNFα and TNFα/IFNγ stimulated cells. # P<0.05, ## P<0.01, ### P<0.001, #### P<0.0001 significant difference between basal non-stimulated and stimulated ASM cells with TNFα or TNFα/IFNγ; * P<0.05, **P<0.01 significant difference between TNFα and TNFα/IFNγ stimulated cells.

3.3.2 Fluticasone dose-dependently inhibits CCL5 and CXCL10 production in ASM cells stimulated with TNFα

The next sets of experiments were designed to confirm the observation that ASM cells stimulated with TNFα alone are sensitive to the inhibitory action of fluticasone. We showed that in these cells, TNFα-induced CXCL10 and CCL5 production was inhibited by fluticasone treatment in a dose dependent manner at concentrations between 0.001 and 10μM (**Figure 3.2**). Both CXCL10 and CCL5 production were inhibited by more than 50%, even at the lowest concentration of fluticasone. At these concentrations, CXCL10 production was reduced to 42.51%±18.2, 11.17%±2.5, 4.27%±11.3, 3.708%±2.6 and 13.94%±5.5, while CCL5 production was reduced to 40.16%±2.9, 30.76%±2.5, 22.77%±2.6, 21.80%±0.1 and 28.04%±3.3 respectively. Since CX3CL1 was not induced by TNFα alone, we did not look at the effect of fluticasone on this chemokine.

3.3.3 Fluticasone failed to inhibit CCL5, CXCL10 and CX3CL1 production in ASM cells stimulated with both TNFα and IFNγ

We then wanted to confirm that ASM cells stimulated with a combination of TNFα and IFNγ become insensitive to the inhibitory action of fluticasone (0.001-10 μM). We showed that in ASM cells stimulated with TNFα and IFNγ for 24 hours, the production of CCL5, CXCL10 and CX3CL1 was completely insensitive to the inhibitory action of fluticasone, even at the highest concentration of 10μM (**Figure 3.3**). Interestingly, chemokine production by TNFα and IFNγ was resistant to fluticasone in cells derived from both healthy and asthmatic subjects (n=3, 2 asthmatics and 1 healthy).
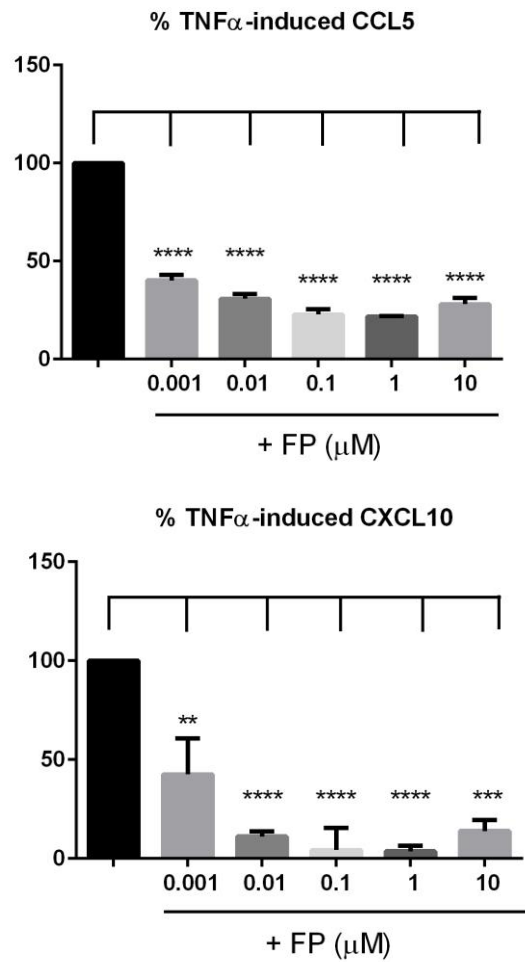
Figure 3.2: Fluticasone inhibits TNFα-induced CCL5 and CXCL10 production in ASM cells. ASM cells were treated with TNFα (10 ng/ml) alone for 24 hours and levels of CCL5 and CXCL10 were measured in the supernatants using an ELISA kit. Data are presented as means ± SEM. Data are expressed as percent inhibition by fluticasone of the net TNFα–induced chemokine production in n = 3 patients, 2 asthmatics and 1 healthy. All experiments were performed in triplicates. Statistical analysis was performed using One-way ANOVA with Bonferroni's post-hoc test. **P<0.01, ***P<0.001, ****P<0.0001
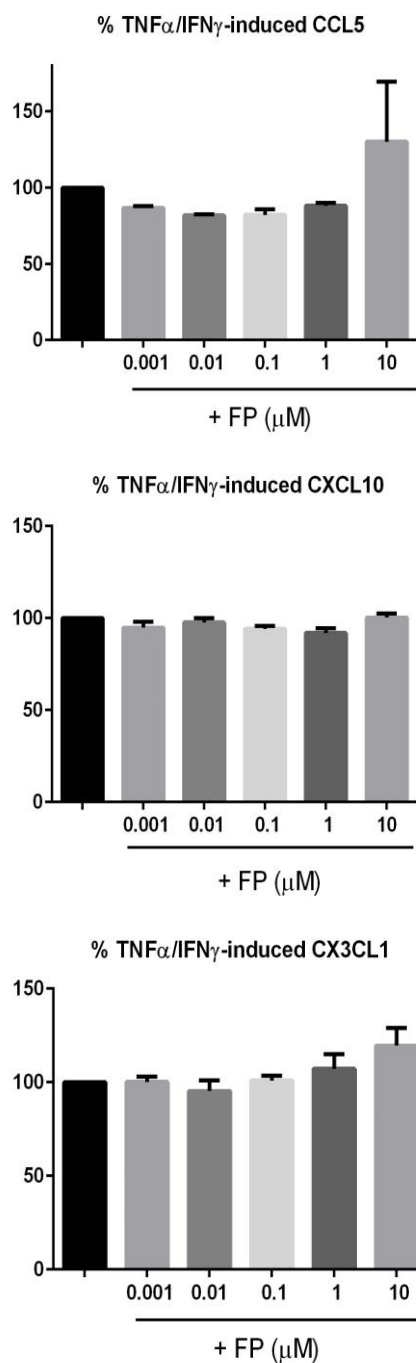
Figure 3.3: Induction of CCL5, CXCL10 and CX3CL1 is insensitive to fluticasone treatment in ASM cells treated with TNFα/IFNγ. ASM cells were treated with TNFα (10 ng/ml) and IFNγ (25 ng/ml) for 24 hours and levels of all chemokines were measured in the supernatants using an ELISA kit. Data are presented as means ± SEM. Data are expressed as percent inhibition by fluticasone of the net TNFα/IFNγ–induced chemokine production in n = 3 patients, 2 asthmatics and 1 healthy. All experiments were performed in triplicates. Statistical analysis was performed using One-way ANOVA with Bonferroni's post-hoc test.

3.3.4 TNFα/IFNγ-induced production of CCL5 and CXCL10 is increased in ASM cells from asthmatic patients compared to healthy individuals

We next looked at whether the induction of chemokines by TNFα/IFNγ is different between the healthy controls and asthmatic cells. Interestingly, cells from asthmatic patients induced increased production of CCL5 (3208±298.5 pg/ml), CXCL10 (20468±680.6 pg/ml) and CX3CL1 (1414±95.78 ng/ml) (**Figure 3.4B right**) when stimulated with TNFα/IFNγ compared to healthy controls (1601±46.61 pg/ml, 13488±204.3 pg/ml and 1197±130.6 ng/ml, respectively) (**Figure 3.4A left**). The production of CCL5 (**Figure 3.5A**) and CXCL10 (**Figure 3.5B**) was significantly increased in asthmatic ASM cells stimulated with TNFα/IFNγ, compared to healthy controls.
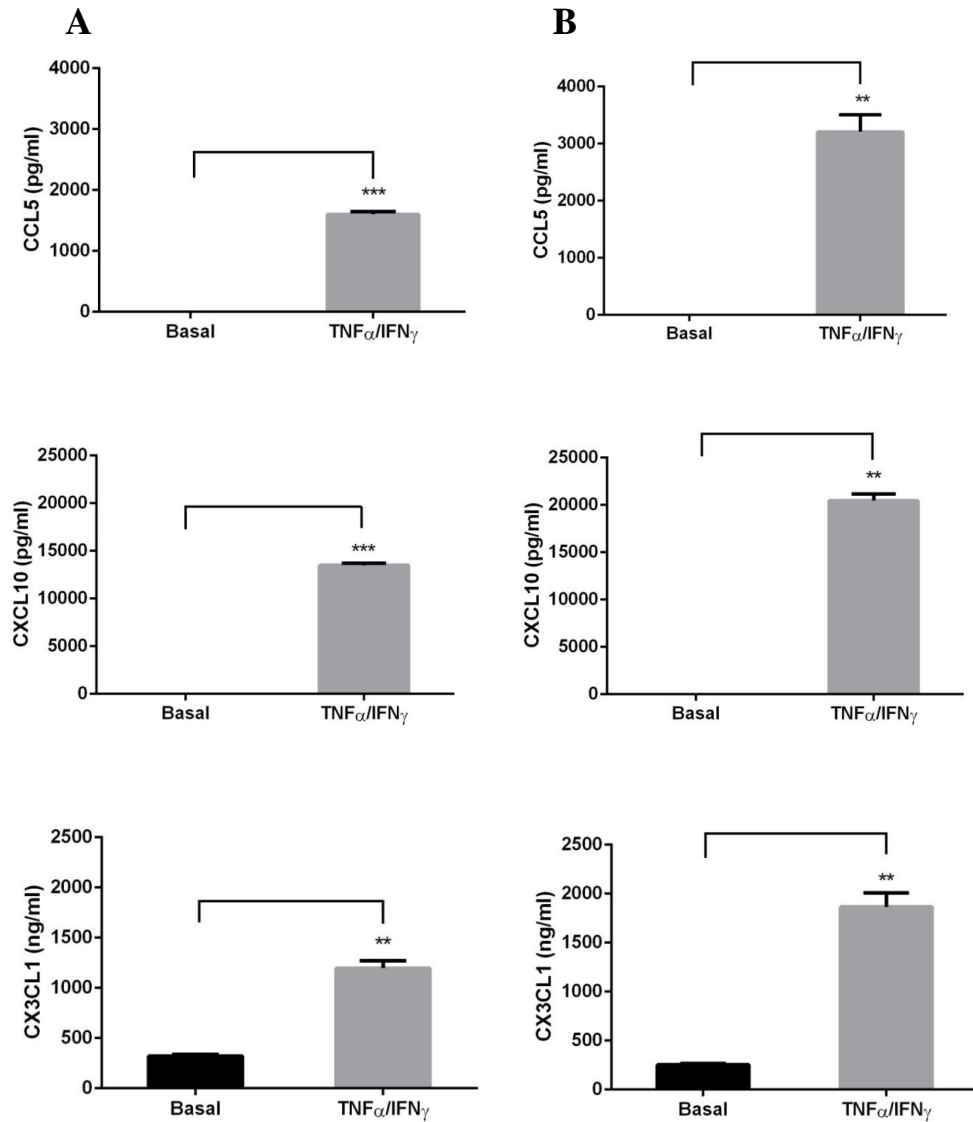
Figure 3.4: Production of chemokines by TNFα/IFNγ in ASM cells from healthy and asthma subjects. Cells were stimulated with TNFα (10ng/ml) and IFNγ (25ng/ml) for 24 h. CCL5, CXCL10 and CX3CL1 levels in the supernatants were assessed by ELISA assays. Data are expressed as means ± SEM of the net TNFα/IFNγ –induced increase of CCL5, CXCL10 and CX3CL1 in (**A left**) n = 4 healthy controls and (**B right**), n = 7 asthmatic patients, all performed in triplicate. Statistical analysis was performed using Student's paired t test. **P<0.01, ***P<0.001
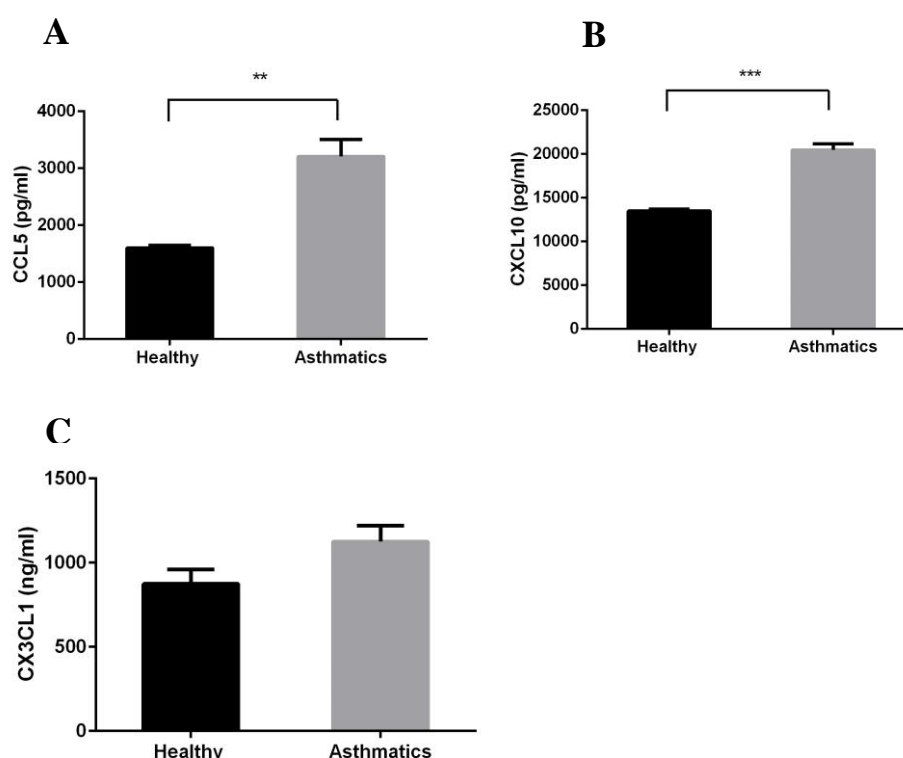
Figure 3.5: Asthmatic ASM cells produce increased levels of CCL5 and CXCL10 when induced by TNFα/IFNγ. Cells were stimulated with TNFα (10 ng/ml) and IFNγ (25 ng/ml) for 24h. CCL5, CXCL10 and CX3CL1 levels in the supernatants were assessed by ELISA assays. Data are expressed as means ± SEM of the net TNFα/IFNγ –induced increase of (**A**) CCL5, (**B**) CXCL10 and (**C**) CX3CL1 in n = 4 healthy controls and n = 7 asthmatic patients, all performed in triplicate. Statistical analysis was performed using Student's unpaired t test. **$P<0.01$, ***$P<0.001$.

3.3.5 CpdA differentially inhibits the expression of fluticasone-resistant chemokines in ASM cells

CpdA was shown to have potent anti-inflammatory properties in a variety of cell types (**Table 1.18**) however its actions in steroid-insensitive conditions have not been investigated. Experiments were designed to look at its effect on fluticasone-resistant chemokine production in our cellular model. We found that the production of CCL5 (**Figure 3.6A**), CXCL10 (**Figure 3.6B**), and CX3CL1 (**Figure 3.6C**) by TNFα/IFNγ was dose dependently inhibited by CpdA at concentrations ranging from 0.1 to 5µM.
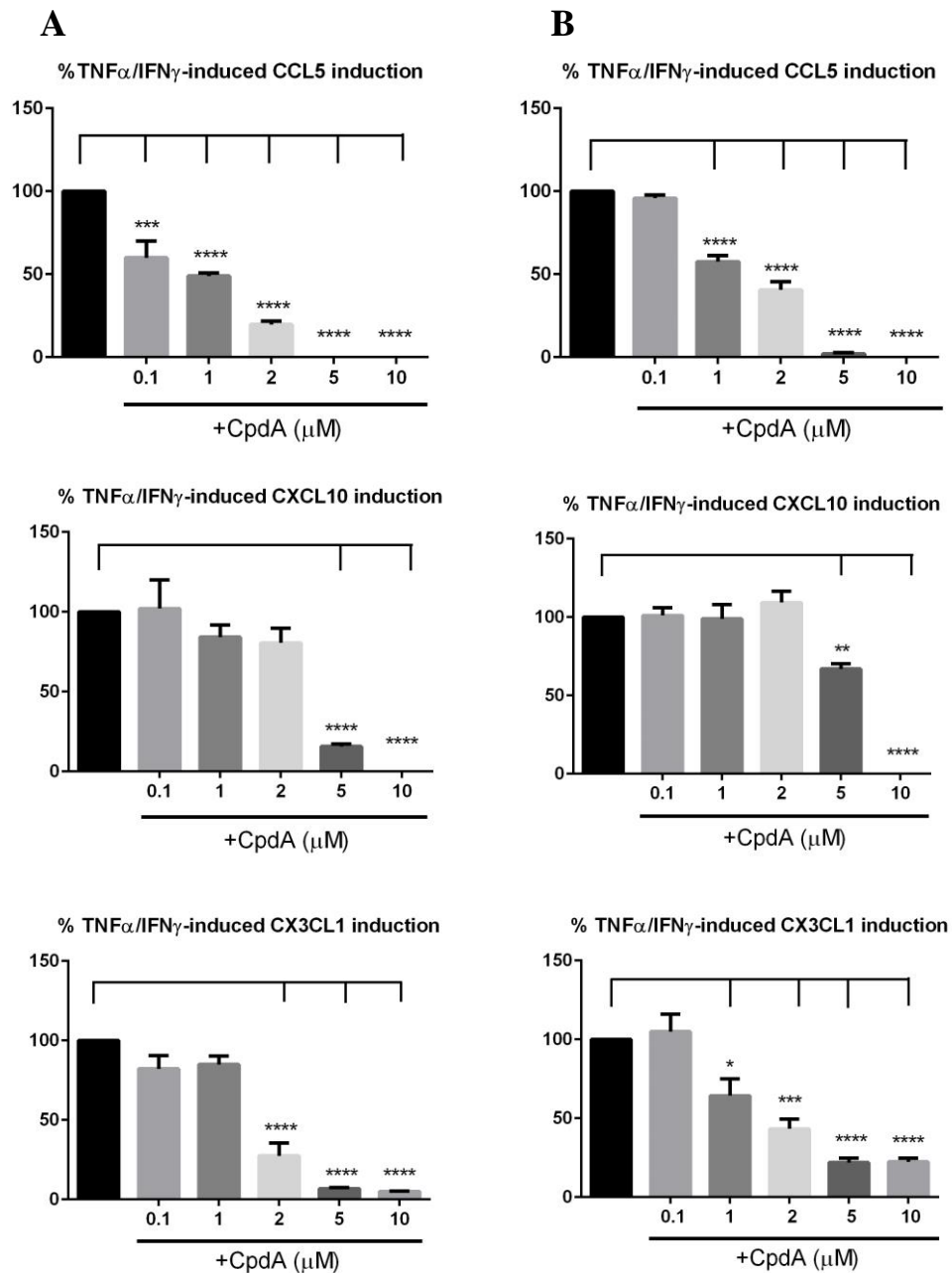
Figure 3.6: Effect of CpdA on CCL5, CXCL10 and CX3CL1 production induced by TNFα/IFNγ in ASM cells from healthy and asthmatic subjects. Cells were pre-treated with different concentrations of CpdA (0.1, 1, 2, 5, 10 μM) for two hours, followed by stimulation with TNFα (10 ng/ml) and IFNγ (25 ng/ml) for 24 h. Chemokine levels in the supernatants were assessed by ELISA assays. Data are expressed as means ± SEM of the net TNFα/IFNγ-induced increase of each chemokine in **(A left)** n = 4 healthy controls and **(B right)** n = 7 asthmatic patients, all performed in triplicate. Data are expressed as %TNFα/IFNγ –induced chemokine production. Statistical analysis was performed using One-way ANOVA with Bonferroni's post-hoc test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

In healthy ASM cells, CpdA inhibited CCL5 production by 40.11%±10 at the lowest concentration of 0.1μM and only by 4.19%±2 in asthmatic cells. The potency of the inhibitory effect of CpdA was reduced on the production of CCL5 in ASM cells from asthmatics, with an inhibition of 42.7%±6.5, 59.42%±4.8 and 97.935%±0.7 at concentrations of 1, 2 and 5μM, compared to 51.11%±1.7, 83.86%±2.1 and 100% in healthy cells. CXCL10 production was also inhibited by CpdA but at higher concentrations. In the healthy ASM cells, 2μM CpdA inhibited CXCL10 production by 19.4%±9.1, and had no effect in the asthmatic ASM cells. At 5μM CpdA, the levels of CXCL10 were inhibited by 84.31%±1.5 in the healthy cells and by 33%±3.1 in the asthmatics cells. CXCL10 was reduced to basal levels at the highest concentration of 10μM in both healthy and asthmatic cells. Finally CX3CL1 production by TNFα/IFNγ was also sensitive to CpdA, with an inhibition seen at 2μM in the healthy cells (72.62%±8.1) and at 1μM in the asthmatic ASM (35.67%±10.7). At higher concentrations of 5 and 10μM, CpdA reduced cytokine-induced CX3CL1 levels to 6.857%±0.5 and 5.049%±0.1 in the healthy cells, compared to 21.97%±2.6 and 22.42%±2.2 in the asthmatics cells. CpdA appeared to be less potent in inhibiting chemokine production in asthmatic ASM cells compared to healthy cells but this could also be explained by our previous finding that ASM cells from asthmatics produce increased levels of chemokines upon stimulation with TNFα/IFNγ, compared to healthy controls. We showed that CpdA was able to inhibit all three different chemokines with different potencies. Therefore, both CX3CL1 and CCL5 were found to be almost completely inhibited by CpdA at 5 μM in contrast to CXCL10 which was reduced by 70% in healthy subjects and by only 30% in asthmatics. This observation clearly shows that CpdA suppressed corticosteroid-resistant genes with different potencies reflecting different mechanisms or the involvement of different intracellular pathways.

3.3.6 CpdA differentially inhibits the mRNA expression of GC-resistant chemokines in ASM cells (CCL5, CXCL10, and CX3CL1)

The effect of CpdA (5µM) on the TNFα/IFNγ-induced mRNA expression of the 3 chemokines was next investigated. Quantitative PCR analyses were performed to determine whether CpdA inhibits TNFα/IFNγ–induced chemokine expression by acting at the transcriptional level. In cells treated with CpdA, the induction of CCL5 (**Figure 3.7A**), CXCL10 (**Figure 3.7B**), and CX3CL1 (**Figure 3.7C**) mRNA expression by TNFα/IFNγ was significantly decreased to 14.9%±2, 32%±10.2, and 21%±5.7, respectively.
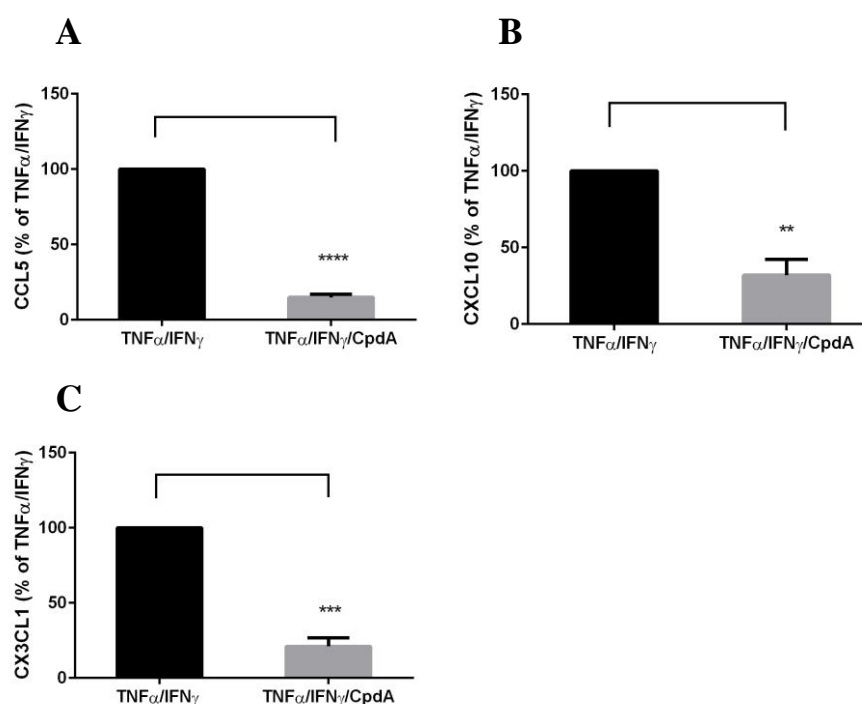
Figure 3.7: CpdA blocks the transcription of chemokine gene expression induced by TNFα /IFNγ in ASM cells. Cells were treated with CpdA (5 µM) for 2 hours followed by stimulation with TNFα (10 ng/ml) and IFNγ (25 ng/ml) for 4 hours. Total RNA was extracted for real-time quantitative PCR. Results are expressed as percentage induction of chemokine expression by calculating the negative inverse of the ΔΔCt value. Expression of the CCL5 **(A)**, CXCL10 **(B)**, and CX3CL1 **(C)** in four donors (asthmatics) is shown. β-actin was used as a house-keeping gene. Statistical analysis was performed using Student's paired t test. **P<0.01, ***P<0.001, ****P<0.0001.

3.3.7   CpdA does not have a cytotoxic effect by MTT assay

Previous work has shown that CpdA has quite a narrow therapeutic window due to its breakdown into highly reactive aziridines which have a cytotoxic potential (345). Therefore, the cytotoxic effect of CpdA was assessed *in vitro* by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay in ASM cells used in the experiment setup. MTT serves as a measure of cell viability, rather than a measure of cell death. This method is based on a reaction catalyzed by mitochondrial enzymes that are active only in living cells, which reduce MTT into a purple colored formazan product. Dead cells lose their ability to convert MTT into formazan, thus color formation serves as a useful and convenient marker for viable cells. The exact cellular mechanisms of MTT reduction into formazan are not well understood, but are thought to involve the reaction with NADH or similar reducing molecules that transfer electrons to MTT. This method's disadvantage is that individual cells cannot be examined, since a minimum cell count is needed in order to arrive at the detection range of formazan. CpdA at concentrations ≤ 5 µM showed no effect on cell viability in the treated conditions, while concentrations ≥ 10 µM had a profound inhibitory effect (>50% death, **Figure 3.8**).
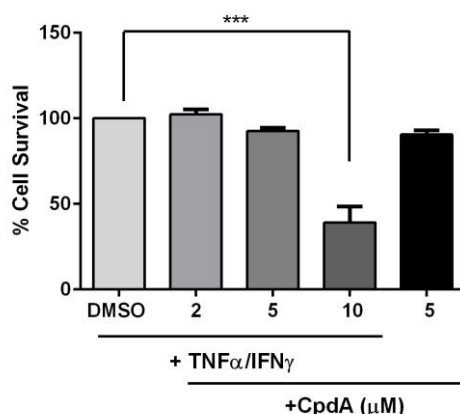
Figure 3.8: Cytotoxic effect of CpdA in ASM cells stimulated with TNFα and IFNγ. Cells from 5 donors (4 asthmatics and 1 healthy) were treated with different concentrations of CpdA (2 μM, 5 μM, 10 μM) for two hours, followed by stimulation with TNFα (10 ng/ml) and IFNγ (25 ng/ml) for 24 hours and assayed by MTT. Statistical analysis was performed using One-way ANOVA with Bonferroni's post-hoc test. ***P<0.001.

3.3.8 CpdA does not have a cytotoxic effect by Annexin V Assay

In addition to this, Annexin V and Propidium Iodide (PI) expression were investigated by flow cytometry. Annexin V is a large protein that binds to phospatidylserine, which in the early stages of apoptosis translocates from the cytoplasmic side of the cell membrane to the exterior. Annexin V can be labelled with various dyes and its binding is Ca^{2+} dependent. Annexin V is a marker for early apoptosis, while PI indicates late necrosis. Supporting the MTT data, there was no indication of apoptotic effect of CpdA at 5μM. Interestingly the higher concentration of CpdA (10μM) did not significantly increase the expression of Annexin V and PI as compared with cells treated just with TNFα/IFNγ (**Figures 3.9 and 3.10**). The discrepancy observed with the MTT assay may not necessarily be due to cell death but rather to decreased cell metabolism. In addition, in comparison to the MTT assay, the Annexin V method permits the analysis of individual cells, looks at cells undergoing

early and late apoptosis, is faster and uses fresh live cells which could explain the differences we see in the effect of CpdA between the two methods.
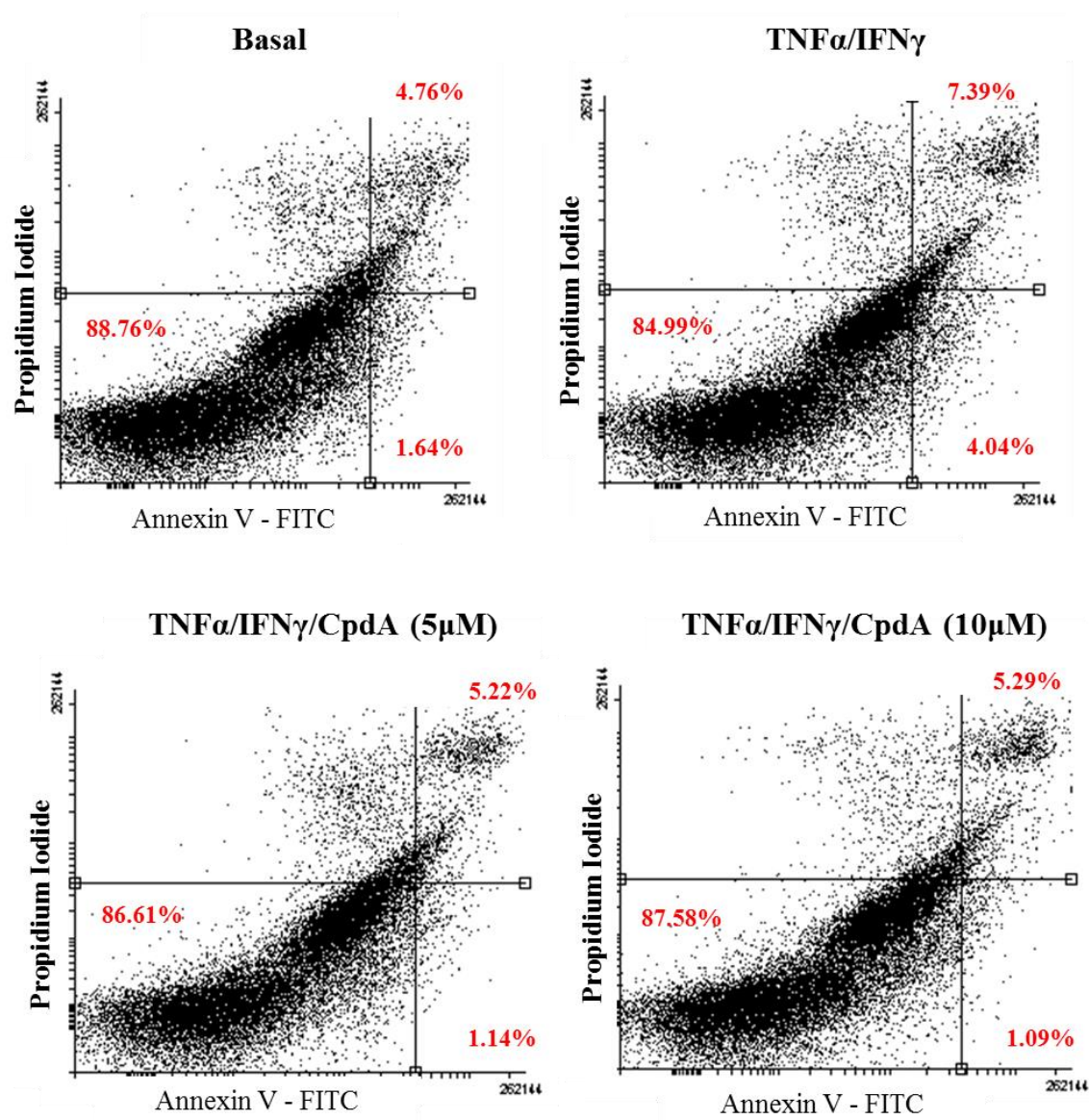
Figure 3.9: **Effect of CpdA on apoptosis in ASM cells stimulated with TNFα and IFNγ.** Representative flow cytometry quadrant plots for ASM staining with Propidium Iodide (PI) and FITC(Fluorescein Isothiocyanate)-labelled Annexin-V assessed by FACScan analysis in untreated cells (Basal), cells stimulated with TNFα and IFNγ and cells pre-treated with CpdA (5 and 10μM) and stimulated with TNFα and IFNγ. Percentages inside the quadrants represent **live cells** (**lower left**), **early apoptotic cells** (**lower right**) and **dead or necrotic cells** (**upper right**) from each condition.
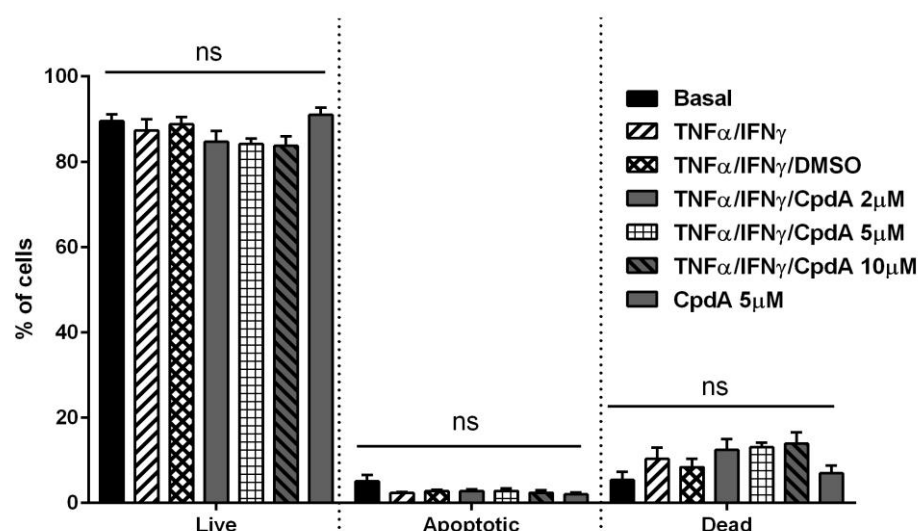
Figure 3.10: Non-cytotoxic effect of CpdA in ASM cells stimulated with TNFα and IFNγ. Cells from 3 donors (asthmatics) were treated with different concentrations of CpdA (2 µM, 5 µM, 10 µM) for two hours, followed by stimulation with TNFα (10 ng/ml) and IFNγ (25 ng/ml) for 24 hours, stained for Annexin V coupled with FITC and PI coupled with PE and assayed by flow cytometry. Statistical analysis was performed using One-way ANOVA with Bonferroni's post-hoc test, comparing between the different stimulations across the three groups of live, early apoptotic and dead cells. ns = not significant

3.3.9 CpdA does not induce GRα nuclear translocation by Immunofluorescence

The mechanisms underlying CpdA inhibitory effects were next investigated. Because CpdA effect is thought to be dependent on GRα in some cell types (336),(339, 341) it was important to first look at whether GRα was required to mediate CpdA suppressive effect on GC-resistant chemokines by looking at its effect on GRα nuclear translocation. The isotype control was used as a negative control for the experiment. As expected, GRα was expressed in the cytoplasm in unstimulated cells (basal), while in cells treated with fluticasone for 2 hours, GRα translocated to the nucleus in almost 100% cells. In contrast, CpdA did not induce GRα nuclear translocation at the two time points tested of 2 hours and 6 hours as shown in **Figure 3.11**, as most of the staining was observed in the cytoplasm.

	GRα	DAPI
Isotype		
Basal		
Fluticasone 2hr		
CpdA 2hr		
CpdA 6hr		
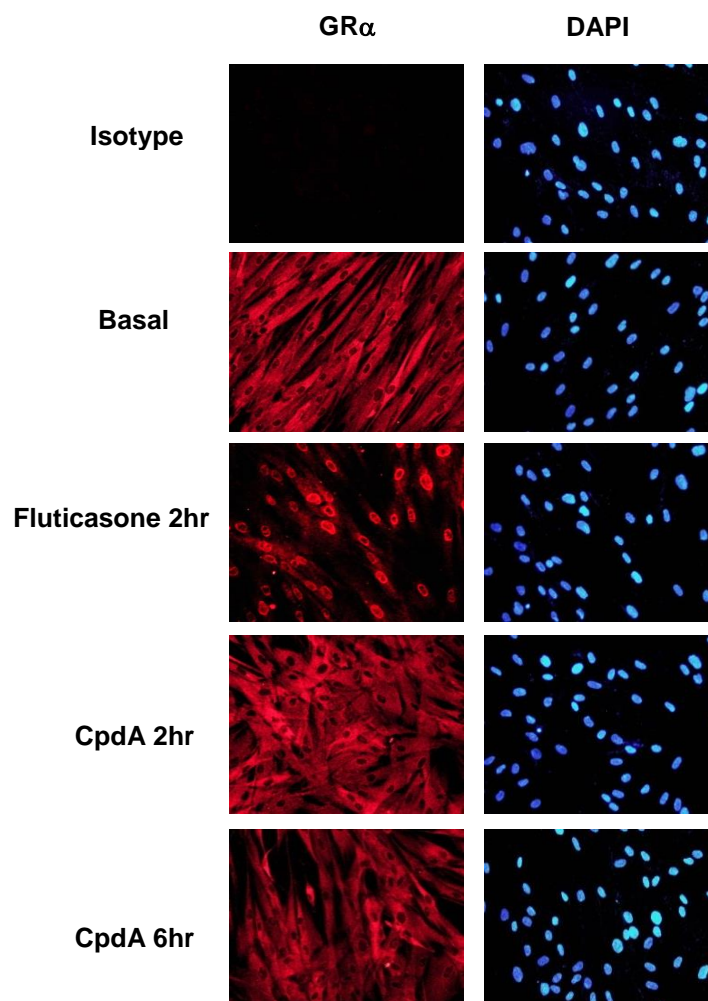
Figure 3.11: CpdA does not induce nuclear translocation of GRα in ASM cells. ASM cells were treated with fluticasone (100 nM) for 2 hours and CpdA (5 μM) for 2 and 6 hours and stained for GRα or for a corresponding isotype-matched antibody. Nuclei were stained with DAPI. Images are representative of data performed in cells from three different donors (2 asthmatics, 1 healthy).

3.3.10 The inhibitory action of CpdA on GC-resistant chemokines is unaffected by the GRα antagonist RU486

The next set of experiments looked at whether GRα was required to mediate CpdA suppressive effect on fluticasone-resistant chemokines by using the receptor agonist RU486. The effect of RU486 was tested on the production of TNFα-induced CCL5, to eliminate the possibility that the antagonist had an inhibitory effect on its

own. At a concentration of 1μM there was no effect of RU486 (4136±414.3 pg/ml compared to vehicle, 5265±403.7 pg/ml), while at concentrations over 3μM, there was an inhibitory effect on the production of CCL5 induced by TNFα (2708±60.89 pg/ml compared to vehicle, 5265±403.7 pg/ml) (**Figure 3.12**).
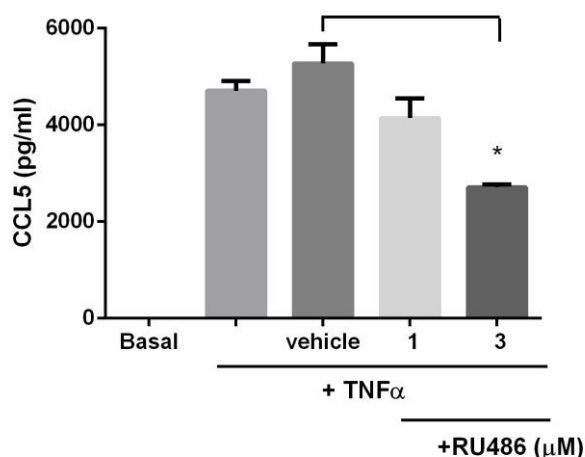
Figure 3.12: Effect of RU486 on TNFα-induced CCL5 production. Cells were pre-incubated with RU486 (1-3 μM) for 10 minutes and then treated with TNFα (10 ng/ml) for 24 hours. 1% ethanol was used as a vehicle. Levels of CCL5 were assayed by ELISA performed in 4 subjects (all asthmatics) in triplicate. Statistical analysis was performed using one-way ANOVA, with Bonferroni post hoc testing. $*P<0.05$.

Figure 3.13 shows that GRα blockade using the receptor antagonist RU486 (1μM) was effective in almost completely reversing the inhibitory action of fluticasone on TNFα-induced CCL5 expression. Fluticasone (100nM) was able to inhibit TNFα-induced CCL5 production by 74.95%±1.4. RU486 at 1μM was able to prevent the inhibitory effect of fluticasone (27.8% inhibition), restoring CCL5 production to 72.12%±4.5. RU486 on its own did not have an inhibitory effect, leading only to 16.24%±2.9 inhibition which was not significant.
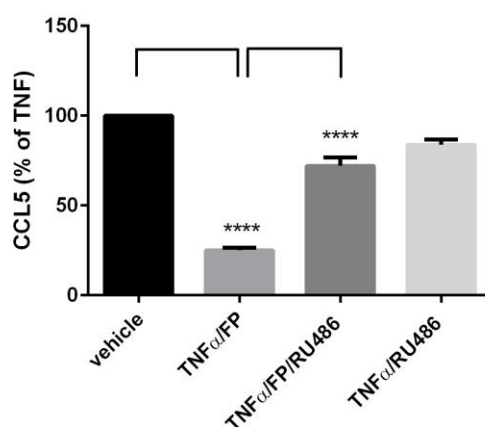
Figure 3.13: RU486 prevented the inhibitory effect of fluticasone on the production of TNFα-induced CCL5 (GC sensitive model). Cells were pre-incubated with RU486 (1 μM) for 10 minutes and then treated with fluticasone (100 nM) for 2 hours followed by TNFα (10 ng/ml) for 24 hours. 1% ethanol was used as a vehicle. Levels of CCL5 were assayed by ELISA performed in 6 subjects (all asthmatics) in triplicate. Statistical analysis was performed using one-way ANOVA, with Bonferroni post hoc testing. ****$P > 0.0001$

In addition to the finding that TNFα-induced CCL5 expression was highly repressed by corticosteroid treatment (**Figure 3.14**), we found that RU486 (1 μM) was unable to affect the inhibitory action seen with a lower concentration of CpdA. At 1μM, CpdA inhibited CCL5 release by 26.33%±5.2. In contrast to what was expected, inhibition of TNFα/IFNγ-induced CCL5 was further increased by the combination of RU486 and CpdA reaching a 43.2%±3.8 blockade of chemokine expression (**Figure 3.14**). RU486 alone did not have an inhibitory effect on TNFα/IFNγ-induced CCL5 production.

The ability of RU486 to affect CpdA on the other two fluticasone-resistant chemokines CX3CL1 and CXCL10 was not investigated as CpdA at such low concentration (1μM) did not inhibit chemokine production (**Figure 3.6**).
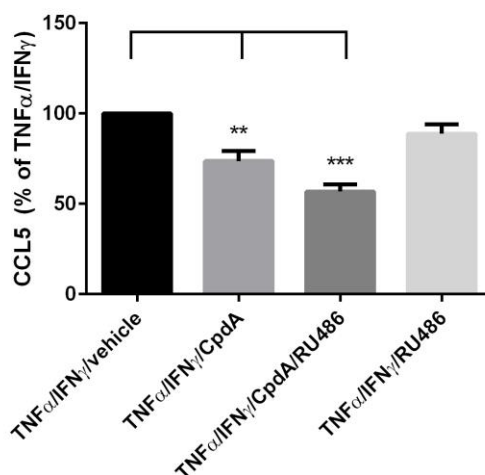
Figure 3.14: RU486 does not prevent CpdA inhibitory effect on TNFα-induced CCL5 production. Cells were pre-incubated with RU486 (1 μM) for 10 minutes and then treated with CpdA (1 μM) for 2 hours followed by TNFα (10 ng/ml) and IFNγ (25 ng/ml) for 24 hours. Ethanol 1% was used as a vehicle control. Levels of CCL5 were assayed by ELISA performed in 5 subjects (all asthmatics) in triplicate. Statistical analysis was performed using one-way ANOVA, with Bonferroni post hoc testing. **P<0.01, ***P<0.001

3.3.11 CpdA differently regulates the expression of GC-Inducible Genes Glucocorticoid-Induced Leucine Zipper (GILZ) and Mitogen-Activated Protein Kinase Phosphatase-1 (MKP-1)

CpdA was shown to have transrepressive properties *in vitro*, without transactivating anti-inflammatory genes (336). It was important to investigate whether CpdA activates known GC-dependent genes such as Glucocorticoid-Induced Leucine Zipper (GILZ) and also the non-conventional GRα-dependent gene Mitogen-Activated Protein Kinase Phosphatase-1 (MKP-1). CpdA at 5μM did not induce the mRNA expression of GILZ at 4 different time points of 2, 4, 6, and 24 hours compared to cells treated with fluticasone for 6 hours which induced GILZ expression by 22.1±2.1-fold (**Figure 3.15A**). On the other hand, CpdA unexpectedly induced MKP-1 mRNA expression at the 2 hours stimulation (5.8±1.8-fold) but not at the later time points of 4,

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6 and 24 hours. By contrast fluticasone, used here as a positive control, induced a 10.2±1.2-fold induction of MKP-1 expression (**Figure 3.15B**).
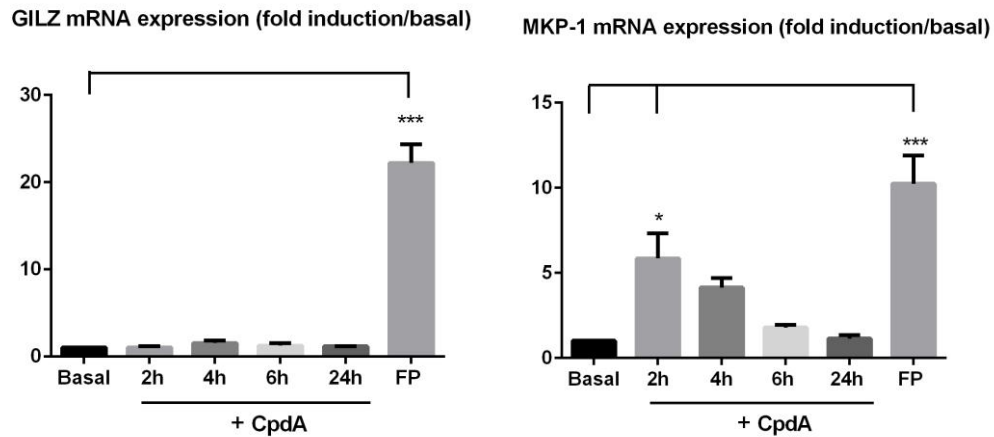
Figure 3.15: Differential induction of glucocorticoid response element–inducible glucocorticoid induced Leucine Zipper (GILZ) and mitogen-activated protein kinase phosphatase 1 (MKP-1) genes by CpdA in ASM cells. Cells were treated with CpdA (5 μM) for 2, 4, 6, and 24 hours or fluticasone (100 nM) for 6 hours. Total RNA was extracted for real-time quantitative PCR for **(A)** GILZ in n = 3 subjects (2 asthmatics, 1 healthy) and **(B)** MKP-1 in n = 4 subjects (3 asthmatics, 1 healthy). Results are expressed as fold induction of chemokine expression by calculating the negative inverse of the ΔΔCt value for each condition. Statistical analysis was performed using one-way ANOVA.*P <0.05, ***P <0.001.

3.4   DISCUSSION

We have previously reported that ASM cells exposed to a combination of TNFα/IFNγ become resistant to the suppressive action of corticosteroids (332), (255), (243), (362), (335). Although the mechanisms leading to this cytokine-induced impaired GC sensitivity are still being investigated, this work demonstrates the implication of pathways sensitive to the natural compound CpdA. As mentioned in the Introduction of this chapter, there are various preclinical studies convincingly showing that CpdA has a strong therapeutic virtue in inhibiting various inflammatory conditions including collagen-induced arthritis (341), experimental autoimmune encephalomyelitis (344, 345), and experimental autoimmune neuritis (346). These studies also demonstrated the lack of side effects typically associated with GRα activation by GC treatment and the equal efficacy of CpdA when compared with dexamethasone. The observation that CpdA effectively blocked allergic responses in a murine model of asthma (349) strongly suggests that CpdA-sensitive pathways are actively involved in the pathogenesis of allergic asthma. Using our ASM model of GC insensitivity, this study found that CpdA differentially inhibits the production of chemokines (CCL5, CXCL10, and CX3CL1) which are all involved in asthma pathogenesis. It was also noticed that the inhibitory profile of CpdA was quite different between all the tested chemokines. The magnitude of CXCL10 inhibition by CpdA was only seen at higher concentration (5μM). In contrast, the suppressive action of CpdA on the other steroid-resistant CX3CL1 and CCL5 was dose-dependent, observed at much lower concentrations (0.1−1μM), and led to a near complete chemokine inhibition. These marked differences in CpdA inhibitory profile suggest that different mechanisms are involved in the expression of GC-insensitive chemokines, and also that CpdA may be regulating the expression of fluticasone-resistant chemokines by acting at different levels. This hypothesis is

supported by previous studies showing that there were different inhibitory potencies and magnitudes in the inhibitory effect of CpdA on the expression of various proinflamamtory mediators. CpdA was shown to inhibit the production of TNFα (341), IL-6 (336, 355, 372), CXCL8 (355) (347), CCL2, CCL5, and CCL11(349) with different potencies. In contrast to some of these studies where dexamethasone was equally effective as CpdA in repressing inflammatory gene expression, the present study is the first to report a therapeutic action of CpdA in steroid-insensitive conditions.

Aiming to delineate the mechanisms involved in CpdA inhibitory actions, we investigated whether GRα was required for CpdA anti-inflammatory effects. Our data is in contrast to current literature, mostly by De Bosscher's group, who described this compound as a fully dissociated GRα ligand (336), (344), (339). Our immunostaining assays in **Figure 3.11** revealed that CpdA did not induce the nuclear translocation of GRα at different time points (2–6h) while fluticasone, used as positive control, stimulated a marked GRα nuclear translocation. This observation contrasts with the vast majority of studies where CpdA was shown to induce GRα nuclear translocation. However some striking differences exist between these studies. Some reports showing the ability of CpdA to induce GR nuclear translocation used much higher concentrations (>10μM) of this compound and despite this reported a much weaker effect when compared to dexamethasone. Furthermore, in some cases these observations were made in cells treated with various pro-inflammatory stimuli (eg. IL-1β) (336, 341, 342, 349, 359). Also the kinetic of CpdA-induced GRα nuclear translocation occurred at different time points including 30 minutes, and 3 to 6 hours post-stimulation. Moreover, one study using prostate cancer cells, showed that CpdA only partially affected GRα conformation, and these changes were not sufficient for receptor nuclear translocation (359). This is not surprising since the process of hormone-induced GRα nuclear

translocation is complex and involves a variety of processes such as phosphorylation, changes in the GRα-chaperone complex, and recruitment of the transport protein dynein (373). On the other hand, adding to this controversial area of research, one recent study supports our findings by showing that in CEM and K562 leukaemia cells, CpdA did not cause significant GRα translocation as compared with flucinolone acetonide (374). To further explore the possibility that CpdA acted independently of the GRα in repressing GC-resistant chemokines, we used the known steroid receptor antagonist RU486 to investigate if we could prevent the inhibitory effect of CpdA on the production of cytokine-induced CCL5. We showed that in the steroid-insensitive model of cells treated with CpdA at 1μM and TNFα/IFNγ, RU486 potentiated the inhibitory effect of CpdA on the production of cytokine-induced CCL5 rather than preventing CpdA inhibitory response. **Figure 3.14** shows that GRα blockade using the GC antagonist RU486 (1μM) was unable to prevent CpdA-induced 26.4% reduction of CCL5 in response to TNFα/IFNγ. In contrast, RU486 completely reversed the inhibitory action of fluticasone on CCL5 expression when induced by TNFα alone and used as a GC-sensitive condition (**Figure 3.13**). Contrary to this, previous work showed that RU486, although used at much higher concentrations (20μM), could prevent the inhibitory effects of CpdA in BWTG3 cell lines (339). Supporting our study is evidence from the latest study by De Bosscher's group. The GRα-independent effect of CpdA was also observed in their study, showing that RU486 could not reverse CpdA-mediated effects on cytokines secreted by PBMCs stimulated with Staphylococcus aureus–derived enterotoxin B protein. In fact, the authors noted a partial agonistic property of RU486 when combined to CpdA in the repression of IFNγ, IL-10, IL-17 and IL-1β (375). Their study suggested that CpdA either binds outside of the GRα ligand-binding pocket or acts independently of GRα itself.

Quantitative PCR experiments looked at whether CpdA had any transactivation activity by assessing the expression of the GC-inducible genes MKP-1 and glucocorticoid-induced Leucine Zipper (GILZ) (247). These experiments confirmed that CpdA had no transactivation potential by its lack of inducing expression of GILZ gene (**Figure 3.15A**). Interestingly, a transient induction of MKP-1, another GC-inducible early gene, was observed. This could be easily explained by the implication of GRα-independent mechanisms since MKP-1 could be induced by different stimuli including TNFα (376), sphingosine 1-phosphate (377), and β2 agonists (378). Not much is known about the exact mechanisms of CpdA action, with a few studies describing an inhibitory effect on various transcription factors (i.e. NF-κB and AP-1 by TNFα or IL-1β (359)) but with often quite contradicting results.

Together, this chapter shows the first evidence that CpdA sensitive pathways are differentially involved in the induction of chemokines under GC insensitive conditions. The present chapter also confirms the complexity of the mechanisms involved in the anti-inflammatory actions of CpdA that appear to be highly dependent on the gene type. This study suggests that elucidating the pathways that are sensitive to CpdA could help to identify novel targets for suppressing GC insensitive features in ASM in severe asthma. The following chapter will aim to address this.

CHAPTER 4

Investigating Compound A sensitive pathways: role of MAPK kinases

4.1   INTRODUCTION

Although the precise underlying mechanisms of CpdA action in ASM cells have not been investigated, the previous chapter demonstrated marked differences in CpdA inhibitory profile for the different chemokines tested (CCL5, CXCL10 and CX3CL1). This suggests that mechanisms differentially affected by CpdA may regulate the expression of GC-insensitive chemokines. We confirmed that CpdA has no transactivation potential by its failure to induce the well-known GC-inducible gene GILZ although it was interesting to observe the transient induction of another gene, MKP-1. Since MKP-1 is known to dephosphorylate and deactivate members of the mitogen-activated protein kinase (MAPK) pathway, this observation raised the possibility that the MAPK signalling pathway may be involved in mediating CpdA inhibitory effects.

As previously mentioned in the **Introduction** (**Section 1.6**), the MAPK is a family of signalling molecules that play a role in a variety of cellular functions important for asthma pathogenesis (i.e. proliferation, migration and synthesis of fibrotic and inflammatory proteins (201), (202)). The three members of this family, p38 MAPK, JNK and ERK, can be activated by a variety of stimuli, including cytokines, chemokines, growth factors, as well as environmental stresses (i.e. allergens and respiratory viruses). Their activation requires ATP-dependent phosphorylation at the threonine and tyrosine residues of their activation loop. Furthermore, since MAPK kinases act on a variety of substrates (**Figure 4.1**), their wide effects range from the level of gene expression of pro-fibrotic proteins and inflammatory cytokines, to the regulation of proliferative pathways by increasing levels of cell cycle proteins to induce cell growth.
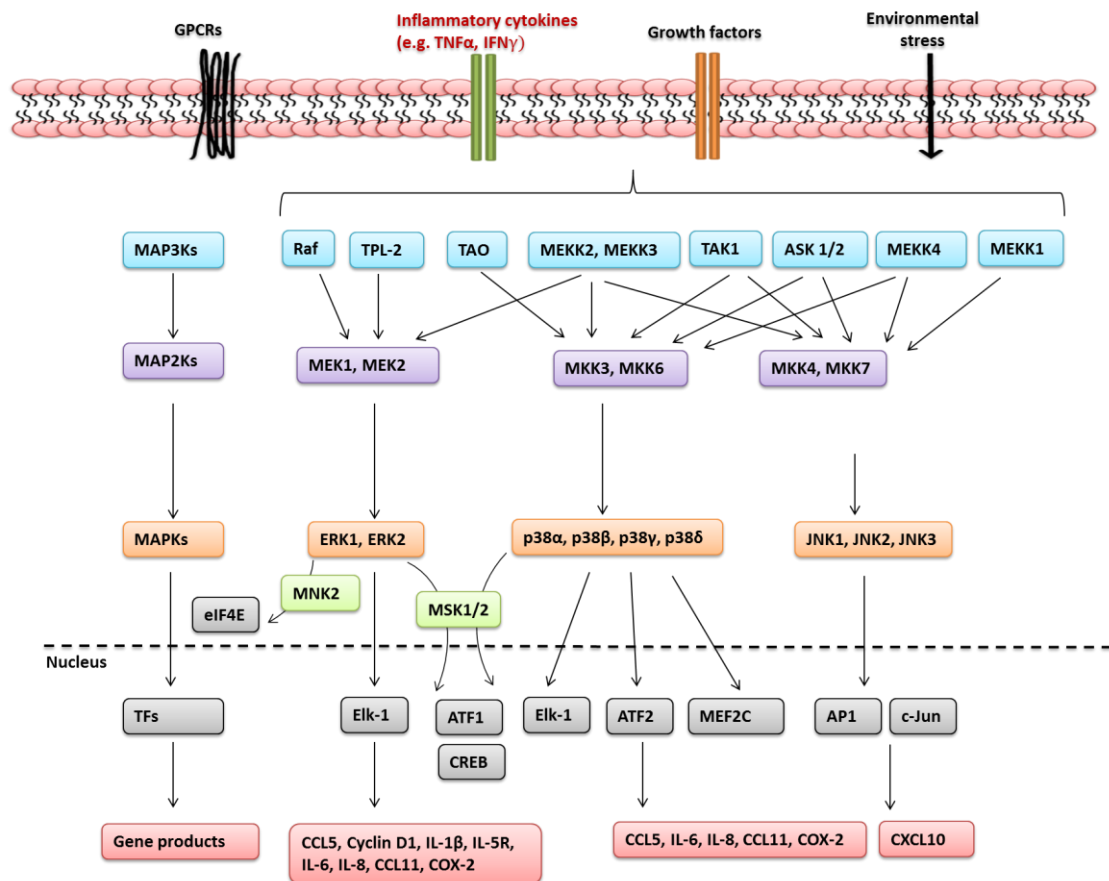
Figure 4.1: MAPK signalling cascades in ASM cells. Extracellular stimuli activate the MAPK pathways through mechanisms mediated by G protein coupled receptors (GPCRs) and tyrosine kinase receptors. First the MAP3Ks such as Raf, MEK and TAK are activated and they phosphorylate MAP2Ks on their 2 serine residues. MAP2Ks in turn activate the MAPKs by phosphorylating them on both threonine and tyrosine residues. Activated MAPKs can translocate to the nucleus or act in the cytoplasm where they can phosphorylate various transcription factors, such as ternary complex factor (TCF) family members, activating transcription factors (ATFs), and components in the activator protein 1 (AP1) complexes. Most of these transcription factors lead to the expression of genes involved in the inflammatory process (Adapted from (379), (380), (381) and (382)).

Current research also supports the notion that activation of MAPKs plays an essential role in modulating contractile, proliferative, and synthetic responses in ASM cells. For example, regulation of MMP-1 expression by Tenascin-C implicated all three MAPKs and regulated ASM contraction (383). One study showed that ERK inhibitors

reduced ASM proliferation and migration induced by leukotriene B4 (LTB4) (384). Work by another group showed that IL-1β and TNFα induction of thymic stromal lymphopoietin (TSLP), a cytokine triggering dendritic cell-mediated Th2 inflammatory responses, was mediated by ERK and p38MAPK (385). Furthermore, ERK and JNK were shown to mediate the mitogenic activity of TGF-β on ASM cells, while p38 was a negative regulator as its inhibition augmented TGF-β1 expression (386). One interesting observation from all these different studies emphasises that, depending on the stimuli used, not all MAPK kinases are equally involved.

More importantly for our work, the MAPK pathways were also shown to be involved in driving steroid insensitivity within the airways by regulating GRα phosphorylation on different serine residues (387). Studies in PBMCs and lung macrophages from patients with severe asthma demonstrated that in these patients, GCs had a reduced inhibitory effect on the production of cytokines induced by LPS and the relative insensitivity to treatment correlated with augmented activation of p38 MAPK (286, 302).

Considering the plethora of evidence to support the role of the MAPK pathway in driving pro-inflammatory responses in a variety of cells including ASM cells, inhibition of this pathway has emerged as an attractive strategy for reversing inflammation and remodelling in asthma. This study will aim to investigate for the first time whether MAPK signalling also drives steroid insensitive pathways in the model of ASM cells stimulated with TNFα and IFNγ. Two strategies were used to test this hypothesis: modulation of MKP-1 up-regulation and pharmacological inhibition of the MAPK members.

MKP-1 is an important regulator of the MAPK pathway that acts as a crucial negative anti-inflammatory mechanism to limit MAPK signalling, resulting in the

inhibition of expression of MAPK-dependent inflammatory mediators (303). The observation from the previous chapter that MKP-1 was up-regulated by CpdA was not surprising and is supported by other reports showing that MKP-1 up-regulation in ASM cells can be due to a variety of stimuli (**Table 4.1).** Various mechanisms involved in the anti-inflammatory potential of MKP-1 up-regulation in ASM have been described (**Table 4.2),** supporting the view that modulating this molecule could be beneficial in the treatment of inflammatory diseases.

Table 4.1: MKP-1 inducers in ASM cells

Inducers	Reference
Dexamethasone ± IL-1β	(388)
Dexamethasone/Fluticasone propionate	(389)
Mitogenic stimulation in asthmatics cells (10% FBS)	(390)
Dexamethasone ± TNFα	(391)
Proteasome Inhibitor MG-132	(200)
TNFα	(376), (392)
Dexamethasone ± Formoterol	(393)
The sphingolipid Sphingosine 1-phosphate (S1P)	(377, 394, 395)
Salmeterol/Fluticasone propionate	(378)
Salbutamol/Formoterol	(396)
MicroRNA-708 (miR-708)	(397)
Phosphodiesterase 4 inhibitors (PDE4) (ilomilast, piclamilast, rolipram) with formoterol	(398)
Compound A	(333)

Table 4.2: Effects of MKP-1-induction in ASM cells

Effects	Mechanism	Reference
Dexamethasone (Dex) and Fluticasone propionate (FP) inhibit TNFα-induced IL-6 protein secretion	MKP-1 up-regulation inhibits p38 MAPK-mediated IL6 mRNA stability	(389)
Asthmatics ASM cells proliferate faster than healthy cells	MKP-1 up-regulation inhibits ERK activation, resulting in a shift to the PI3-kinase proliferative pathway	(390)
Dex inhibits TNFα–induced CD38 expression	MKP-1 up-regulation inhibits all three MAPKs: p38, ERK and JNK	(391)
Proteasome inhibition using MG-132 leads to the	MKP-1 up-regulation inhibits p38 MAPK	(200)

inhibition of TNFα-induced IL-6 secretion (but not IL-8)		
Dex inhibits S1P-induced IL-6 secretion	Up-regulation of MKP-1 blocks the activation of MSK1 and phosphorylation of histone H3	(395)
Salmeterol increases FP-induced MKP-1 expression, resulting in an additive suppressive effect on TNFα – induced IL-8, but not on IL-6 production	Salmeterol transiently upregulates MKP-1, inducing IL-6 release. FP induces a sustained increase in MKP-1 protein. Salmeterol inhibits TNFα-induced IL-8 in combination with FP	(378)
miR-708 inhibits TNFα-induced CD38 expression	MKP-1 up-regulation inhibits phosphorylation of JNK	(397)
PDE4 inhibits inhibition of TNFα-induced IL-8	PDE4 upregulates MKP-1 by increasing β2-agonist-induced cAMP	(398)
Active tristetraprolin (TTP) reduces TNFα-induced IL-6	temporal bi-phasic regulation between MKP-1 and p38	(392)

The second approach was to directly target the MAPK kinases using small molecule inhibitors for p38MAPK, ERK and JNK (**Table 4.3**). This strategy has been used for treating inflammation and remodelling in a variety of cellular models and mouse models of chronic inflammatory conditions such as inflammatory bowel disease (399), rheumatoid arthritis (400), COPD (401) and asthma (202).

Table 4.3: Small molecule inhibitors

Inhibitor	Target	Concentration	References
SB203580	p38 MAPK	1μM	(402), (403), (404)
SP600125	JNK	1μM	(405), (406)
U0126	MEK1/2	1μM	(407), (408), (403)

Furthermore, various clinical trials are currently investigating the potential of using MAPK inhibitors (**Table 4.4**). Since many of these orally administered inhibitors have been poorly tolerated due to side effects, the development of inhaled therapies is thought to optimise the therapeutic index by increased pulmonary versus systemic exposure.

Table 4.4: Clinical studies using kinase inhibitors

Drug	Target	Disease	Outcome
Vertex, Pamapimod	p38MAPK	Rheumatoid arthritis	Failed due to cytotoxicity and lack of efficacy
PH-797804, losmapimod	p38MAPK	COPD	Well tolerated (24-week duration) but with minimal effects on lung function
PH-797804	p38MAPK	Acute neutrophilic airway inflammation	↓airway neutrophil numbers and airway inflammatory biomarkers in healthy subjects after LPS challenge
JNK-401(CC-401)	JNK	Asthma	Completed Phase I trial in healthy volunteers
CNI-1493	JNK	Rheumatoid arthritis, Crohn's disease	Phase II
ARRY‑438162	ERK	Rheumatoid arthritis	Reduced efficacy

4.2   AIM

This chapter aimed to investigate a potential role of the MAPK pathway in modulating the production of fluticasone-resistant chemokines in our model of cytokine induced GC insensitivity (ASM cells exposed to TNFα/IFNγ). Studies were designed first to address whether the up-regulation of MKP-1 was important for the inhibitory effects of CpdA. Second, this chapter aimed to investigate the potential implication of inhibiting members of the MAPK family in the modulation of steroid-resistant chemokines. To test this hypothesis, the following questions were addressed:

(1) Is MKP-1 up-regulation by CpdA important for CpdA inhibitory effects?

(2) Does the combination of TNFα and IFNγ activate the MAPK pathways?

(3) Does CpdA modulate the activation of MAPK kinases by TNFα/IFNγ?

(4) Do MAPK pathways play a role in mediating TNFα/IFNγ-induced chemokine production?

(5) Do MAPK pathways play a role in modulating the sensitivity of chemokines (CCL5, CXCL10 and CX3CL1) to fluticasone in ASM cells stimulated with TNFα/IFNγ?

4.3   RESULTS

4.3.1   Time course of MKP-1 expression induced by CpdA

Experiments were designed to determine the role of MKP-1 in our model of cytokine induced GC insensitivity. In the previous chapter we showed that CpdA led to a transient induction of MKP-1 at mRNA level. To confirm that MKP-1 was also affected at the protein level, ASM cells were stimulated with 5µM CpdA for the different time points of 2, 4, 6, and 24 hours. Immunoblot experiments showed that the induction of MKP-1 by CpdA was transient and seen only at 2 hours, with an increase of 159%±21.7 when compared to basal (**Figure 4.2**). Levels of MKP-1 then decreased but remained sustained at the later time points of 4, 6, and 24 hours, to 90.23%±17.92, 70.93%±10.53 and 65.74%±9.966 respectively.
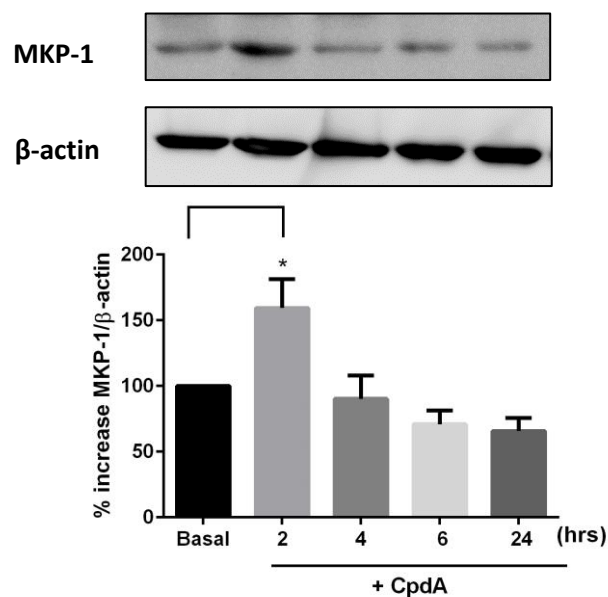
Figure 4.2: CpdA induces a transient MKP-1 up-regulation at protein level in ASM cells. Cells were stimulated with CpdA (5µM) for the indicated time points (2-24 hours). Total cell lysates were assayed for MKP-1 and β-actin by immunoblot assays. Data are expressed as % of MKP-1 expression over basal (untreated cells). Results are presented as means ± SEM in cells from n=5 donors (4 asthmatics and 1 healthy). Statistical analysis was performed using one-way ANOVA with Bonferroni's post hoc test. *$P<0.05$

4.3.2 Assessing the effect of MKP-1 silencing on cytokine production: effect on CXCL10

Silencing experiments using small interfering RNA (siRNA) were performed to look at the effect of knocking down MKP-1 on the production of steroid-resistant chemokines. Following transfection, ASM cells were lysed and assayed for MKP-1 and β-actin by immunoblot assays to confirm MKP-1 silencing at the protein level. MKP-1 was silenced by 52%±14.33 (**Figure 4.3**) which was in line with other studies using the same method (389) and in line with the manufacturer's recommendation of 63% efficiency for ASM cells (using Amaxa). Secondly, following transfection, ASM cells were stimulated with TNFα/IFNγ for 16 hours with or without CpdA pre-treatment for 2 hours and the production of CXCL10, CX3CL1 and CCL5 in the supernatants was assessed by ELISA. Interestingly, knocking down MKP-1 (**Figure 4.3**) completely prevented CpdA from inhibiting cytokine-induced CXCL10 production (**Figure 4.4**) but had no effect on CCL5 (**Figure 4.5A**) and CX3CL1 (**Figure 4.5B**). MKP-1 knockdown completely prevented the 21.47%±4.5 inhibition of cytokine-induced CXCL10 by CpdA. These data suggest that MKP-1 up-regulation by CpdA was required in its inhibitory action on the expression of the GC insensitive chemokine CXCL10 in ASM cells. These data strongly implicate MKP-1–sensitive MAPK pathways in the regulation of CXCL10 expression in ASM cells. Because the effects of CpdA on the other chemokines were not affected by MKP-1 silencing, this suggests that CpdA sensitive but MKP-1-independent pathways are also required for the induction of GC-resistant chemokines.
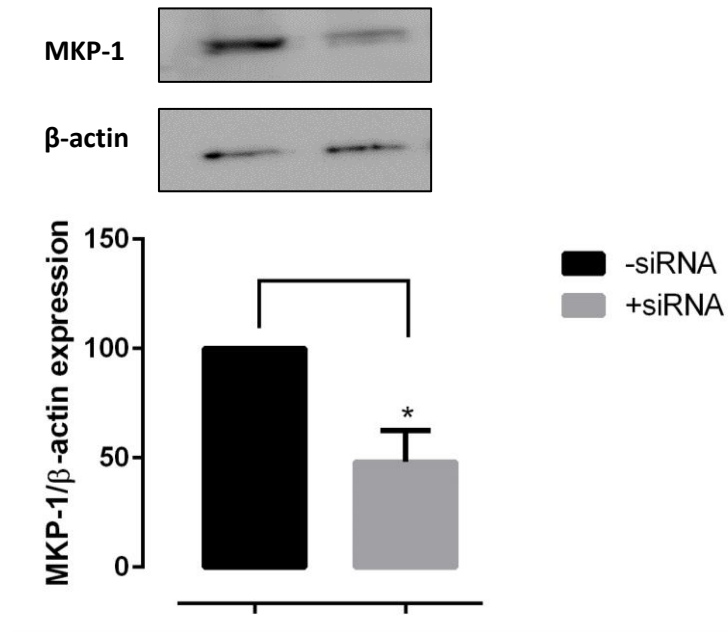
Figure 4.3: MKP-1 silencing in ASM cells using small interfering siRNA. Cells were transfected with Silencer Pre-designed siRNA MKP-1 oligonucleotides or non-silencing control scrambled siRNA (μg). Following transfection, cells were lysed and assayed for MKP-1 and β-actin by immunoblot assays. Data are expressed as % of MKP-1 induction over basal. Results are presented as means ± SEM in cells from n=3 donors (healthy) performed in duplicate. Statistical analysis was performed using the student unpaired t test. *P<0.05
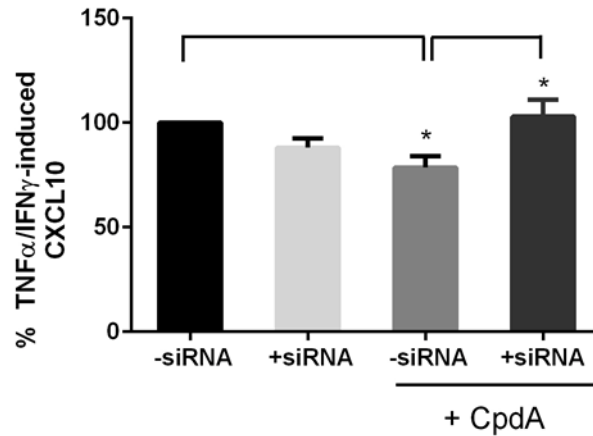
Figure 4.4: MKP-1 silencing mediates the inhibition of cytokine-induced CXCL10 by CpdA in ASM cells. Cells were transfected with Silencer Pre-designed siRNA MKP-1 oligonucleotides or non-silencing control scrambled siRNA (1µg). Following transfection cells were stimulated with TNFα/IFNγ for 16 hrs in the presence or absence of CpdA (5µM) added 2 hr before. CXCL10 levels in the supernatants were assessed by ELISA assays. Data are expressed as % of cytokine-induced CXCL10 expression. Results are presented as means ± SEM in cells from n=4 donors (healthy) performed in duplicate. Statistical analysis was performed using ANOVA with Bonferroni's post hoc test. *P<0.05
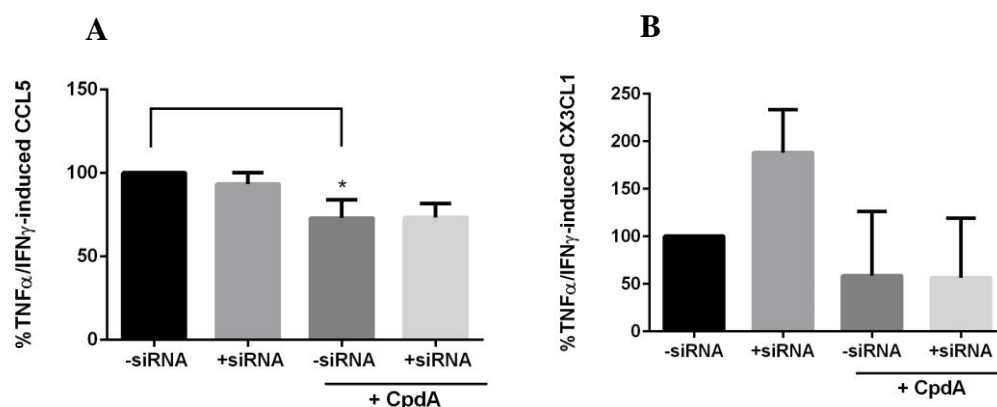
Figure 4.5: MKP-1 silencing does not affect cytokine-induced CCL5 and CX3CL1 by CpdA in ASM cells. Cells were transfected with Silencer Pre-designed siRNA MKP-1 oligonucleotides or non-silencing control scrambled siRNA (1μg). Following transfection cells were stimulated with TNFα/IFNγ for 16 hrs in the presence or absence of CpdA (5μM) added 2 hr before. Protein levels of CCL5 **(A)** and CX3CL1 **(B)** in the supernatants were assessed by ELISA assays. Data are expressed as % of cytokine-induced expression. Results are presented as means ± SEM in cells from n=4 donors (healthy) performed in duplicate. Statistical analysis was performed using ANOVA with Bonferroni's post hoc test. *$P < 0.05$

Targeting the MAPK cascade is an attractive strategy for reversing steroid insensitivity in asthma and a better understanding of how this pathway regulates pro-asthmatic responses in ASM is of therapeutic importance. Apart from the up-regulation of the endogenous MAPK deactivator, another strategy for targeting this cascade is the use of small molecule MAPK inhibitors. The next part of this chapter investigated the role of MAPK inhibition in reducing the production of fluticasone-resistant chemokines in ASM cells stimulated with TNFα and IFNγ. In addition, the potential enhancing effect of using GCs in combination with MAPK inhibitors was tested.

4.3.3 Time course of p38 MAPK activation induced by TNFα and IFNγ combination

It is known that MKP-1 dephosphorylates the active phospho-p38MAPK (p-p38) to reduce its activity and therefore interferes with expression of p38-dependent inflammatory mediators. More importantly, p38MAPK has been shown to be involved in mediating *in vitro* corticosteroid insensitivity via the phosphorylation of GRα at serine residues ser226 (409), (410).

First, this study tested whether the inhibitory effect of CpdA on fluticasone-resistant genes may be due to the suppression of p38MAPK activity and if indeed p38MAPK was involved in cytokine-induced expression of corticosteroid resistant chemokines. Western Blot experiments were performed to look at the time course of p38 phosphorylation (p-p38) following stimulation with TNFα and IFNγ. p38 was found to be maximally phosphorylated at 10 minutes with a 1.53±0.1-fold increase over basal, before p-p38 levels decreased at later time points (0.5, 1, 2, and 4 hours) (0.54±0.19, 0.52±0.09, 0.78±0.32 and 0.59±0.33-fold) (**Figure 4.6**).
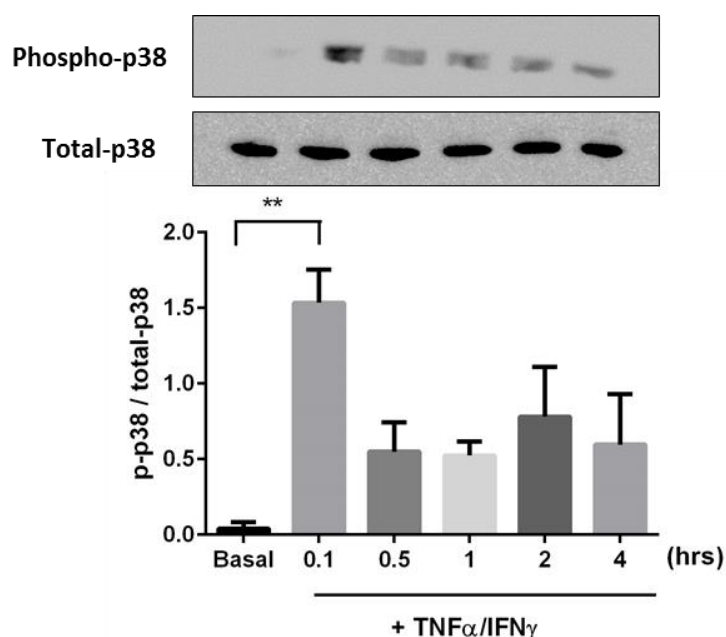
Figure 4.6: Phosphorylation of p38 by TNFα/IFNγ is time-dependent in ASM cells. Cells were stimulated with TNFα (10ng/ml) and IFNγ (25ng/ml) at different time points (0.1, 0.5, 1, 2, and 4 hours). Total cell lysates were assayed for p-p38 and total p38 by immunoblotting assays. Blot picture is representative of 1 donor (asthmatic). Lower panels represent means ± SEM of scanning densitometric measurements using ImageJ of p-p38 expression normalised over total p38 from n=4 (3 asthmatics and 1 healthy) donors. Statistical analysis was performed using one-way ANOVA. **P<0.01

4.3.4   Effect of CpdA on p38 activation by TNFα and IFNγ

The effect of CpdA on the phosphorylation of p38MAPK by TNFα and IFNγ was next investigated. ASM cells were stimulated with TNFα and IFNγ for 10 minutes with or without CpdA (5μM) pre-treatment for 2 hours. P-p38 and total p38 activation were assayed by Western Blotting. We found that CpdA had no inhibitory effect on the phosphorylation of p38 in response to TNFα and IFNγ. Although we observed an inhibitory effect in some of the donors, the combined effect of CpdA from all subjects did not reach statistical significance (**Figure 4.7**). The activation of p-p38 by TNFα and IFNγ was very similar in cells from healthy and asthmatic donors, with no significant effect of CpdA as shown in **Figure 4.8**.

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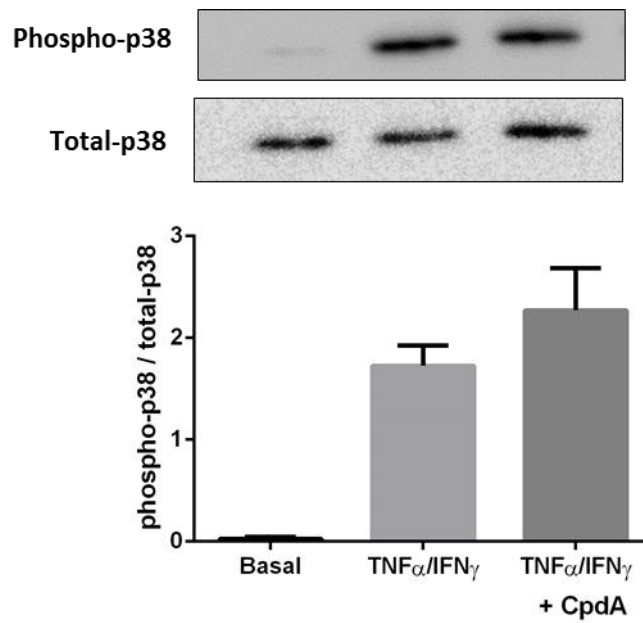
Figure 4.7: CpdA has no effect on phosphorylation of p38 by TNFα and IFNγ in ASM cells. Cells from 9 donors (6 asthmatics and 3 healthy) were either left unstimulated, treated with TNFα (10ng/ml) and IFNγ (25ng/ml) for 10 minutes or pre-treated with CpdA (5μM) for 2 hours and then stimulated with TNFα and IFNγ for 10 minutes. Total cell lysates were assayed for p-p38 and total p38 expression by immunoblotting. Blot picture is representative of 1 donor (healthy). Lower panels represent means ± SEM of scanning densitometric measurements using ImageJ of p-p38 expression normalised over total p38. Statistical analysis was performed using one-way ANOVA.
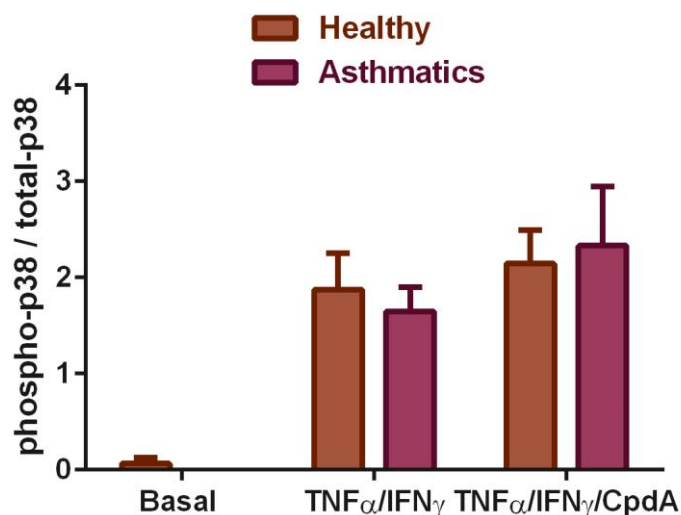
Figure 4.8: CpdA effect on phospho-p38 activation by TNFα and IFNγ in healthy and asthmatic ASM cells. Cells from 3 healthy and 3 asthmatic donors were either left unstimulated, treated with TNFα (10ng/ml) and IFNγ (25ng/ml) for 10 minutes or pre-treated with CpdA (5μM) for 2 hours and then stimulated with TNFα and IFNγ for 10 minutes. Total cell lysates were assayed for p-p38 and total p38 expression by immunoblotting. Data represent means±SEM of scanning densitometric measurements using ImageJ of p-p38 expression normalised over total p38. Statistical analysis was performed using one-way ANOVA.

4.3.5 Effect of p38 inhibitor (SB203580) on cytokine-induced production of CCL5, CXCL10 and CX3CL1

To determine the role of p38MAPK in driving fluticasone-resistant chemokines, ASM cells were pre-treated with the p38 inhibitor SB203580 at different concentrations (1, 3, 5, and 10μM) and stimulated cells with TNFα and IFNγ for 16 hours. ELISA was then used to determine the levels of fluticasone-resistant CCL5, CXCL10 and CX3CL1. SB203580 had a significant inhibitory effect on cytokine-induced CX3CL1, reducing its levels to 3771±288 (5μM) and 2160±1658 (10μM), compared to the vehicle control (8503±288) (ng/ml) (**Figure 4.9C**). SB203580 also had an inhibitory effect on CCL5 production but at the highest concentration of 10μM, with levels being reduced from

3634±16.37 to 2738±253 (pg/ml) (**Figure 4.9A**). SB203580 had no effect on CXCL10

production induced by cytokines (**Figure 4.9B**).
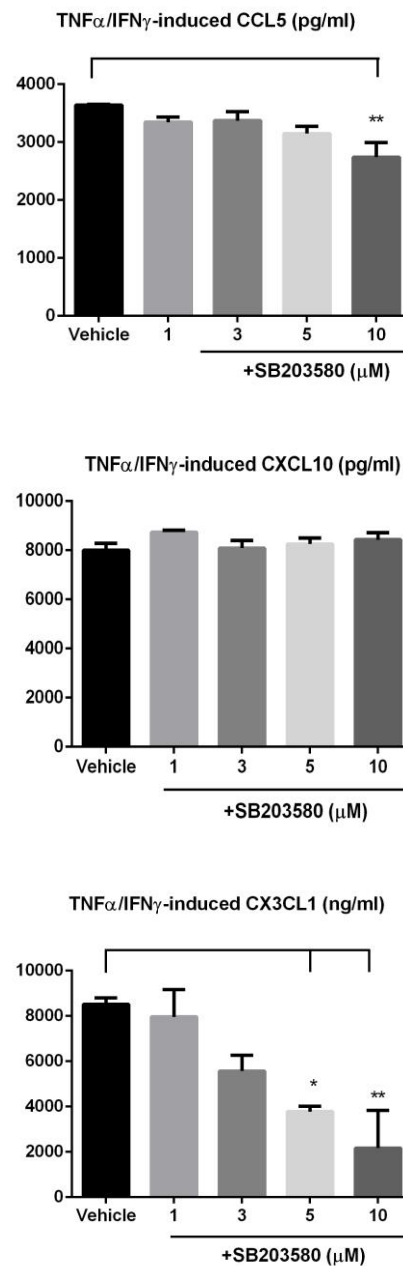
Figure 4.9: Effect of SB203580 on TNFα/IFNγ-induced production of CCL5, CXCL10 and CX3CL1. CCL5 (**A**), CXCL10 (**B**) and CX3CL1 (**C**) production was assessed by ELISA in cells from 5 donors (4 healthy and 1 asthmatic) treated with different concentrations of the p38 inhibitor (1-10 μM) and stimulated with TNFα (10 ng/ml) and IFNγ (25 ng/ml) for 16 hours. Chemokine levels in the supernatants of each subject were performed in triplicate and data are expressed as means ± SEM of TNFα/IFNγ–induced chemokine production. Statistical analysis was performed using one-way ANOVA. *P < 0.05, **P < 0.01

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Since the effect of the p38MAPK inhibitor was seen only at the highest concentration, we wanted to exclude a cytotoxic action of this compound. We tested the cytotoxicity of the p38 inhibitor by MTT assays and showed that it didn't affect the viability of the cells (**Figure 4.10**).
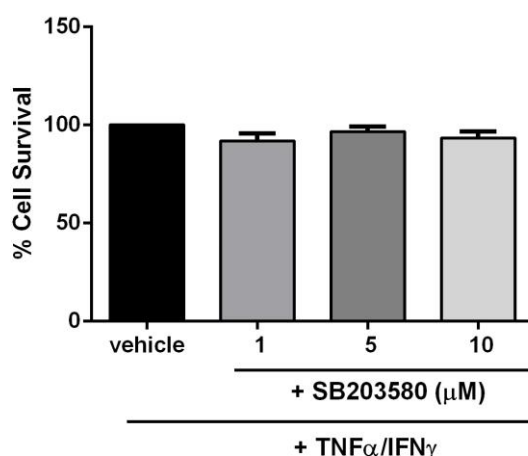
Figure 4.10: Cytotoxic effect of SB203580 in ASM cells stimulated with TNFα and IFNγ. Cells from 3 donors (2 healthy and 1 asthmatic) were treated with different concentrations of SB203580 (1 µM, 5 µM, 10 µM), followed by stimulation with TNFα (10 ng/ml) and IFNγ (25 ng/ml) and assayed by MTT. Statistical analysis was performed using One-way ANOVA with Bonferroni's post-hoc test.

4.3.6 Effect of p38 inhibitor (SB203580) on sensitivity to fluticasone treatment in ASM cells treated with TNFα and IFNγ

Further work looked at whether SB203580 could modulate the sensitivity of ASM cells to fluticasone that was lost when cells were treated with a combination of TNFα and IFNγ. Although the inhibitor on its own had little or no significant inhibitory effect on the production of cytokine-induced chemokines (**Figure 4.11A-C**), the presence of both SB203580 and fluticasone led to a dramatic inhibition of CCL5 by 51.17%±4.4 at 1µM, 51.61%±3.7 at 5µM and 48.56%±2.2 at 10µM (**Figure 4.11A**). A

similar effect, albeit less pronounced, was observed on the production of CXCL10 at 1μM which was reduced by 18.92%±6.24 and on the production of CX3CL1 at 10μM which was reduced by 34.95%±4.573 (**Figure 4.11B-C**). It is also worth mentioning that the effect of fluticasone combination with the p38 inhibitor appeared to have a less dramatic inhibitory effect on CX3CL1 production, compared to using the inhibitor alone which was effective at both 5 and 10μM (**Figures 4.9C** and **4.11C**).
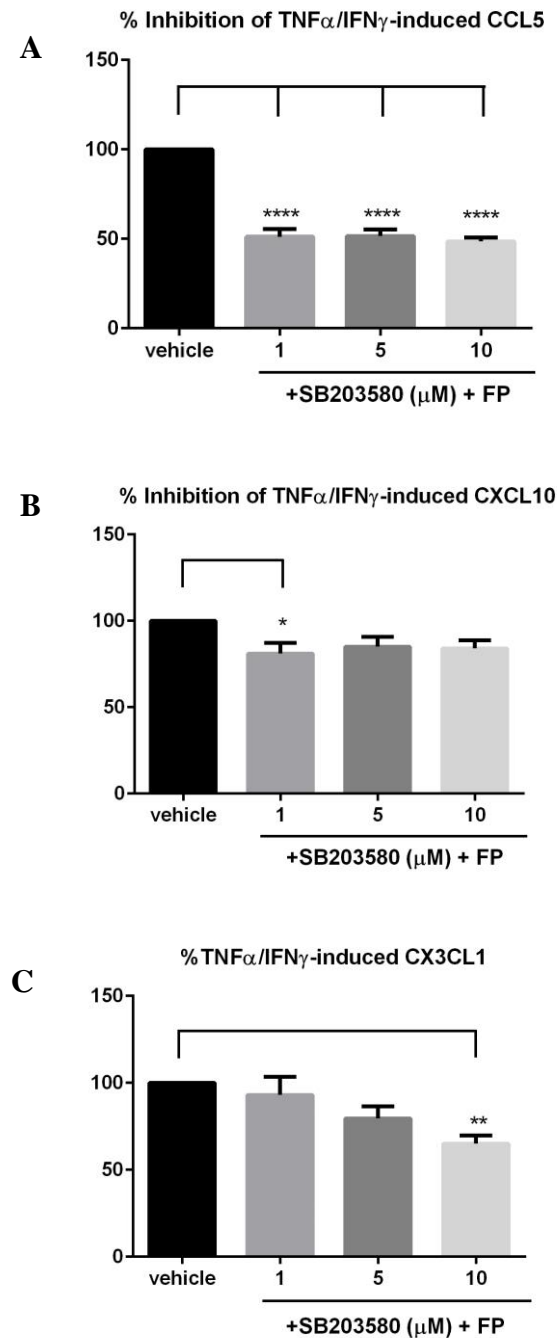
Figure 4.11: Effect of SB203580 in combination with fluticasone on the production of TNFα/IFNγ-induced CCL5, CXCL10 and CX3CL1. CCL5 (**A**), CXCL10 (**B**) and CX3CL1 (**C**) production was assessed by ELISA in cells from 5 donors (4 healthy and 1 asthmatic) treated with different concentrations of the p38 inhibitor (1-10μM) and fluticasone (FP) (100nM) and stimulated with TNFα (10ng/ml) and IFNγ (25ng/ml) for 16 hours. Chemokine levels in the supernatants of each subject were performed in triplicate and data are expressed as means±SEM as % of TNFα/IFNγ–induced chemokine production. Statistical analysis was performed using one-way ANOVA. *P < 0.05, **P < 0.01, ***P<0.001, ****P<0.0001

4.3.7 Time course activation of SAPK/JNK by TNFα/IFNγ

As previously mentioned, in another study using ASM cells, JNK was shown to be activated by a combination of TNFα, IFNγ and IL-1β (411). Similar to our data, this study showed that the cytomix induced CXCL10 expression, which was halved when using JNK and NF-kB inhibitors. The authors also showed that both JNK isoforms P-54 and P-46 were rapidly phosphorylated following cytomix stimulation, however their activation was markedly reduced in asthmatic ASM cells compared to non-asthmatics. In addition, the JNK inhibitor SP600125 reduced the early P-54 phosphorylation more than P-46 phosphorylation in the asthmatic cells, while there was no difference in the non-asthmatic cells.

Western blot experiments showed a time course activation of JNK by TNFα and IFNγ. Similarly to p38, JNK activation occurred at an early time point (10 minutes) and decreased at later time points (0.5, 1, 2 and 4 hours) **(Figure 4.12).** It is important to mention that basal activation of JNK was observed in some donors. Due to the small number of samples used for these experiments it is hard to compare the phosphorylation patterns between asthmatics and healthy donors.
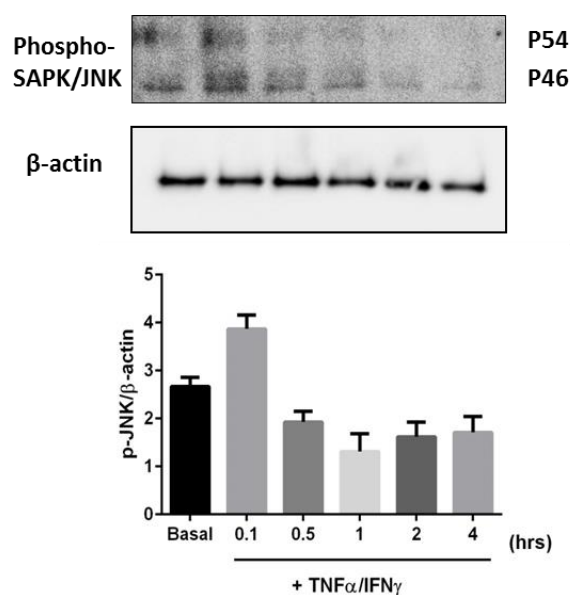
Figure 4.12: Phosphorylation of JNK by TNFα/IFNγ is time-dependent in ASM cells. Cells were stimulated with TNFα (10ng/ml) and IFNγ (25ng/ml) at different time points (0.1, 0.5, 1, 2, and 4 hours). Total cell lysates were assayed for p-JNK and β-actin by immunoblotting assays. Blot picture is representative of 1 donor (healthy). Lower panels represent means ± SEM of scanning densitometric measurements using ImageJ of p-JNK expression normalised over β-actin from n=3 (2 asthmatics and 1 healthy) donors. Statistical analysis was performed using one-way ANOVA. **P<0.01

4.3.8 Assessing the effect of CpdA on SAPK/JNK activation

Next, ASM cells were stimulated with TNFα and IFNγ for 10 min in the presence or absence of CpdA treatment (5μM) for 2 hours and total cell lysates were assayed for phospho-JNK (p-JNK) by immunoblotting. CpdA had no inhibitory effect on cytokine-induced JNK activation at 10 minutes (**Figure 4.13**). No significant differences were observed in the TNFα/IFNγ-induced JNK phosphorylation between healthy and asthmatics ASM cells. The effect of CpdA was also not significantly different between the two groups (**Figure 4.14**).
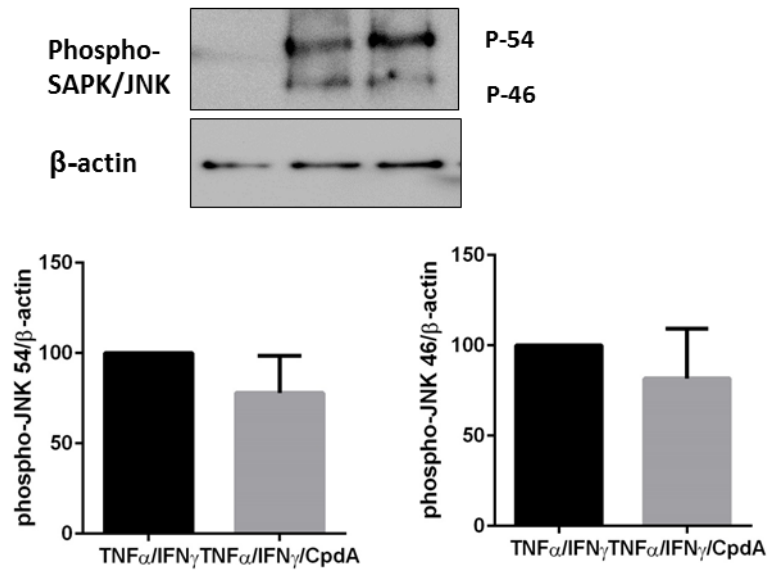
Figure 4.13: CpdA has no effect on phosphorylation of SAPK/JNK by TNFα/IFNγ in ASM cells. Cells from 6 donors (3 healthy and 3 asthmatics) were either left unstimulated, treated with TNFα (10ng/ml) and IFNγ (25ng/ml) for 10 min or pre-treated with CpdA (5μM) for 2 hours and then stimulated with TNFα and IFNγ for 10 min. Total cell lysates were assayed for p-JNK activation by immunoblotting. Blots are representative of 1 donor (asthmatic). Pictures were quantified using ImageJ as the ratio of each JNK phosphorylated site: P-54 **(bottom left)** and P-46 **(bottom right)** over β-actin and expressed as % of TNFα/IFNγ-induced activation. Statistical analysis was performed using student's paired t-test.

Figure 4.14: CpdA effect on phosphorylation of JNK by TNFα and IFNγ in healthy and asthmatic ASM cells. Cells from 3 healthy and 3 asthmatic donors were either left unstimulated, treated with TNFα (10ng/ml) and IFNγ (25ng/ml) for 10 minutes or pre-treated with CpdA (5μM) for 2 hours and then stimulated with TNFα and IFNγ for 10 minutes. Total cell lysates were assayed for p-JNK activation by immunoblotting. Pictures were quantified using ImageJ as ratio of p-JNK over β-actin and expressed as % of TNFα/IFNγ for the two phosphorylated sites (P-54 and P-46). Statistical analysis was performed using one way ANOVA.

4.3.9 Effect of JNK inhibitor (SP600125) on cytokine-induced production of CCL5, CXCL10 and CX3CL1

To determine the role of JNK pathways in driving steroid resistant chemokines, we used the known JNK inhibitor SP600125 (**Table 4.3**). The TNFα/IFNγ-induced production of CCL5 and CX3CL1 was inhibited by SP600125 with different potencies. Cytokine-induced CCL5 production was significantly decreased from 11115±1900 (vehicle treated cells), to 5501±1166 (5μM), and 3157±519.1 (pg/ml) (10μM) (**Figure 4.15A**). Cytokine-induced CX3CL1 production was reduced at higher concentrations of 10μM, from 3526±436.6 to 1805±95.94 (ng/ml) (**Figure 4.15C**). CXCL10 production induced by TNFα/IFNγ was not affected by using the JNK inhibitor (**Figure 4.15B**). The use of MTT assays revealed that SP600125 had no cytotoxic effect, with no changes observed in cell viability at the concentrations tested (**Figure 4.16**).
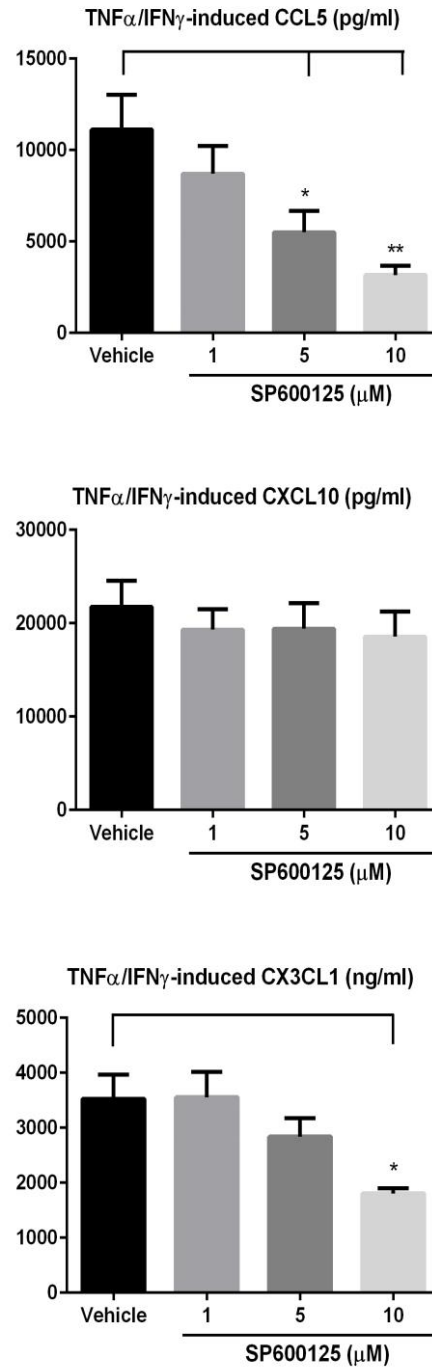
Figure 4.15: Effect of SP600125 on the production of TNFα/IFNγ-induced CCL5, CXCL10 and CX3CL1. CCL5 (**A**), CXCL10 (**B**) and CX3CL1 (**C**) production was assessed by ELISA in cells from 5 donors (4 healthy and 1 asthmatic) treated with different concentrations of the JNK inhibitor (1-10μM) and stimulated with TNFα (10ng/ml) and IFNγ (25ng/ml) for 16 hours. Chemokine levels in the supernatants of each subject were performed in triplicate and data are expressed as means ± SEM as % of TNFα/IFNγ–induced chemokine production. Statistical analysis was performed using one way ANOVA. *$P < 0.05$, **$P < 0.01$.

Figure 4.16: Cytotoxic effect of SP600125 in ASM cells stimulated with TNFα and IFNγ. Cells from 3 donors were treated with different concentrations of SP600125 (1μM, 5μM, 10μM), followed by stimulation with TNFα (10ng/ml) and IFNγ (25ng/ml) and assayed by MTT. Statistical analysis was performed using One-way ANOVA with Bonferroni's post-hoc test.

4.3.10 Time course activation of ERK by TNFα and IFNγ

This is the first study to show the activation and role of ERK in response to TNFα and IFNγ in ASM cells. Although a few studies have looked at ERK activation by TNFα alone in ASM cells (412), (413), (414), the only other study investigating its activation in response to TNFα and IFNγ combination was performed in human endothelial cells. The authors showed that a combination of both cytokines induced a rapid activation of ERK seen within 15 minutes. More importantly, blocking ERK activation using U0126 reduced CXCL10 production in response to TNFα and IFNγ (415).

Firstly, the time course activation of ERK following stimulation with TNFα/IFNγ was investigated. ASM cells were stimulated with both cytokines for different time points (0.1, 0.5, 1, 2 and 4 hours) before ERK phosphorylation was assessed in total cell lysates by Western Blotting. Phosphorylation of ERK was found

to be maximal at 10 minutes post-stimulation (**Figure 17**), which remained sustained up to 0.5-1 hours and decreased at later time points of 2-4 hours.
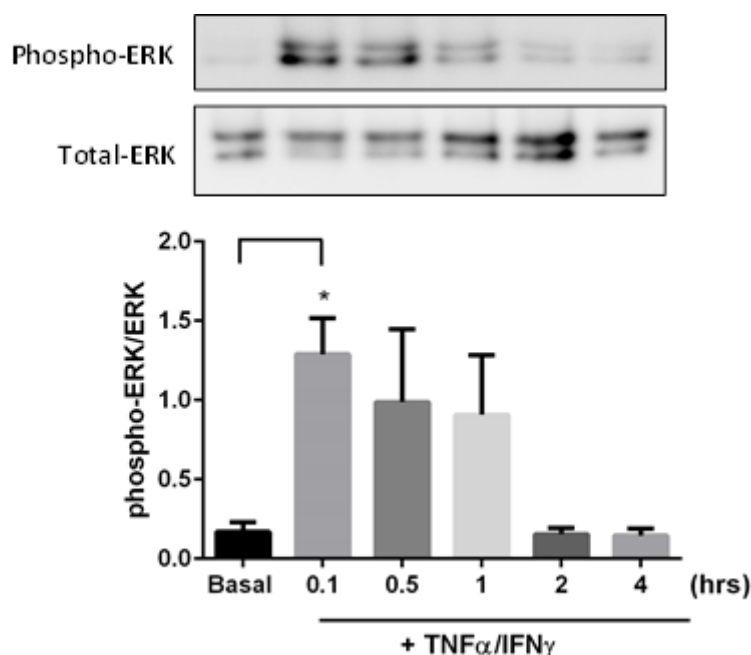
Figure 4.17: Kinetics of ERK phosphorylation by TNFα/IFNγ in ASM cells. Cells from 4 donors (3 asthmatics and 1 healthy) were stimulated with TNFα (10ng/ml) and IFNγ (25ng/ml) at different time points (0.1/0.5/1/2/4 hrs). Total cell lysates were assayed for p-ERK and total ERK by immunoblotting assays. Blot picture is representative of 1 donor (asthmatic). Lower panels represent means ± SEM of scanning densitometric measurements using ImageJ of p-ERK expression normalised over total ERK. Statistical analysis was performed using one-way ANOVA. *P<0.05.

4.3.11 Effect of CpdA on ERK activation by TNFα and IFNγ

Next, Western Blot experiments were performed to determine the potential effect of CpdA on cytokine-induced ERK activation. ASM cells were stimulated with TNFα/IFNγ for 10 minutes with or without CpdA pre-treatment and whole cell lysates were isolated for Western Blotting. Interestingly, there was an inhibitory effect of CpdA on ERK activation by TNFα/IFNγ (**Figure 4.18**).
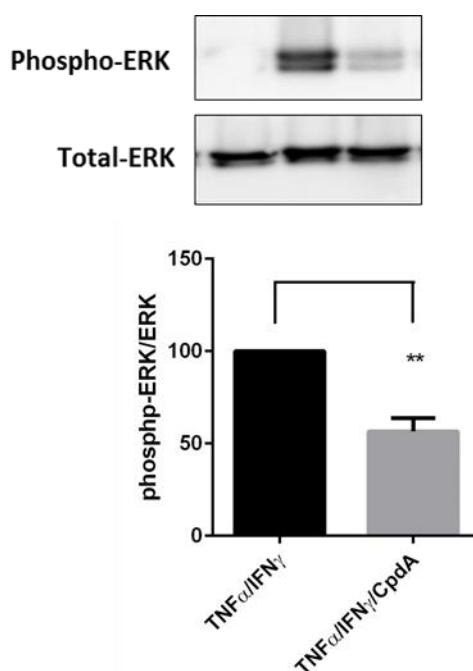
Figure 4.18: CpdA inhibits phosphorylation of ERK induced by TNFα and IFNγ in ASM cells. Cells from 6 donors (3 healthy and 4 asthmatics) were either left unstimulated, treated with TNFα (10ng/ml) and IFNγ (25ng/ml) for 10 minutes or pre-treated with CpdA (5μM) for 2 hours and then stimulated with TNFα and IFNγ for 10 minutes. Total cell lysates were assayed for p-ERK and total ERK expression by immunoblotting. Blot picture is representative of 1 donor (healthy). Lower panels represent means ± SEM of scanning densitometric measurements using ImageJ of % of TNFα/IFNγ–induced p-ERK normalised over total ERK. Statistical analysis was performed using the student's paired t test. **P<0.01.

When comparing the phosphorylation patterns of ERK upon stimulation with TNFα and IFNγ, there appeared to be increased activation in the asthmatics compared to healthy controls. Moreover, CpdA had a higher potency in inhibiting ERK activation in the healthy subjects compared to that in asthmatic controls, which could be explained by the increased ERK phosphorylation in the asthmatics (**Figure 4.19**). Increased immunostaining for phosphoERK1/2 was previously reported in asthmatic biopsies compared to healthy controls and activated ERK expression was observed particularly in the airway epithelium and ASM cells (416).
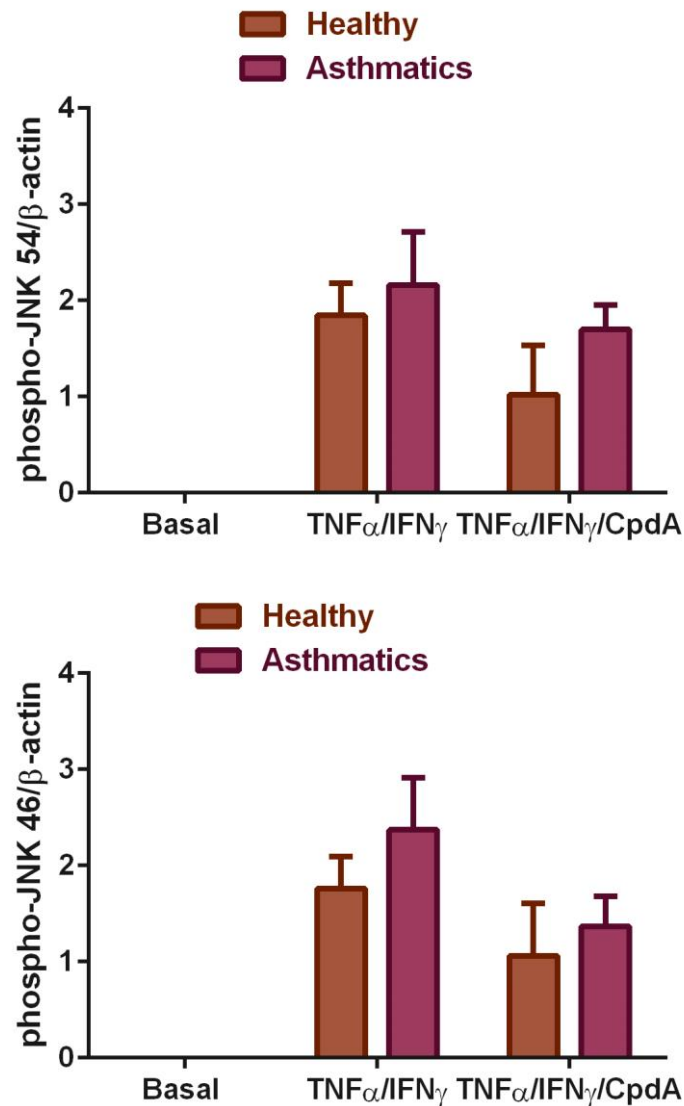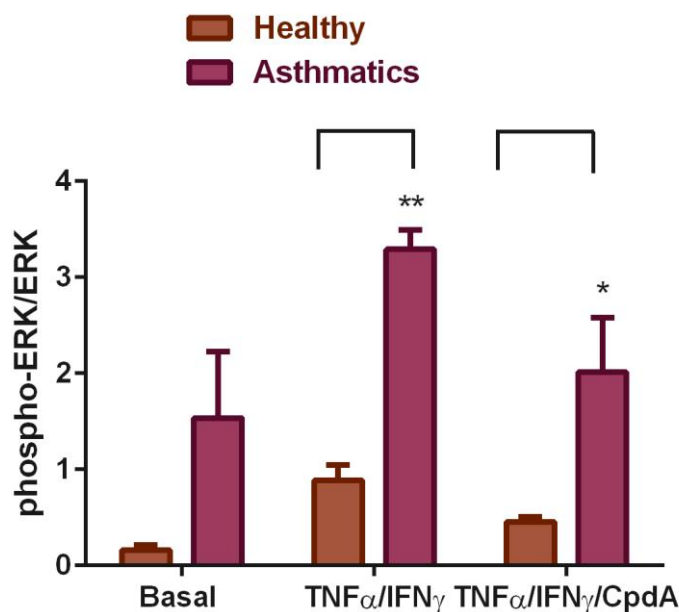
Figure 4.19: CpdA effect on phospho-ERK activation by TNFα and IFNγ in healthy and asthmatic ASM cells. Cells from 3 healthy and 3 asthmatic donors were either left unstimulated, treated with TNFα (10ng/ml) and IFNγ (25ng/ml) for 10 minutes or pre-treated with CpdA (5µM) for 2 hours and then stimulated with TNFα and IFNγ for 10 minutes. Total cell lysates were assayed for p-ERK and total ERK expression by immunoblotting. Data represent means±SEM of scanning densitometric measurements using ImageJ of p-p38 expression normalised over total p38. Statistical analysis was performed using one-way ANOVA.

4.3.12 Effect of the ERK inhibitor (U0126) on cytokine-induced production of CXCL10, CCL5 and CX3CL1

Contrary to a previous report showing an involvement of ERK in driving CXCL10 production in other cell types (415), in our study, CXCL10 production was not affected by the ERK inhibitor U0126 (**Figure 4.20B**). Also the other 2 chemokines were unaffected, with an inhibition, although not significant, on CX3CL1 production by TNFα/IFNγ observed between 1µM and 10µM (**Figure 4.20A, C**).
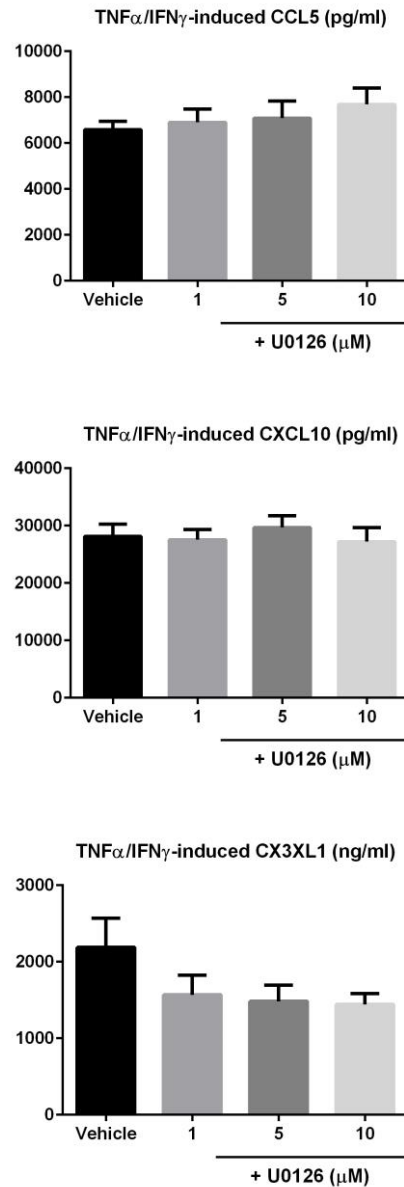
Figure 4.20: Effect of U0126 on the production of CCL5, CXCL10 and CX3CL1 by TNFα/IFNγ. CCL5 (**A**), CXCL10 (**B**) and CX3CL1 (**C**) production was assessed by ELISA in cells from 4 donors (all healthy) treated with different concentrations of the ERK inhibitor (1-10μM) and stimulated with TNFα (10ng/ml) and IFNγ (25ng/ml) for 16 hours. Chemokine levels in the supernatants of each subject were performed in triplicate and data are expressed as means ± SEM as % of TNFα/IFNγ–induced chemokine production. Statistical analysis was performed using one way ANOVA with Bonferroni's post-hoc test.

4.3.13 Effect of ERK inhibitor (U0126) on sensitivity to fluticasone treatment in ASM cells treated with TNFα and IFNγ

We next investigated the potential of the ERK inhibitor to modulate cell sensitivity to fluticasone in steroid resistant conditions (cells stimulated with TNFα and IFNγ). The presence of both U0126 and fluticasone led to the inhibition of CCL5 production by cytokine combination, by $31.25\% \pm 2.1$ at 1μM, $27.01\% \pm 5.54$ at 5μM and $22.95\% \pm 4.39$ at 10μM (**Figure 4.21**). Interestingly, in contrast, U0126 in combination with fluticasone led to an increase in the production of cytokine-induced CX3CL1, significant at 1μM. There was no effect on cytokine-induced CXCL10 production using this combination. Finally we tested against a cytotoxic effect of U0126 and showed that the ERK inhibitor did not affect cell viability by MTT assays (**Figure 4.22**).
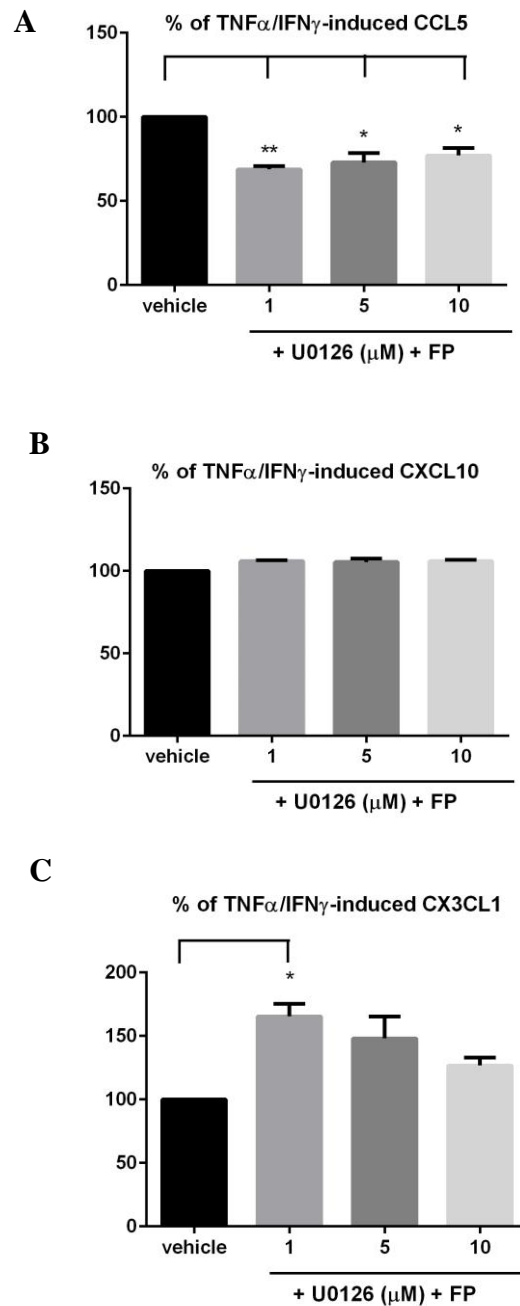
Figure 4.21: Effect of U0126 in combination with fluticasone on the production of CCL5, CXCL10 and CX3CL1 induced by TNFα/IFNγ. CCL5 (**A**), CXCL10 (**B**) and CX3CL1 (**C**) production was assessed by ELISA in cells from 4 donors (all healthy) treated with different concentrations of the ERK inhibitor (1-10μM) and fluticasone (FP) (100nM) and stimulated with TNFα (10ng/ml) and IFNγ (25ng/ml) for 16 hours. Chemokine levels in the supernatants of each subject were performed in triplicate and data are expressed as means±SEM as % of TNFα/IFNγ–induced chemokine production. Statistical analysis was performed using one way ANOVA with Bonferroni's post-hoc test. *P < 0.05, **P < 0.01
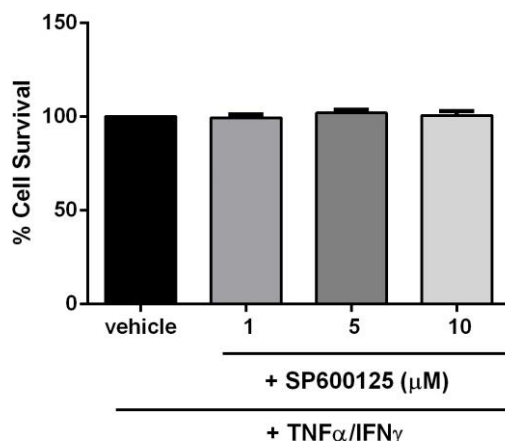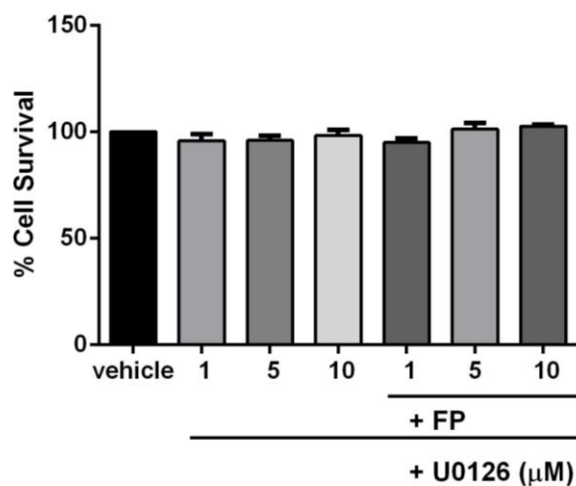
Figure 4.22: Cytotoxic effect of U0126 in ASM cells stimulated with TNFα and IFNγ. Cells from 3 donors were treated with different concentrations of U0126 (1μM, 5μM, 10μM) with or without pre-treatment with fluticasone (100nM), followed by stimulation with TNFα (10ng/ml) and IFNγ (25ng/ml) for 16 hours and assayed by MTT. Data are expressed as means±SEM as % of cell survival. Statistical analysis was performed using One-way ANOVA with Bonferroni's post-hoc test.

4.4   DISCUSSION

This chapter provides novel findings to support the implication of the MAPK signalling pathway in the production of fluticasone-resistant chemokines in our cellular model. Firstly, the finding that CpdA was able to transiently up-regulate MKP-1 at both mRNA and protein levels has functional relevance. Experiments using small interfering RNA (siRNA) showed that knocking down MKP-1 (**Figure 4.3**) completely prevented CpdA from inhibiting cytokine-induced CXCL10 production (**Figure 4.4**) but had no effect on CCL5 (**Figure 4.5A**) and CX3CL1 (**Figure 4.5B**). These data suggest that MKP-1 up-regulation by CpdA is playing a key role in inhibiting cytokine-induced CXCL10 in ASM cells. These data also define an extra mechanism by which MKP-1 up-regulation has anti-inflammatory properties in ASM cells. A few other mechanisms employing MKP-1 have been described and have been summarised in **Table 4.2**. For example dexamethasone was shown to inhibit the expression of the inflammatory chemokine growth-related oncogene protein-α (GRO-α) via the induction of MKP-1 (417). Also, MKP-1 up-regulation was critical in the suppressive effect of dexamethasone and fluticasone on TNFα-induced IL-6 production. This was via the inhibition of p38MAPK which affected IL-6 mRNA stability (389).

These findings are extremely important in the search for strategies aimed at harnessing the endogenous MAPK inhibitor MKP-1. Since the induction of MKP-1 is transient, other approaches have been suggested and involve the design of specific molecules that block the proteosomal degradation of MKP-1, such as MG132. However the potential clinical benefit of these molecules is still being investigated (200).

Interestingly, the only kinase directly inhibited by CpdA in cells stimulated with TNFα/IFNγ was ERK. A role of ERK in driving steroid insensitive pathways is supported by studies showing that activation of the MEK/ERK pathway by superantigen

or T-cell co-stimulation may drive a loss of GRα function (204, 287, 418). Our study is the first one to show ERK activation by TNFα/IFNγ in ASM cells. Another report showed that ERK was involved in CXCL10 expression by TNFα/IFNγ but in human endothelial cells. In this study they used a novel PPARγ ligand, rosiglitazone (RGZ), which inhibited ERK activation, with an effect similar to CpdA. Moreover, CXCL10 production was further reduced when RGZ was used in combination with U0126, suggesting that this ligand also exerted an inhibitory effect on other pathways involved in CXCL10 stimulation (i.e. NF-kB) (415). We propose that this could also be the case for CpdA. In contrast to our study, another report showed a partial involvement of JNK in CXCL10 production, although ASM cells were stimulated with IL-1β in addition to the TNFα/IFNγ combination. Furthermore, another group pointed out some contradicting observations showing that JNK inhibition had no effect on CXCL10 production from HRV-16-infected epithelial BEAS-2B cell lines however it had a significant inhibitory effect in human bronchial epithelial cells. This adds to the complexity of the mechanisms involved, showing that there may be different pathways involving posttranscriptional or posttranslational mechanisms (419). This finding also supports the idea that different stimuli and the cell types used will impact on the involvement of the different kinase pathways.

It this study it was surprising to notice that despite the inhibitory effect of CpdA on ERK activation by TNFα/IFNγ, the ERK inhibitor had no effect on CXCL10 production by the same cytokine stimulation. Although this finding does not support the implication of ERK in the inhibitory action of CpdA via induction of MKP-1, a few explanations can be drawn from our study design and from the literature. First of all it is important to mention that the ERK pathway is composed of two kinases ERK1/2 and ERK5 and U0126 is able to inhibit both. It would be interesting in further studies to

investigate which of these pathways was inhibited by CpdA and whether this was important in regulating TNFα/IFNγ-induced CXCL10 expression (420). Furthermore, the mechanism of ERK inhibition using U0126 does not fully recapitulate the deactivation of the ERK pathway by MKP-1. While MKP-1 is a dual-phosphatase that dephosphorylates both phosphotyrosine and phosphothreonine residues on ERK to inactivate it (421), U0126 is rather a selective inhibitor of MEK1 and MEK2 (420), both molecules upstream of ERK which prevent its activation. Also, there is a feedback loop by which ERK was shown to act back on MKP-1 to induce its expression and stabilise its protein against degradation as shown in RAW 264.7 macrophages stimulated with LPS (422). Surprisingly this finding was not replicated in ASM cells where ERK did not contribute to the up-regulation of MKP-1 by MG-132 (200). Although we showed that CpdA inhibits the phosphorylation of ERK by TNFα/IFNγ, it is possible that CpdA may have a different effect of ERK activity or the downstream signalling cascade. The mechanisms of diminished ERK activation in the presence of CpdA may involve the interactions of this kinase with its downstream target proteins, the direct effects of CpdA on the downstream proteins or indirect effects on the other kinases. For example, threonine 168 has been identified as a third phosphorylation site targeted by ERK and it would be interesting to determine whether phosphorylation at this new residue plays a role in TNFα/IFNγ-stimulated MKP-1 stabilization (423). In addition, although it appears that MKP-1 is required for inhibiting cytokine-induced CXCL10 production, there may also be MKP-1 independent mechanisms involved.

The reason why other MAPK pathways were not affected by CpdA is not clear and this area of research warrants additional studies. One question to address would be whether MKP-1 acts preferentially on certain MAPK and ultimately whether there are any other substrates, apart from p38, JNK and ERK on which MKP-1 acts as a

phosphatase. Answers to this question are given in studies looking at the binding of MKP-1 to the MAPK kinases. These reports have identified a kinase interaction motif (KIM) within the N-terminus of the MKPs, found to be responsible for their interaction (424), (425). It was interesting to notice that for MKP-1, the KIM was only required for interaction with ERK and p38, but not JNK as an MKP-1 mutant (with Arg53–55 substituted by Ala) failed to bind to either ERK2 or p38, but still interacted with and inactivated JNK both *in vitro* and *in vivo* just as efficiently as the wild-type protein (426). This alludes to the complexity and the differential binding of MKP-1 to the MAPKs.

Another novel observation using our cellular model was that inhibition of p38 and JNK activation led to marked suppression of cytokine-induced CCL5 and CX3CL1 production. Since no effect was observed on cytokine-induced CXCL10 production, it appears that the effect of MAPK inhibition is gene-specific. Other studies have looked at the implication of the MAPK kinases in the production of CCL5 and CX3CL1 in ASM cells, however under different stimulation conditions. For example, TNFα-induced CCL5 production was p38MAPK-dependent but ERK-independent (427). By contrast, IL-1β-induced CCL5 production was insensitive to p38MAPK inhibition but was reduced by inhibition of MKK1, a molecule upstream of ERK (428). When inhibitors for p38 and JNK were used together, an additive effect was observed on the production of CCL5 induced by platelet activating factor (PAF) (414), supporting the view that these pathways may be acting in parallel and in a synergistic manner. It would be interesting to also test this hypothesis in our model. In the case of CX3CL1, our finding replicates work by another group, showing that JNK and p38 inhibition significantly attenuated TNFα/IFNγ-induced CX3CL1 protein synthesis in ASM cells (334). There is also evidence from other cell types of the potential role of inhibiting

these pathways in steroid insensitive conditions. Previous reports clearly showed the relationship between p38MAPK and GC insensitivity especially in PBMCs, where inhibition of p38MAPK prevented steroid insensitivity induced by IL-2 and IL-4 (410). IL-13 also reduced steroid sensitivity via activation of p38MAPK in PBMCs from severe asthmatics (409). More recently, one study using PBMCs and alveolar macrophages showed that oxidants and LPS promoted the induction of histone 3 at serine 10 (H3-Pser10) which was not inhibited by dexamethasone. Reducing H3-Pser10 using the selective p38 MAPK inhibitor, SB239063, and the IKK-2 inhibitor, TPCA-1, was more effective than dexamethasone at controlling the expression of inflammatory mediators (IL-6, TNFα, CCL2 and CXCL8) in cells from severe asthmatics (429). In addition, the importance of p38 MAPK inhibition in the treatment of asthma has been highlighted in a mouse model of asthma demonstrating reduced inflammation and AHR in the lungs (188). Despite the beneficial anti-inflammatory effects, these initial *in vivo* studies also alerted to the potential cytotoxic effects in the liver of such inhibitors (430). As for JNK inhibition, similar beneficial effects were reported. JNK inhibition was shown to reduce ASM hyperplasia and inflammatory cytokine release in mice chronically exposed to allergens (431). Furthermore, in rhinovirus-infected epithelial cells, the combination of JNK and IKK2 inhibitors totally restored dexamethasone's ability to suppress IL-8 release, to induce MKP-1 gene expression, and to restore GRα nuclear translocation (432). An implication of JNK is also supported by earlier reports showing that in severe asthma there was an increase of bronchial mucosal c-jun and JNK phosphorylation in patients who were insensitive to high-dose therapy with prednisolone (433). The clinical relevance of using p38MAPK and JNK inhibitors in the treatment of pro-asthmatic responses is currently being tested in clinical trials. p38MAPK inhibitors are also being tested for the treatment of steroid insensitive

conditions but it is widely acknowledged that more studies are necessary to fully assess their safety and efficacy.

Another strategy used in this study was to investigate the potential enhancing effect of using GCs in combination with MAPK inhibitors. Firstly this study made the novel observation that fluticasone used in combination with the p38 inhibitor SB203580 reduced the production of GC-resistant chemokines with different potencies. SB203580 used in combination with fluticasone, reduced cytokine-induced CCL5 production by 48.83% at the lowest concentration of 1μM, compared to 12.53% when using the inhibitor alone. At the same concentration, CXCL10 production, which was not affected by the inhibitor alone, was also reduced by 18.92% when fluticasone was added. These findings suggest that combining p38MAPK inhibitors and GCs may represent a good therapeutic strategy to inhibit GC-insensitive genes in ASM cells. The mechanisms behind this enhanced effect are not really understood. It could be that fluticasone and the p38 inhibitor affect ligand-induced GRα phosphorylation, a process known to alter GC sensitivity since it regulates GRα nuclear translocation and decreases GRα transcriptional activity. A previous study indicated that p38MAPK can differentially phosphorylate GRα at specific residues, in the absence or presence of fluticasone. In this study, treatment with SB203580 increased basal and fluticasone-induced GRα phosphorylation at ser211 by 50 and 30%, had no effect on ser226 and reduced phosphorylation at ser203 by 50 and 35%, respectively (387). One can hypothesize that, in our model of cytokine-induced GC insensitivity where GRα phosphorylation at ser211 is impaired, p38MAPK activity is actually playing a role in inhibiting ligand-induced GRα phosphorylation. Since Bouazza *et al* looked at the effect of p38MAPK on GRα phosphorylation in ASM cells in the absence of cytokine combination, it would be interesting to also look at whether p38MAPK activation by TNFα/IFNγ leads to a

dysregulation of GRα phosphorylation and to determine if the combined inhibitory effect of SB203580 with fluticasone on the production of CCL5, CXCL10 and CX3CL1 is due to restored GRα phosphorylation on ser211. p38MAPK was also shown to be key in GRα phosphorylation at ser226, inhibiting its function in PBMCs (409, 410). Furthermore, reports by other groups indicate that p38MAPK is not the only kinase that can influence GRα phosphorylation. For example, JNK was also shown to induce GRα phosphorylation at ser226 in human lung A549 cells (434), HeLa and COS-7 cells (435, 436). The controversial action of the different kinases on GRα phosphorylation may be explained by the use of different experimental conditions, such as using immune cells versus structural cells, transformed cells versus primary cells, and different stimuli.

The observation that there was an enhanced effect of ERK inhibition in combination with fluticasone on the production of TNFα/IFNγ-induced CX3CL1 is perhaps not surprising considering the evidence from another study showing that CX3CL1 production by TNFα/IFNγ was enhanced by another GC, dexamethasone, while the ERK inhibitor had no effect (437). The CX3CL1 up-regulation by dexamethasone in ASM cells stimulated with TNFα/IFNγ was thought to be due to increased mRNA transcription (437) and probably fluticasone employs the same mechanism. It would be interesting to perform a parallel experiment in ASM cells stimulated with TNFα/IFNγ to compare the up-regulation of CX3CL1 by fluticasone alone and in combination with the ERK inhibitor to confirm if there are any differences in the magnitude of the responses.

To conclude, we expected to see differences in the extent of regulation of cytokine secretion achieved by CpdA, especially considering the stimulus-dependent differences in MAPK signalling pathways within ASM cells. This study revealed that MKP-1, as well as all three MAPK kinases have very specific effects on the regulation

of fluticasone-resistant chemokines. In addition to the findings from this study, a full understanding of how MAPK signalling is involved in steroid resistant pathways will require knowledge about the characterization of all directly phosphorylated substrates. Finally, the finding that MKP-1-independent pathways could be involved in the inhibition of fluticasone-resistant chemokines by CpdA is intriguing and the following chapter will aim to investigate this further.

CHAPTER 5

Role of IRF-1 in glucocorticoid resistance in asthma

5.1   INTRODUCTION

ASM is a target and a source of different pro-asthmatic factors which contribute to the orchestration and perpetuation of chronic inflammation within the airways (438), (85), (439), (440). Of particular importance in the regulation of immune-modulatory functions of ASM is the interferon (IFN) signaling pathway. The two major components of the IFN signaling pathway are the Janus tyrosine kinases (JAKs) and the signal transducers and activators of transcription (STATs) factors (441). STATs interact with various transcription factors to regulate gene transcription, including the transcription factor Interferon Regulatory Factor-1 (IRF-1). This factor is of particular importance to our study and its relevance in asthma pathogenesis and potential implication in corticosteroid resistance will be discussed in the following sections.

IRF-1 is able to regulate many immuno-modulatory genes by physically binding and activating DNA binding elements called Interferon-Sensitive Response Elements (ISRE) that are normally recognized by STAT1/STAT2 heterodimers (442), (443), (444). This strongly suggests that the joint activation of IRF-1 and STATs by different types of cytokines may represent a key mechanism to regulate an overlapping set of pro-inflammatory genes (e.g. VCAM-1, inducible nitric oxide synthase (iNOS), IL-12, IL-15, COX 2, angiotensin II type 2 receptor, and IL-1β converting enzyme (445), (446). IRF-1 can be induced by viral infection, leukocyte inhibitory factor, and LPS (447), (435). The first evidence of a role of IRF-1 in asthma comes from one study showing increased IRF-1 expression in the airway epithelium of asthmatic patients, compared to healthy controls and this correlated with STAT1 up-regulation (448). The authors proposed that the constitutive activation of STAT1 induced overexpression of a subset of immuno-regulatory genes, including IRF-1, leading to persistent airway inflammation. Although the exact role of IRF-1 was not elucidated, it was proposed that

it may drive the expression of inducible nitric oxide synthase (iNOS) in airway epithelial cells. This suggestion was based on evidence from a previous study showing that macrophages from IRF-1 knockout mice produced little or no nitric oxide (NO) and synthesized barely detectable iNOS in response to stimulation with IFNγ and LPS. Moreover, the iNOS promoter had two adjacent IRF-1 response elements which resulted in more severe infection in knockout mice infection with Mycobacterium bovis (BCG) compared to wild type (449).

Our lab has provided the initial observations describing the role of IRF-1 in ASM cells. Short exposure of human ASM cells to TNFα/IFNγ led to the synergistic expression of different pro-asthmatic genes, including CD38, CXCL10, CX3CL1 and CCL5 but also to impaired cell sensitivity to GCs (243, 364, 450, 451). One proposed mechanism for this synergic induction of inflammatory genes was the enhanced activation of the JAK/STATs pathway, in part via the autocrine secretion of IFNβ and the enhanced activation of transcription factors such as IRF-1, STAT1 and STAT2 (364). The inhibitory effect of TNFα and IFNγ on GC signalling in ASM cells was shown to partially involve IRF-1, via the competition with GRα for the transcriptional coactivator GRIP-1, leading to decreased GC-induced transcriptional activity of anti-inflammatory genes such as MKP-1. Our group also showed that through this mechanism, IRF-1 was playing a key role in the synergistic expression of pro-inflammatory genes such as CD38 (451) by promoting its transcriptional activation (362).

The aim of this chapter was to elucidate whether IRF-1 was playing a central role in the transcription of GC-resistant genes and whether inhibiting IRF-1 was a mechanism by which CpdA suppressed the expression of fluticasone-resistant genes. In addition, expression of IRF-1 was examined *in vivo* in biopsies from severe asthma

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patients. Studies from other cell types and mouse models are supporting a role of IRF-1 in modulating asthma-relevant immune pathways, as summarized in **Table 5.1.**

Table 5.1: IRF-1 modulation of asthma-relevant immune pathways

Cell type	Finding	References
Airway epithelial cells	EGFR activation by viruses (influenza, rhinovirus, RSV) reduced IRF-1 dependent CXCL10 production.	(452)
	HRV-induced CXCL10 transcription depends on activation of NF-κB and IRF-1.	(453)
	NO inhibits HRV-16-induced production of CXCL10 by inhibiting viral activation of IRF-1.	(454)
Human bronchial epithelial cells (HBECs)	LPA inhibits TNFα/IFNγ-induced CCL5 production by blocking the binding of IRF-1 to the CCL5/RANTES promoter via the $G_{(i)}$ receptor and PI3K activation.	(455)
Human ASM cells	Accumulation of IRF-1 decreased GRα transactivation via depletion of GRIP-1 from the GR transcriptional regulatory complex.	(362)
	IRF-1 mediates steroid dysfunction induced by TNFα/IFNγ.	(255)
Macrophages	IFNγ–induced IRF-1 protein expression is reduced in smokers as compared with non-smokers	(456)

Moreover, population studies have investigated IRF-1 gene polymorphisms and their associations with asthma features such as IgE and atopy. These studies revealed that polymorphisms in IRF-1 gene were associated with an increased risk of childhood asthma however with sometimes contradictory results depending on the population studied. In a Taiwanese population, the relationship between asthma and polymorphisms in IFNγ and IRF-1 was investigated in 348 subjects (adults and children). This revealed that polymorphisms in IFNγ (CA repeats) and IRF-1 (GT repeats) influenced the risk of childhood asthma (457). Using a similar approach, another study showed that polymorphisms in the IRF-1 gene were less likely to play a substantial role in the development of atopy and asthma in a Japanese population (458).

Furthermore, in a sex-stratified linkage analysis in an European cohort, a male-specific linkage polymorphism was detected on the 5q31.1 chromosome locus which includes IRF-1, and this correlated with lung function (FEV1/Height^2) and allergen polysensitization (459). EVE Asthma Genetics Consortium used similar genome-wide scans for genotyping sex interactions and revealed that the most significant association was at the IRF-1 locus at the same region (5q31.1), which included single-nucleotide polymorphisms previously associated with asthma. This suggests that the IRF1 locus is a strong candidate region for male-specific asthma susceptibility (460). Finally, IRF-1 was shown to correlate with IgE regulation and atopy (461), strongly supporting its role in asthma pathogenesis.

5.2   AIM

Evidence from our lab reported that IRF-1 is a novel player involved in the expression of some GC-resistant proasthmatic proteins such as CD38 (255). Whether IRF-1 also regulates the expression of other GC resistant chemokines and mediates the inhibitory effects of CpdA is not known. This chapter aimed to investigate a potential role of IRF-1 in driving steroid insensitive genes in ASM cells. The specific questions we addressed were as follows:

(1) Does CpdA have an effect on IRF-1 activation by TNFα/IFNγ?

(2) Does CpdA have an effect on IRF-1 nuclear translocation by TNFα/IFNγ?

(3) Is CpdA acting at transcriptional or post-transcriptional level?

(4) Does IRF-1 regulate the expression of corticosteroid resistant-chemokines?

(5) Is IRF-1 expressed in biopsies from healthy and severe asthma patients (pre and post treatment with GCs)?

(6) Does IRF-1 expression correlate with any clinical parameters?

5.3   RESULTS

5.3.1   Kinetics of IRF-1 induction by TNFα/IFNγ

It was shown previously that IRF-1 is involved in mediating cytokine-induced GC insensitivity in ASM cells from healthy subjects (255, 362, 451). Here we showed that IRF-1 activation by TNFα and IFNγ occurs at 2 hours (0.6452±0.2211-fold) compared to basal levels (0.06779±0.01167-fold). The IRF-1 activation induced by cytokines remained sustained up to 5 (0.8241±0.1575) and 6 hours (1.045±0.05908-fold) (**Figure 5.1**).
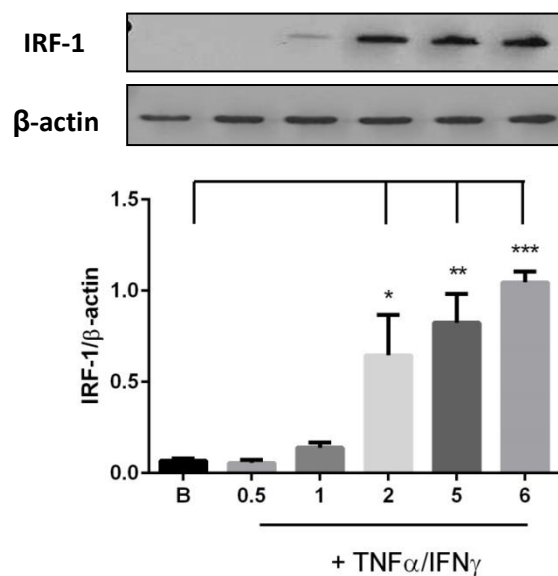
Figure 5.1: Time-course of IRF-1 activation following stimulation with TNFα and IFNγ. Cells from three donors (asthmatics) were stimulated with TNFα (10ng/ml) and IFNγ (25ng/ml) at different time points (0.5, 1, 2, 5 and 6 hrs). Total cell lysates were assayed for IRF-1 expression by immunoblotting. The nitrocellulose membrane was stripped and blotted again for β-actin. Blots are representative of one donor. Pictures were quantified using Image J and expressed as ratio of IRF-1 over β-actin (bottom graph). Statistical analysis was performed using one-way ANOVA with Bonferroni's post hoc test. *P<0.05, **P<0.01, ***P<0.001.

5.3.2 Activation of IRF-1 by TNFα and IFNγ is inhibited by CpdA

The effect of CpdA on cytokine-induced IRF-1 activation by TNFα and IFNγ was next examined. ASM cells were stimulated with TNFα and IFNγ for 2 hours, with or without pre-treatment with CpdA (5μM) and total cell lysates were assayed by Western Blotting. Cytokine-induced IRF-1 activation was found to be reduced by CpdA by more than 30% (34.6%±9) (**Figure 5.2**).
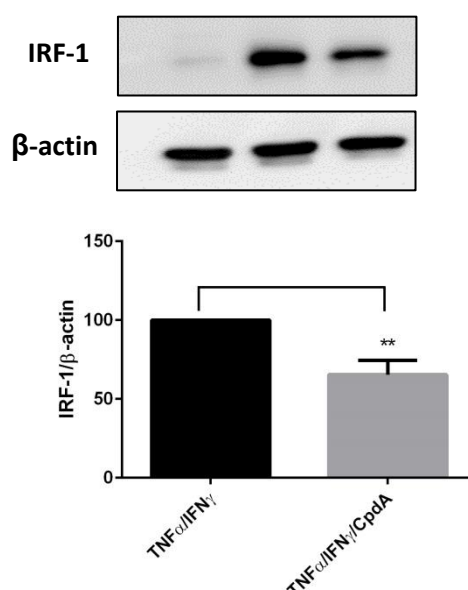
Figure 5.2: CpdA inhibits IRF-1 activation following stimulation with TNFα and IFNγ. Cells from 12 donors (5 healthy and 7 asthmatics) were either left unstimulated (basal), stimulated with TNFα (10ng/ml) and IFNγ (25ng/ml) for 2 hours or pre-treated with CpdA (5μM) and then stimulated with TNFα and IFNγ for 2 hours. Total cell lysates were assayed for IRF-1 expression by immunoblotting. The nitrocellulose membrane was stripped and blotted again for β-actin (bottom gel). Blots are representative of one donor. Pictures were quantified using Image J and expressed as % inhibition of TNFα/IFNγ-induced IRF-1 expression. Statistical analysis was performed using Student's paired t test. **P<0.01.

5.3.3 CpdA does not inhibit cytokine-induced IRF-1 induction by acting at a transcriptional level

We next examined whether the inhibitory effect of CpdA on IRF-1 activation occured at a transcriptional level, by looking at the effect on mRNA expression. Similar with the previous experiment, ASM cells were stimulated with TNFα and IFNγ for 2 hours, with or without pre-treatment with CpdA (5μM) and total RNA was isolated and used for cDNA synthesis and ultimately for RT-PCR. Surprisingly, CpdA had no inhibitory effect on cytokine-induced IRF-1 mRNA expression (**Figure 5.3**).
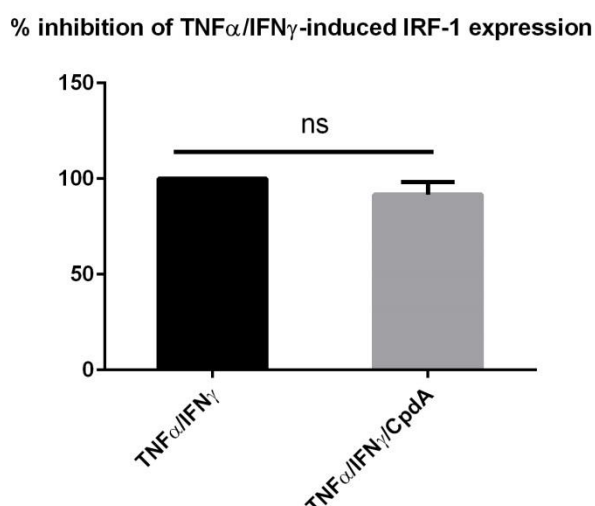
Figure 5.3: CpdA has no effect on IRF-1 mRNA expression following stimulation with TNFα and IFNγ. Cells from 5 donors (4 asthmatics and 1 healthy) were either left unstimulated (basal), stimulated with TNFα (10ng/ml) and IFNγ (25ng/ml) for 2 hours or pre-treated with CpdA (5μM) and then stimulated with TNFα and IFNγ for 2 hours. Total RNA was extracted for real-time quantitative PCR. Results are expressed as percentage inhibition of TNFα/IFNγ-induced IRF-1 expression by calculating the negative inverse of the ΔΔCt value. β-actin was used as a house-keeping gene. Statistical analysis was performed using Student's paired t test.

5.3.4 CpdA modulates IRF-1 expression by affecting mRNA stability

Actinomycin D chase experiments were designed to investigate whether CpdA affects IRF-1 mRNA stability. Actinomycin D is an RNA polymerase inhibitor that prevents synthesis of new mRNA transcripts. Growth-arrested ASM cells were treated with vehicle or CpdA for 2 hours (5μM) and TNFα and IFNγ for 2 hours. Cells were then washed and incubated with actinomycin D (5μg/ml) to inhibit further transcription (389). Total RNA was extracted following 0, 0.5, 1, 2, 4 and 6 hours incubation with actinomycin D and IRF-1 mRNA expression was quantified by real-time RT-PCR. Results are presented as % mRNA remaining (i.e. in comparison to steady state levels of mRNA expression following 2 hours of treatment with TNFα and IFNγ) after actinomycin D treatment. CpdA was found to affect IRF-1 mRNA stability, significantly reducing its levels by 22.02% at 30 minutes, and 20.62% at 1 hour, an effect that was not present at later time points (**Figure 5.4**).
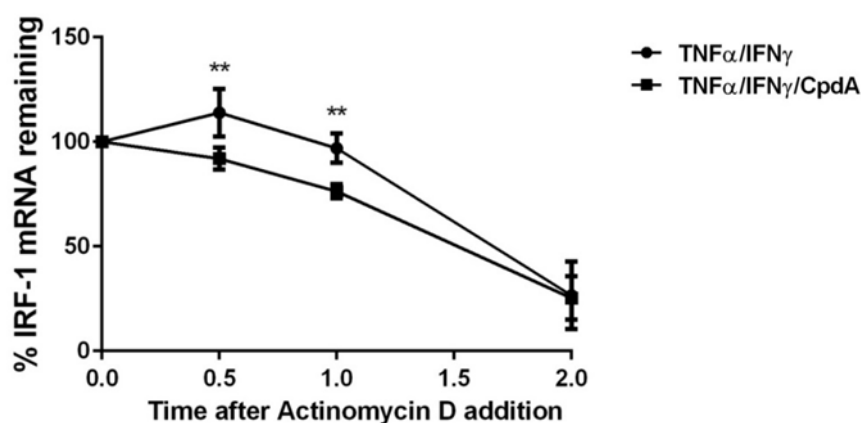
Figure 5.4: CpdA affects IRF-1 mRNA stability following stimulation with TNFα and IFNγ. Cells were either left untreated or pre-treated with CpdA (5μM) for 2 hours, followed by stimulation with TNFα/IFNγ for 2 hours. Actinomycin D was added after the 2 hours stimulation with TNFα/IFNγ and total RNA was extracted at different time points (0, 0.5, 1, 2, 4 and 6 hours). IRF-1 mRNA stability was measured by actinomycin D chase using real-time RT-PCR and results expressed as % mRNA remaining over time. Data are mean±SEM values from n=3 subjects (2 asthmatics and 1 healthy), performed in duplicates. Statistical analysis was performed using 2-way ANOVA with Bonferroni's post hoc test. **$P < 0.01$

5.3.5   CpdA inhibits TNFα and IFNγ induced IRF-1 nuclear translocation

Immunofluorescence staining was next performed to look at the effect of CpdA on IRF-1 nuclear translocation in response to TNFα/IFNγ (**Figure 5.5**). TNFα/IFNγ induced IRF-1 nuclear translocation which was inhibited in the presence of CpdA (5μM) by 68.3±14.7% when compared to non-treated cells (**Figure 5.5**) suggesting that in addition to a reduced expression of IRF-1 at the protein level (**Figure 5.2**), CpdA also interferes with IRF-1 movement to the nucleus.
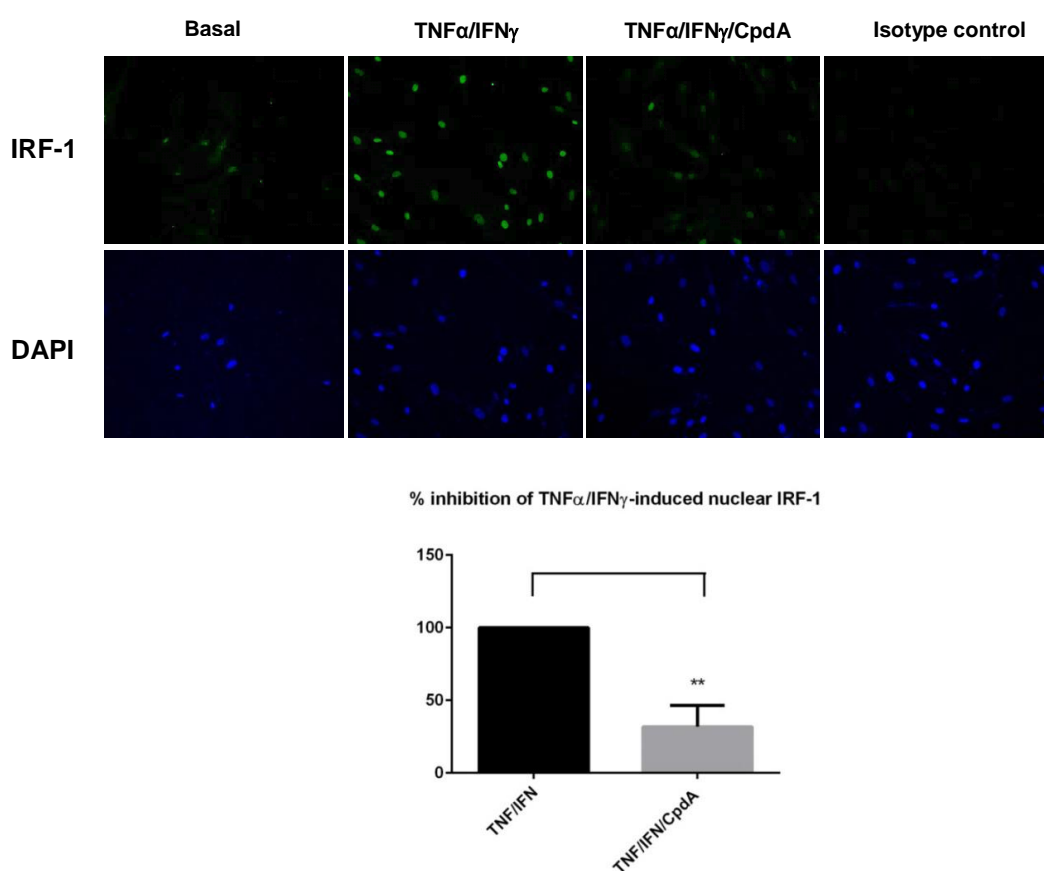
Figure 5.5: CpdA inhibits IRF-1 nuclear translocation following stimulation with TNFα and IFNγ. Cells from 7 donors (3 asthmatics and 4 healthy) were either left unstimulated, stimulated with TNFα (10ng/ml) and IFNγ (25ng/ml) for 2 hours or pre-treated with CpdA (5μM), and then stimulated with TNFα (10ng/ml) and IFNγ (25ng/ml) for 2 hours. An isotype control was used as negative control. 100 cells were analysed for each treatment and the intensity of the staining was measured using ImageJ. Data are expressed as means ± SEM. Statistical analysis was performed using Student's unpaired t test. **P<0.01.

5.3.6 IRF-1 regulates TNFα/IFNγ-induced CCL5 and CX3CL1 expression

As mentioned in the **Introduction** (**Section 5.1**), it was previously reported that IRF-1 is an important factor mediating the expression of some GC-resistant pro-asthmatic proteins such as CD38 (255). Whether IRF-1 also regulates the expression of GC resistant chemokines is not known. Therefore, silencing experiments were used to determine whether the inhibitory effect of CpdA on fluticasone-resistant chemokines was IRF-1-dependent. Following transfection of ASM cells with small interfering RNA oligonucleotides IRF-1 mRNA expression was successfully reduced by 58.7%±7.6 (**Figure 5.6**). We then investigated whether IRF-1 silencing had any effect on the expression of CCL5, CXCL10 and CX3CL1. We discovered that silencing IRF-1 had no effect on mRNA expression of CX3CL1 and CXCL10 (**Figure 5.7B-C**), however it resulted in significant suppression of CCL5 mRNA expression by 60%±7.3 (**Figure 5.7A**). This suggests that CCL5 inhibition by CpdA may occur via the inhibition of IRF-1, while inhibition of CX3CL1 and CXCL10 may rely on IRF-1-independent mechanisms (**Figure 5.7B-C**). We also looked at the effect of IRF-1 knockdown on chemokine expression at the protein levels by Western Blotting. Interestingly, IRF-1 knockdown also led to a suppression of TNFα/IFNγ–induced CCL5 and CX3CL1 at the protein level by 45.4%±5.1 (Figure 5.8A) and 47.1%±14.2 (**Figure 5.8C**), respectively. IRF-1 silencing had no effect on cytokine-induced CXCL10 (**Figure 5.8B**).
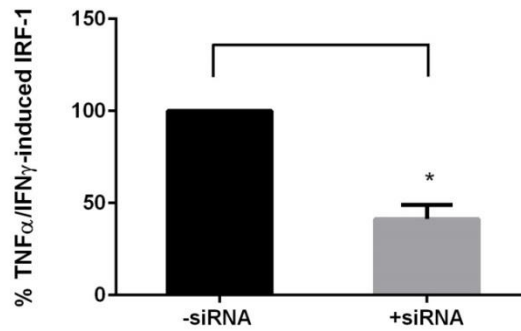
Figure 5.6: Silencing IRF-1 using small interfering RNA oligonucleotides. ASM cells from 3 donors (2 healthy and 1 asthmatic) were transfected with 300nM of nonsilencing small interfering RNA (siRNA) control or with 300 nM of Silencer Pre-designed siRNA IRF-1 oligonucleotides for 18 hours before TNFα (10 ng/ml) and IFNγ (500 IU/ml) were added for 2 hours. Total mRNA (500ng) was subjected to RT-PCR with IRF-1 and β-actin primers. Data are expressed as means±SEM and expressed as % of controls. Statistical analysis was performed using Student's unpaired t test. *P<0.05.
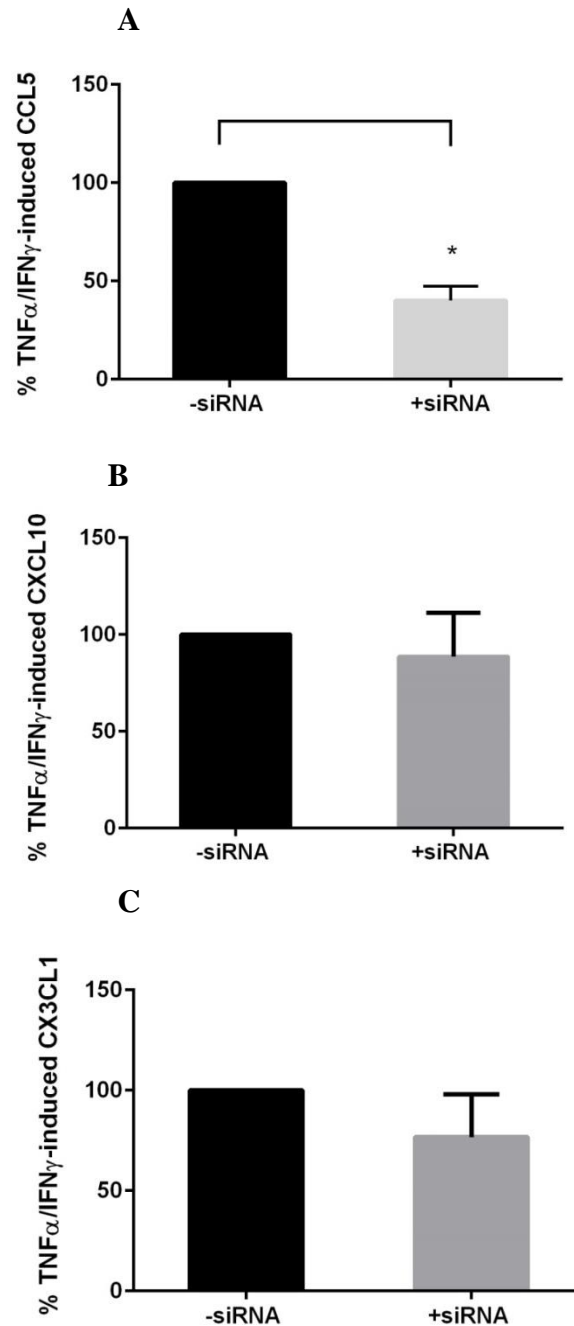
Figure 5.7: IRF-1 silencing reduces TNFα/IFNγ–induced mRNA expression of CCL5 but not CXCL10 and CX3CL1. Cells were transfected with Silencer Pre-designed small interfering RNA (siRNA) IRF-1 oligonucleotides or nonsilencing control scrambled siRNA (300nM). After transfection, cells were serum deprived and stimulated with TNFα/IFNγ for 2 hours. RNA was isolated and assayed for CCL5 **(A)**, CXCL10 **(B)**, and CX3CL1 **(C)** using real-time RT-PCR. Results are expressed as percentage mRNA remaining compared with controls (-siRNA). Data are means±SEM from 3 subjects (2 healthy and 1 asthmatic) performed in duplicate. Statistical analysis was performed using Student's paired t test. *P<0.05.

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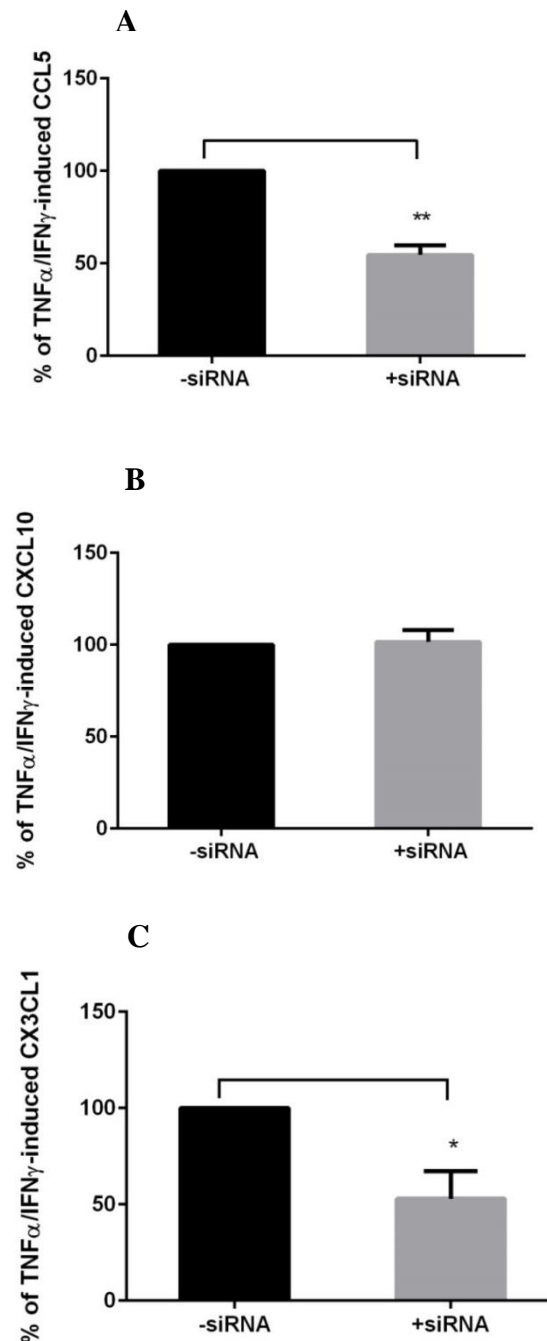
Figure 5.8: IRF-1 silencing reduces TNFα/IFNγ–induced protein expression of CCL5 and CX3CL1 but not CXCL10. Cells were transfected with Silencer Pre-designed small interfering RNA (siRNA) IRF-1 oligonucleotides or nonsilencing control scrambled siRNA (300nM). After transfection, cells were serum deprived and stimulated with TNFα/IFNγ for 2 hours. Chemokine levels of CCL5 (**A**), CXCL10 (**B**) and CX3CL1 (**C**) in the supernatants of scrambled (-siRNA) and IRF-1 siRNA-transfected cells incubated with TNFα/IFNγ for 24 hours were assessed by ELISA assays. Data are means±SEM from four donors (all healthy) performed in duplicate. Statistical analysis was performed using Student's paired t test. *P<0.05, **P<0.01.

5.3.7 IRF-1 is expressed within the ASM in human lung biopsies

Although the role of IRF-1 in driving GC resistance has been demonstrated *in vitro* using ASM cells, there are no studies that have examined whether IRF-1 is expressed in ASM tissues *in vivo*. To assess this we used glycol methacrylate (GMA) embedded bronchial biopsies from healthy subjects (n=8), severe asthmatics prior steroid treatment (n=14) and severe asthmatics post-steroid treatment (n=20). The clinical characteristics of healthy controls and subjects with severe asthma before and after steroid treatment are shown in **Table 5.2**. Subjects were classed as severe asthmatics according to the GINA guidelines (steps 4 and 5). Groups were well matched for age and smoking history. Subjects with severe asthma had impaired lung function, with decreased FEV1 and FEV1% predicted values, which did not improve following the 2 week treatment with oral prednisolone. PC_{20}, a marker of AHR, was also reduced in severe asthma and did not improve upon steroid treatment. IgE serum levels were elevated in severe asthma subjects compared to healthy controls. There was evidence of high eosinophilic inflammation as severe asthma patients had high sputum eosinophil counts which were reduced following steroid treatment. It was also interesting to observe the presence of high neutrophilic inflammation, with high neutrophil sputum counts in the severe asthmatics, which were not affected by steroid treatment.

Table 5.2: Demographics of patients used for immunohistochemistry experiments

	Healthy	Severe Asthma pre-treatment	Severe Asthma post-treatment
Number	8	14	20
Gender, F/M/unknown	1/6/1	1/10/3	4/11/5
Age (years)	42.25±4.712	48.07±2.885	47±2.57
FEV1	3.944±0.2953	**2.744±0.2249**	**2.845±0.2033**
FEV1 % predicted	107±2.244	**79.02±4.66**	N/A
FVC	4.994±0.344	4.075±0.2621	**3.975±0.2363**
ICS	0	**1446±670.4**	**1122±480.3**
Receiving oral corticosteroids (no.)	0	0	20
PC$_{20}$ (mg/ml)	>16	**4.977±1.877**	**5.707±1.684**
Serum IgE (IU/ml)	N/A	**1840±819.1**	N/A
Smokers (no.)	0	1	3
Ex-smokers (no.)	1	6	9
Non-smokers (no.)	7	7	8
Sputum eosinophils (%)	ND	**21.39±6.721**	1.876±0.4868
Sputum neutrophils (%)	ND	**48.38±9.207**	**58.69±7.321**

Data are expressed as means±SEM. Values in bold indicate significance when compared to healthy controls. The statistical tests used were: unpaired t-tests for comparing between 2 groups and ANOVA or the Kruskal-Wallis tests to compare between more than 2 groups; IU=International units

Sequential 3μm sections were cut from these bronchial biopsies and immunostained using a rabbit monoclonal anti-human IRF-1 (2μg/ml) antibody. The sections were stained for α-smooth muscle actin (α-SMA) (1μg/ml) and Nuclear Factor-1 (NF-1) (1μg/ml) as positive controls for airway smooth muscle staining and nuclear staining, respectively. The isotype control rabbit IgG (2μg/ml) was used as a negative control. The staining was performed using EnVision™ FLEX Mini Kit (DAKO) as described in the **Methods** section.

The immunohistochemistry revealed that ASM bundles expressed IRF-1 *in vivo* as evidenced by the marked DAB staining which was mainly localised within the nucleus. Nuclear staining within the sections was confirmed using the NF-1 antibody and no staining was observed when the rabbit IgG isotype control was used (Figure 5.9). Interestingly, positive IRF-1 staining was observed in all biopsies, in both healthy and severe asthmatics pre- and post-steroid treatment. Positive expression was also seen within the nuclei of epithelial cells.
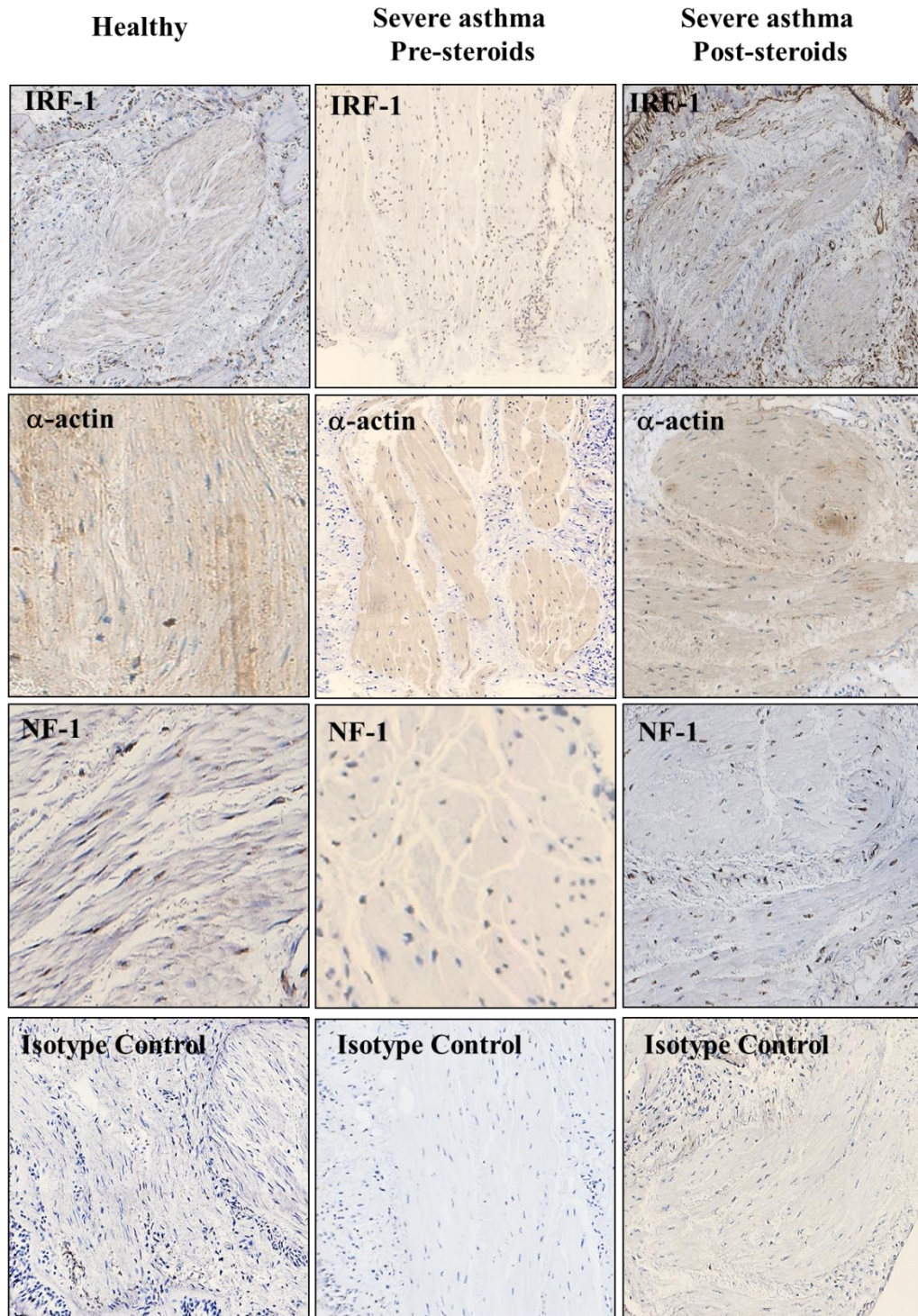
Figure 5.9: Immunohistochemistry staining of IRF-1, NF-1 and α-actin in human bronchial smooth muscle bundles. Bronchial biopsies in acetone fixed GMA embedded sections were stained with the following antibodies: rabbit monoclonal IRF-1 (2μg/mL), rabbit monoclonal α-actin (1μg/mL), rabbit monoclonal NF-1 (1μg/mL) and rabbit IgG isotypes (2μg/mL). Positive staining was visualised with DAB detection and hematoxylin counter stain. Magnification was at x20.

5.3.8 IRF-1 expression is upregulated in biopsies from severe asthma patients

The nuclear expression of IRF-1 within the ASM bundle was quantified with ImageJ analysis using the IHC Profiler. This open space plugin allows the quantitative evaluation and automated scoring of immunohistochemistry images of human tissue samples (462). In short, the ASM areas were delineated from the whole biopsy and the intensity of the DAB staining within the nuclear region was quantified as a % of the total ASM area (in pixels). Data analysis revealed that within the ASM layer, IRF-1 staining was significantly up-regulated in biopsies from subjects with severe asthma pre-steroid treatment, compared to healthy controls (3.07%±0.51, p= 0.0021 compared to 0.62%±0.15). More importantly, this up-regulation was maintained in subjects with severe asthma post-steroid treatment (2.681%±0.5720, p = 0.0341) (**Figure 5.10**).
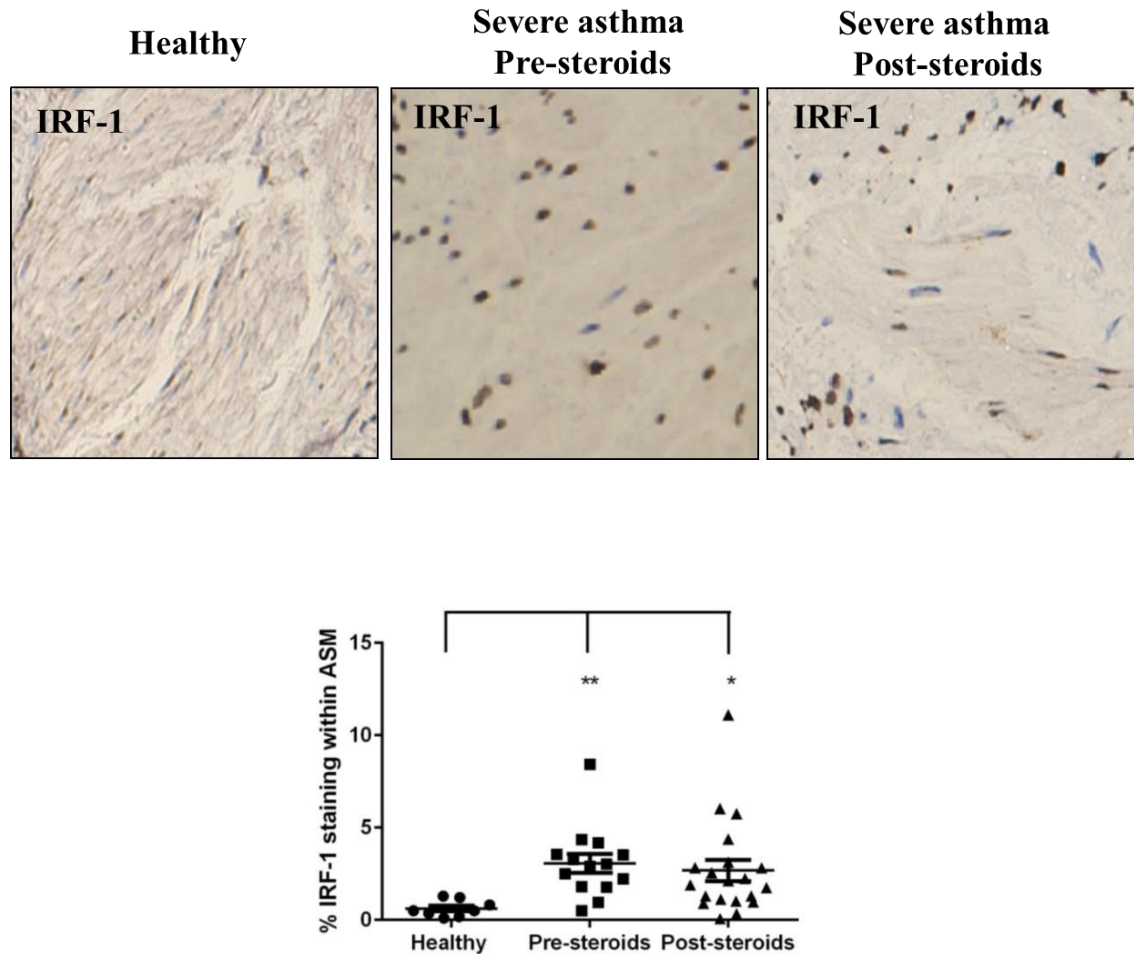
Figure 5.10: Expression of IRF-1 is upregulated in bronchial smooth muscle bundles from severe asthma patients compared to healthy controls. Bronchial biopsies were stained with the rabbit monoclonal IRF-1 antibody (2μg/mL) in acetone fixed GMA embedded sections. Positive staining was visualised with DAB detection and hematoxylin counter stain. Quantitative analysis was performed using ImageJ with the IHC Profiler plugin. Each point represents the % of nuclear IRF-1 DAB staining (pixels) within the whole ASM area (pixels). The upper photos represent higher magnification of IRF-1 nuclear staining, representative of one donor. Data are expressed as means±SEM from n=8 healthy donors, n=14 severe asthma pre-steroids and n=20 severe asthma post-steroids. Statistical analysis was performed using unpaired t tests to compare between the healthy and the two severe asthma groups and paired t tests were used to compare between the pre-steroids and post-steroids severe asthma groups. *P<0.05, **P<0.01.

We next investigated the clinical profile of the severe asthma patients in terms of their sputum eosinophil and neutrophil counts. Increased sputum eosinophil counts have been described in subjects with asthma and were associated with decreased asthma control and increased exacerbations (463). Treatment with ICSs was shown to decrease sputum eosinophilia and improve asthma control in most subjects, and sputum eosinophil counts above 3% were shown to respond to treatment (464), (211). Here we used a cut-off point of >3% to define high eosinophilic asthma. Previous research also indicates that increased neutrophils can correlate with asthma severity (465). A distinct subgroup of patients with mild to moderate asthma were shown to have predominantly neutrophilic airway inflammation and responded less well to treatment with inhaled corticosteroids (466). Most studies looking at abnormally high neutrophil counts have set a threshold between 61% to 93%, with some ranging from as low as 49% (467) to as high as 93% (468). Here we defined an abnormally high sputum neutrophil count as a percentage >61%. Moreover, studies looking at both clinical phenotypes and inflammatory cell counts identified four distinct sputum clusters: eosinophilic, neutrophilic, paucigranulocytic and mixed granulocytic asthma. These clusters were shown to cover the whole severity spectrum, from mild-to-moderate allergic asthma characterised by sputum eosinophilic inflammation to moderate-to-severe asthma with complex neutrophil predominant or mixed granulocytic inflammation. In this study however, the thresholds for sputum eosinophils and neutrophils were slightly less, >2% and >40% respectively (469).

We wanted to see whether IRF-1 expression correlated with these two groups of asthma patients, with either high eosinophilic or high neutrophilic, using the thresholds of >3% for sputum eosinophils and >61% for sputum neutrophils. We showed that severe asthma patients pre-steroid treatment were mostly eosinophilic (50%), and less

neutrophilic (14.28%), with a small percentage (14.28%) with both high eosinophil and neutrophil counts. Following steroid treatment, most patients shifted to being more neutrophilic (40%) and less eosinophilic (10%), with only 1 donor exhibiting both. We also noticed that overall, following treatment, the sputum eosinophil counts were reduced from 21.39%±6.721 to 1.876%±0.4868, while the sputum neutrophil counts slightly increased with treatment (48.38%±9.207 to 58.69%±7.321) (**Table 5.2**). This suggests that steroid treatment is able to reduce eosinophilia, while neutrophils are somewhat resistant to treatment. Although there was no change in the expression of IRF-1 between the pre- and post-steroid treated samples, there was a change in the profile of eosinophilic and neutrophilic inflammation. Also there was a trend of reduction in IRF-1 levels following steroid treatment, however not significant (**Figure 5.10**).
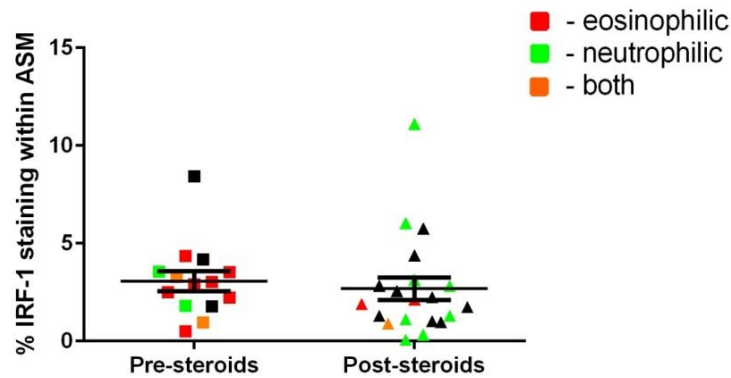
Figure 5.11: Distribution of high eosinophilic or high neutrophilic asthma. To define high eosinophilic and neutrophilic asthma we used a threshold of >3% sputum eosinophils and >61% sputum neutrophils. A higher proportion of severe asthma patients have high eosinophilic asthma pre-steroid treatment and this shifts to a high neutrophilic pattern following treatment. Each point represents the % of nuclear IRF-1 DAB staining (pixels) within the whole ASM area (pixels). Data are expressed as means±SEM from n=14 severe asthma pre-steroids and n=20 severe asthma post-steroids. Black represents patients with neither eosinophilic nor neutrophilic patterns.
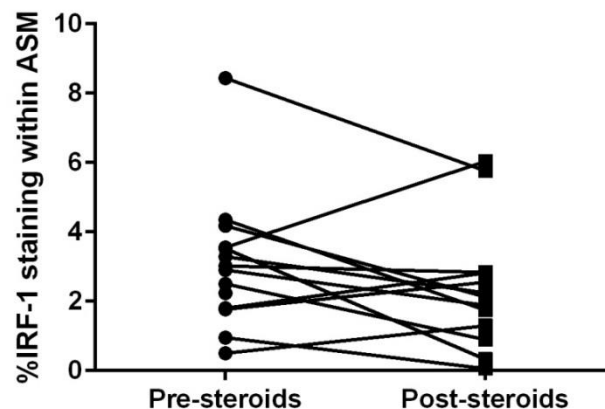
Figure 5.12: IRF-1 expression is insensitive to steroid treatment. Following treatment with steroids, IRF-1 expression was still present in biopsies from severe asthmatics. Each point represents the % of nuclear IRF-1 DAB staining (pixels) within the whole ASM area (pixels). Data is representative of n=13 severe asthma pre-steroids and n=13 severe asthma post-steroids. Statistical analysis was performed using a paired t test to compare between pre-steroid and post-steroid severe asthma groups.

We next looked at the correlations of IRF-1 expression with various clinical parameters known to drive pathological features of asthma. We did not see any correlations with markers of lung function such as FEV1, FEV1% predicted and FVC. However, IRF-1 expression within the ASM bundle was found to correlate positively with serum IgE levels pre-treatment with steroids (**Figure 5.13**).
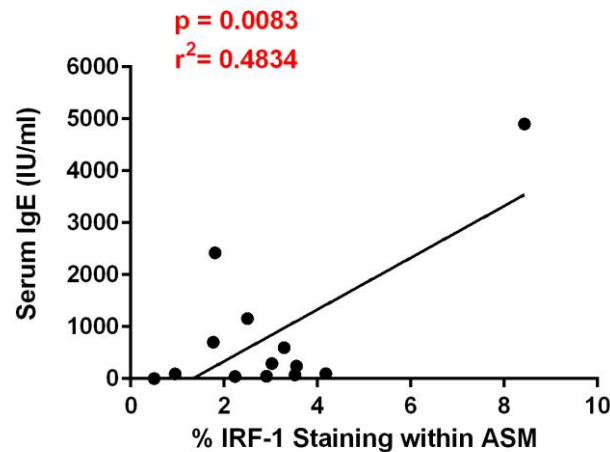
Figure 5.13: IgE levels correlate with IRF-1 expression within biopsies of severe asthmatics pre-steroid treatment. IRF-1 expression was quantified in human biopsies from n=13 severe asthma subjects before treatment with oral steroids. Each point represents the % of nuclear IRF-1 DAB staining (pixels) within the whole ASM area (pixels). This was correlated with serum IgE levels measured before treatment. Statistical analysis was performed using Pearson correlation test. P=0.0083 and Pearson r value=0.4834.

Other significant correlations were observed. For example, IRF-1 expression positively correlated with alveolar exhaled nitric oxide (aENo) levels in biopsies from severe asthmatics post-steroid treatment (**Figure 5.14**). Adding to the evidence that ENo production could be orchestrating eosinophilic inflammation, we here showed that IRF-1 expression also correlated with % sputum eosinophils pre-treatment with steroids (**Figure 5.15**). Furthermore, there was a positive correlation between sputum eosinophils and ENo levels both before and after treatment in these patients (**Figure**

5.16). This suggests a potential relationship between increased IRF-1 levels, increased eosinophils and increased ENo levels. Following steroid treatment this relationship was lost and this could be due to the lower number of eosinophils following GC treatment (**Table 5.2**).
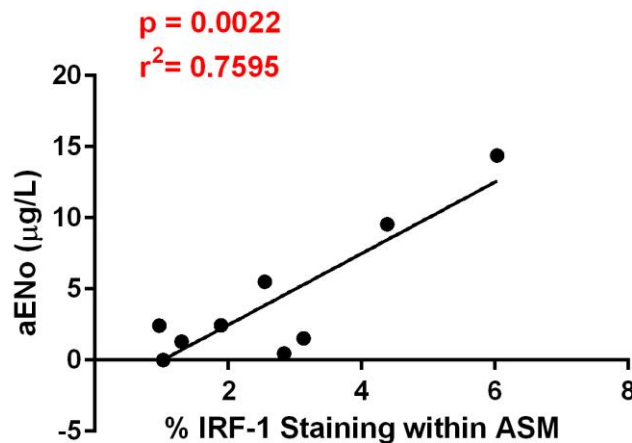
Figure 5.14: Serum aENo levels correlate with IRF-1 expression within biopsies of severe asthmatics post-steroid treatment. IRF-1 expression was quantified in human biopsies from n=9 severe asthma subjects following treatment with oral steroids. Each point represents the % of nuclear IRF-1 DAB staining (pixels) within the whole ASM area (pixels) and IRF-1 expression was correlated with alveolar ENo levels. Statistical analysis was performed using Pearson correlation test. P=0.0022 and Pearson r value=0.7595.

Figure 5.15: IRF-1 expression correlates with % sputum eosinophils in biopsies of severe asthmatics pre-steroid treatment. IRF-1 expression was quantified in human biopsies from n=12 severe asthma subjects prior to treatment with oral steroids. Each point represents the % of nuclear IRF-1 DAB staining (pixels) within the whole ASM area (pixels) and its expression correlated with % eosinophils present in sputum. Statistical analysis was performed using Pearson correlation test. P=0.0211 and Pearson r value=0.4277.
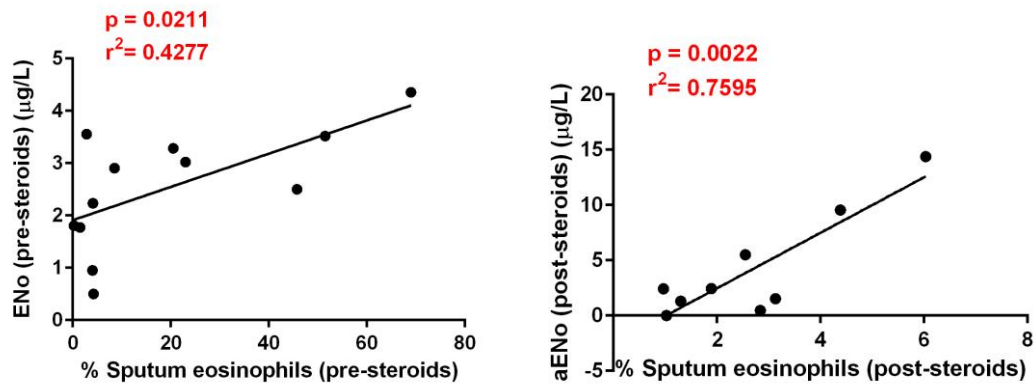
Figure 5.16: Correlation of sputum eosinophils with exhaled nitric oxide levels before and after treatment with steroids in human biopsies. Sputum eosinophil counts correlate with ENo levels pre-treatment with steroids in n=12 subjects **(left)** and sputum eosinophil counts correlate with alveolar ENo post-treatment with steroids **(right)** in n=9 subjects. Statistical analysis was performed using Pearson correlation test.

We looked at all potential correlations between IRF-1 expression within the ASM bundle in human bronchial biopsies from subjects with severe asthma pre- and post-steroid treatment. The correlations between IRF-1 expression with cell counts (in sputum, BAL, BW) and lung function parameters are summarised in **Tables 5.3** and **5.4**, respectively. All values in **bold*** show statistically significant differences using a Pearson correlation test.

Table 5.3: Correlations of IRF-1 expression with cell counts

Measurements	Cells	Pre-steroids		Post-steroids	
		Pearson r value	p-value	Pearson r value	p-value
Sputum	Macrophages	0.0004166	0.9498	0.008742	0.7305
	Eosinophils	**0.4277***	**0.0211***	0.1007	0.2312
	Neutrophils	0.0008945	0.9265	0.02052	0.5966
	Lymphocytes	0.03718	0.5482	0.08257	0.2805
	Epithelial	0.2057	0.1612	0.01546	0.6464
BAL	Macrophages	0.09034	0.3987	0.1608	0.1965
	Eosinophils	8.971e-005	0.9793	0.001634	0.9007
	Neutrophils	0.04459	0.5581	0.2798	0.0770
	Lymphocytes	0.001021	0.9302	0.003878	0.8475
	Epithelial	0.1670	0.2411	0.08707	0.3518
BW	Macrophages	0.07783	0.3799	0.02785	0.5367
	Eosinophils	0.0077	0.7863	0.001322	0.8937
	Neutrophils	0.1179	0.2746	0.05741	0.3714
	Lymphocytes	0.1788	0.1708	0.05480	0.3829
	Epithelial	0.1474	0.2179	0.01468	0.6549

Table 5.4: Correlations of IRF-1 expression with parameters of lung function

Clinical parameters	Pre-steroids		Post-steroids	
	Pearson r value	p-value	Pearson r value	p-value
FEV1	0.07158	0.3768	0.01729	0.5805
FEV1% predicted	0.1615	0.1543	N/A	N/A
FVC	0.1507	0.1702	0.06045	0.2961
PC20	0.2780	0.0956	0.005877	0.7700
IgE (IU/ml)	**0.4834***	**0.0083***	N/A	N/A
Blood eosinophils	0.01481	0.7215	0.01636	0.6369
ENo (µg/L)	0.2831	0.0921	0.002179	0.8637
aENo (µg/L)	0.05893	0.6940	**0.7595***	**0.0022***

Finally, to address whether IRF-1 truly drives the expression of known steroid-resistant chemokines, staining for CCL5 was performed in the same biopsies. The numbers used for these experiments were considerably smaller, especially for healthy controls, with n=4 healthy, n=14 severe asthmatics pre-steroids and n=13 severe asthmatics post-steroids (**Figure 5.17**). Firstly, we observed CCL5 nuclear expression in the severe asthma biopsies but very little staining in the healthy biopsies. CCL5 was significantly upregulated in biopsies from subjects with severe asthma before and after treatment compared to healthy controls (**Figure 5.18**). Surprisingly, the *in vivo* expression of CCL5 in ASM has not been extensively investigated, with only two studies reporting this in asthmatic human biopsies (63, 470). These results are interesting but we have to acknowledge the limitation of using a small number of healthy donors for these comparisons. Also, similarly to IRF-1, CCL5 expression was

not different between the two severe asthma groups, suggesting that in these severe

asthma biopsies CCL5 expression is also insensitive to treatment (**Figure 5.19**).
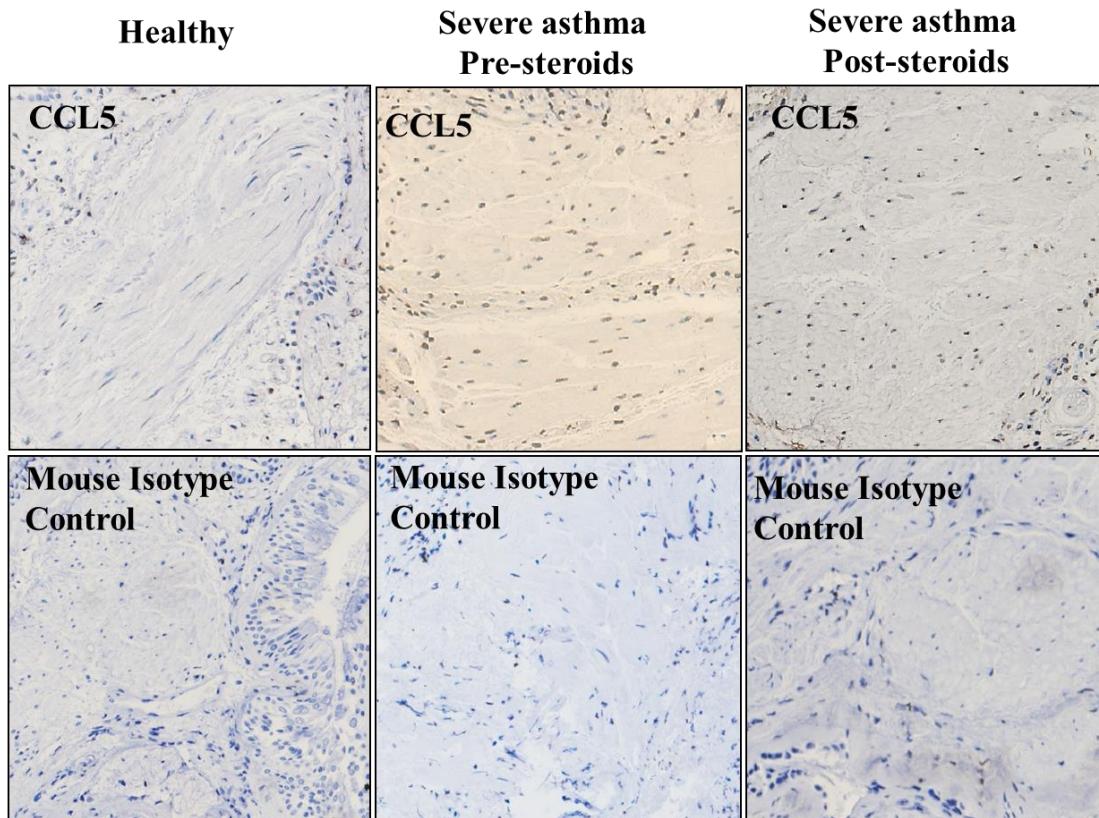
Figure 5.17: Immunohistochemistry staining of CCL5 in human bronchial smooth muscle bundles. Bronchial biopsies in acetone fixed GMA embedded sections were stained with the mouse monoclonal CCL5 antibody (2µg/mL) and as a negative control the mouse IgG isotype control (2µg/mL). Positive staining was visualised with DAB detection and hematoxylin counter stain. Magnification used was 20x.
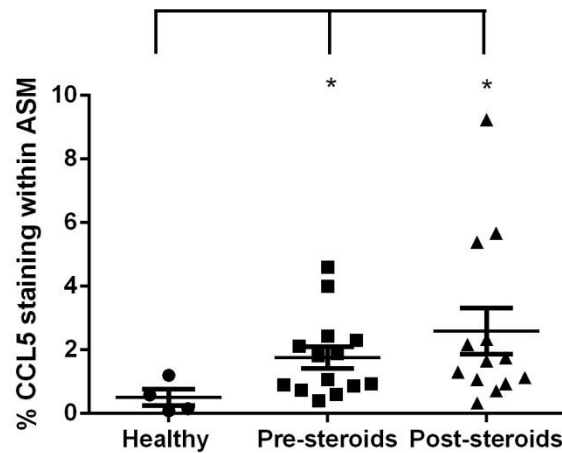
Figure 5.18: Expression of CCL5 is upregulated in bronchial smooth muscle bundles from severe asthma patients compared to healthy controls. Bronchial biopsies were stained with the mouse monoclonal CCL5 antibody (2μg/mL) in acetone fixed GMA embedded sections. Positive staining was visualised with DAB detection and hematoxylin counter stain. Quantitative analysis was performed using ImageJ with the IHC Profiler plugin. Each point represents the % of nuclear CCL5 DAB staining (pixels) within the whole ASM area (pixels). Data are expressed as means±SEM from n=4 healthy donors, n=14 severe asthma pre-steroids and n=13 severe asthma post-steroids. Statistical analysis was performed using Mann-Whitney tests to compare between the healthy and the two severe asthma groups. *P<0.05
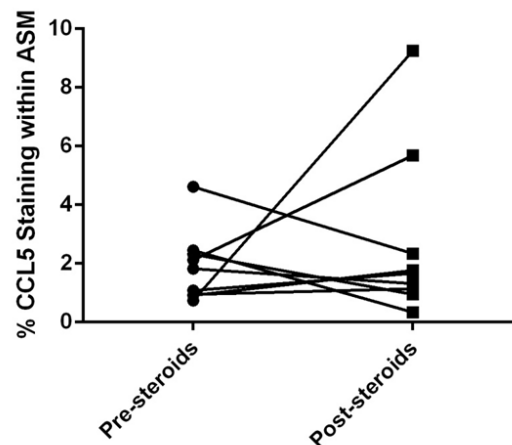
Figure 5.19: CCL5 expression is insensitive to steroid treatment. Following treatment with steroids, CCL5 expression is still present in biopsies from severe asthmatics. Each point represents the % of nuclear CCL5 DAB staining (pixels) within the whole ASM area (pixels). Data are representative of n=8 severe asthma pre-steroids and n=8 severe asthma post-steroids. Statistical analysis was performed using a paired t test to compare between pre-steroids and post-steroids severe asthma groups.

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We found that there was no relationship between IRF-1 and CCL5 expression in the severe asthma group pre-steroid treatment. On the other hand, we showed that in biopsies from severe asthmatics following steroid treatment there was a positive correlation between IRF-1 and CCL5 expression (**Figure 5.20**).
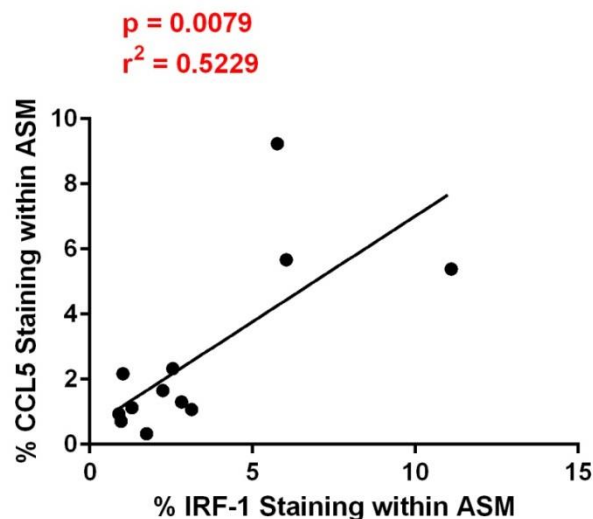
Figure 5.20: IRF-1 expression correlates positively with CCL5 expression within the ASM bundles of human bronchial biopsies of subjects with severe asthma post-steroid treatment. Following treatment with steroids, IRF-1 and CCL5 expression was quantified as % of nuclear IRF-1 or CCL5 DAB staining (pixels) within the whole ASM area (pixels) from n=12 subjects with severe asthma post-steroids. Statistical analysis was performed using a Pearson correlation test. P=0.0079 and r value=0.5229.

We noticed that, similarly to IRF-1, CCL5 expression correlated with % epithelial cells in the bronchial wash (BW) (**Figure 5.21**) and with % eosinophils in the bronchial alveolar lavage (BAL) fluid before steroid treatment (**Figure 5.22**), as well as with aENo levels (**Figure 5.23**) post steroid treatment.
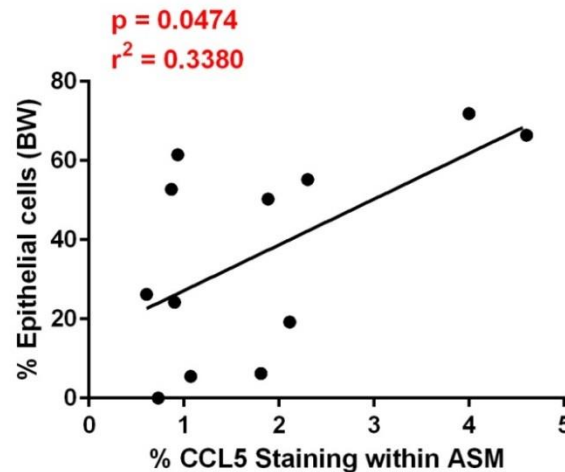
Figure 5.21: The percentage of epithelial cells in the bronchial wash (BW) correlates with CCL5 expression within biopsies of severe asthmatics pre-steroid treatment. CCL5 expression was quantified in human biopsies from n=12 severe asthma subjects before treatment with oral steroids. Each point represents the % of nuclear CCL5 DAB staining (pixels) within the whole ASM area (pixels) and its expression was correlated with the % of epithelial cells in the bronchial wash. Statistical analysis was performed using Pearson correlation test. P=0.0474 and Pearson r value=0.3380.
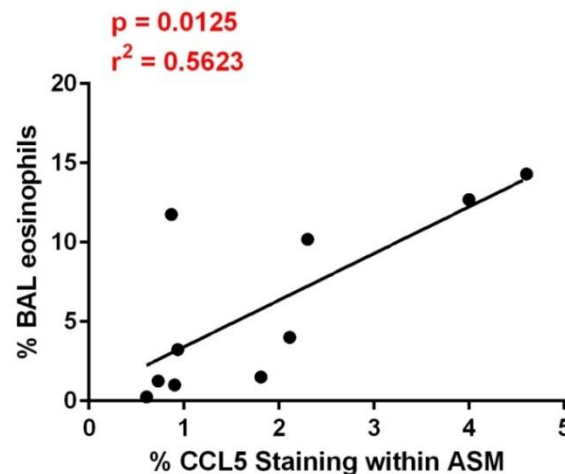
Figure 5.22: CCL5 expression correlates with % eosinophils in the bronchial alveolar lavage (BAL) fluid in biopsies of severe asthmatics pre-steroid treatment. CCL5 expression was quantified in human biopsies from n=10 severe asthma subjects before treatment with oral steroids. Each point represents the % of nuclear CCL5 DAB staining (pixels) within the whole ASM area (pixels) and its expression was correlated with the % of BAL eosinophils. Statistical analysis was performed using Pearson correlation test. P=0.0125 and Pearson r value=0.6523.
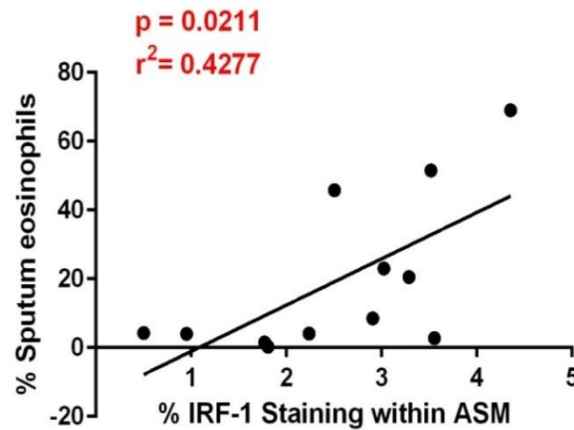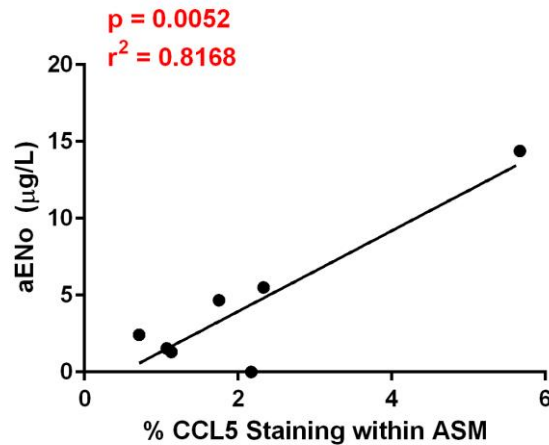
Figure 5.23: aENo levels correlate with CCL5 expression within biopsies of severe asthmatics post-steroid treatment. CCL5 expression was quantified in human biopsies from n=7 severe asthma subjects following treatment with oral steroids. Each point represents the % of nuclear CCL5 DAB staining (pixels) within the whole ASM area (pixels) and its expression was correlated with aENo. Statistical analysis was performed using Pearson correlation test. P=0.0052 and Pearson r value=0.8168.

We also looked at the correlations between CCL5 expression with cell counts (in sputum, BAL, BW) and lung function parameters as summarised below (**Tables 5.5** and **5.6**).

Table 5.5: Correlations of CCL5 expression with cell counts

Measurements	Cells	Pre-steroids		Post-steroids	
		Pearson r value	p-value	Pearson r value	p-value
Sputum	Macrophages	0.2490	0.0986	0.1859	0.2466
	Eosinophils	0.08590	0.3552	0.1626	0.2818
	Neutrophils	0.1710	0.1815	0.2658	0.1554
	Lymphocytes	0.2751	0.0800	0.04606	0.5792
	Epithelial	0.04817	0.4931	0.1364	0.3280
BAL	Macrophages	0.2702	0.1235	0.06796	0.5329
	Eosinophils	**0.5623***	**0.0125***	0.03157	0.6738
	Neutrophils	0.08065	0.4265	0.2234	0.2369
	Lymphocytes	0.08363	0.4177	0.02720	0.6963
	Epithelial	0.01996	0.6970	0.2260	0.2338
BW	Macrophages	0.04505	0.5078	0.1297	0.3067
	Eosinophils	0.1467	0.2192	0.04727	0.5463
	Neutrophils	0.04385	0.5136	0.3021	0.0998
	Lymphocytes	5.800e-006	0.9941	0.004964	0.8466
	Epithelial	**0.3380***	**0.0474***	0.07115	0.4563

Table 5.6: Correlations of CCL5 expression with parameters of lung function

Clinical parameters	Pre-steroids		Post-steroids	
	Pearson r value	p-value	Pearson r value	p-value
FEV1	0.1587	0.2250	0.2365	0.1089
FEV1% predicted	0.0002640	0.9600	N/A	N/A
FVC	0.01051	0.7272	0.01705	0.6707
PC20	0.1549	0.2947	0.0005636	0.9448
IgE (IU/ml)	0.01372	0.7032	N/A	N/A
Blood eosinophils	0.1124	0.3135	0.3491	0.0556
ENo (µg/L)	0.08373	0.3881	0.0002211	0.9675
aENo (µg/L)	0.07592	0.6537	**0.8168***	**0.0052***

5.4   DISCUSSION

Previous reports showed that CpdA repressed the activity of transcription factors such as NF-κB and AP-1 (374),(360),(372),(344). Work from the previous chapter revealed that in our model, CpdA inhibited GC resistant genes via mechanisms independent of GRα. This chapter now shows that in GC insensitive state, activation of IRF-1 (assessed by looking at its induction and nuclear translocation) was repressed by CpdA. This observation further supports the potential role of IRF-1 in the regulation of CG-insensitive chemokines in ASM cells.

Our silencing experiments confirmed the role of IRF-1 in ASM cells in driving the expression of cytokine-induced CCL5 and CX3CL1 but not CXCL10. Knocking down IRF-1 led to a marked suppression of both mRNA and protein of CCL5 (**Figure 5.7A** and **5.8A**). The effect of the IRF-1 silencing was different for the other chemokines, with no effect on CXCL10 expression and an inhibition of protein levels of CX3CL1 induced by cytokines. This indicates that the effect of IRF-1 acts at both transcriptional (CCL5) (**Figure 5.7A** and **5.8A**) and post-transcriptional levels (CX3CL1) (**Figure 5.8C**). Experiments in mouse embryonic fibroblasts lacking IRF-1 showed almost complete reduction of CXCL10 and CCL5 induced by IL-1β, whereas expression of IL-6 was diminished to a lesser extent and this observation was also replicated *in vivo* in IRF1$^{-/-}$ knockout mice (471). Earlier studies demonstrated that the murine CCL5 promoter contains a putative IRF binding site. Interestingly, mutating this site led to the reduction in the promoter's activity in cells stimulated with TNFα and IFNγ (472). Experiments using vascular smooth muscle cells (473) and bronchial epithelial cells (455) provided additional evidence for a role of IRF-1 in CCL5 expression. Studies looking at the involvement of IRF-1 in CXCL10 gene expression have led to conflicting observations. Reports from primary human endothelial cells

stimulated with IFNβ showed that induction of CXCL10 expression depended on an IFN-stimulated response element (ISRE) within the CXCL10 promoter as a double point mutation of the putative IRF1/2 binding site abolished IFN-β-induced promoter activity (474) (471). IRF-1 was also involved in CXCL10 expression when induced by rhinovirus in epithelial cells (453) but played a negative role in CXCL10 production in pancreatic cells when induced by cytokines (IL-1β, TNFα, and IFNγ) (475). It may be that the transcriptional induction of IRF-1 and the stabilisation of its protein are not sufficient for activation of IRF-1 dependent genes such as CXCL10. This is supported by studies in mouse embryonic fibroblasts, where IFNγ-induced expression of CXCL10 was shown to require further activation of IRF1, via phosphorylation by IKKβ (471). We could not find published evidence of IRF-1 binding regions in the promoter of CX3CL1. However, the post-transcriptional role of IRF-1 has been highlighted before in vascular endothelial cells in the synergistic induction of CX3CL1 by the same cytokine combination (TNFα/IFNγ) (476). We can also not completely exclude a role of IRF-1 in driving the expression of CXCL10 and CX3CL1 as our silencing efficacy was only effective at knocking down 60% of the protein. Moreover, IRF-1-dependent expression of CXCL10 and CCL5 was required for the effective recruitment of mononuclear cells into sites of sterile inflammation. It is clear from these data that the implication of IRF-1 in the regulation of inflammatory genes is highly gene specific. Nonetheless, our present report supports our initial hypothesis that IRF-1 acts as a central player in the transcription of some GC-resistant chemokines CX3CL1 and CCL5 in addition to the previously published CD38 (243), (255).

One potential mechanism by which IRF-1 blocks GC sensitivity may derive from its ability to blunt fluticasone-induced transactivation by competing with GRα for the common coactivator GRIP-1 (Introduction to this chapter (**Section 5.1**))

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(255), (362). GRIP-1 is one of 3 homologues belonging to the p160 family of nuclear receptor activators that can interact with the GC-GRα complex and facilitate gene transcription via the recruitment of histone acetyltransferases and methyltransferases. These coactivators also interact and regulate the activity of a number of transcription factors including members of the IRF family (477).

Very interestingly, we observed a lack of inhibitory effect of CpdA on IRF-1 mRNA suggesting post-transcriptional regulation. However, CpdA significantly reduced IRF-1 mRNA stability at an early time point, an observation that could explain its inhibitory effect at protein level. Very few studies have looked at IRF-1 post-transcriptional regulation but one in particular highlighted that polyubiquitination is important in the control of its stability. Therefore, K48-linked (478) and K63-linked polyubiquitination (479) targeted IRF-1 for proteasomal degradation and this was mediated by a molecule called cIAP2. Such events may contribute considerably to the transcriptional induction and stabilization of the IRF-1 protein, and their regulation may influence the activation of IRF-1-dependent genes (479).

This chapter also provides evidence to support an *in vivo* role of IRF-1 in asthma. Firstly, the fact that IRF-1 was expressed mostly in the nuclear region within the ASM bundles in all biopsies suggests a constitutive activation of IRF-1 in severe asthma. Our present *in vitro* findings show that TNFα and IFNγ also led to IRF-1 translocation to the nucleus (**Figure 5.5**). Interestingly, both cytokines have been shown to be elevated in severe asthma contributing to the view that they could be responsible for the IRF-1 activation seen in the ASM of these biopsies. Serum TNFα levels were increased in severe asthma patients and correlated with exhaled NO and with circulating neutrophils (480). IFNγ, commonly known to be induced by viral infections was also found to be elevated in severe asthma patients (326). Only one previous study found

that IRF-1 was upregulated in the airways of asthmatic patients and its expression was identified within the epithelium (448), supporting the hypothesis that this transcription factor is abnormally activated in the lung structural tissues in asthma patients. Furthermore, here we show that IRF-1 expression was insensitive to treatment with oral steroids (**Figure 5.12**). These observations point to two conclusions: **(1)** IRF-1 is up-regulated in severe asthma biopsies in the ASM and **(2)** IRF-1 is a steroid-resistant transcription factor.

The clinical importance of IRF-1 expression in asthma was assessed by looking at possible correlations with clinical parameters. IRF-1 expression correlated with high sputum eosinophils pre-treatment with steroids and this was lost following treatment with prednisolone (**Figure 5.15**, **Table 5.3**). Induced sputum differential counts are useful for measuring the predominance of a specific inflammatory cell phenotype. Higher sputum eosinophil counts are associated with more marked airways obstruction and reversibility, greater asthma severity and atopy (481). Furthermore, there is growing evidence that measures of eosinophilic airway inflammation (which defines "Th2 high" asthma) are linked to a positive response to corticosteroid treatment (464, 482, 483), while a poor response to GCs has been associated with non-eosinophilic asthma (also known as "Th2 low" asthma") (464), (484). Our finding suggests that IRF-1 may be involved in the recruitment of eosinophils. We would argue that CCL5, a potent chemoattractant for eosinophils (485) could drive this process through an IRF-1 dependent mechanism. We did not see a correlation between IRF-1 and CCL5 expression in the pre-treated biopsies, while post-treated biopsies showed that IRF-1 levels highly correlated with CCL5 levels, suggesting this relationship was somewhat influenced by the use of oral steroids. Not surprisingly, we also observed a correlation

of CCL5 staining with eosinophil counts in BAL fluid in the severe asthma biopsies pre-steroid treatment, which was lost following therapy.

There was no correlation of IRF-1 with sputum neutrophils however there was a change in the profile of eosinophilic and neutrophilic inflammation following treatment. This could be explained by that fact that sputum eosinophil counts were reduced by treatment, while sputum neutrophil counts were unaffected (**Table 5.1**). Therefore steroid treatment is able to reduce eosinophilia as expected in "Th2 high" patients, while neutrophils are somewhat resistant to treatment. High sputum neutrophils have been reported previously in severe asthma (465, 486). Moreover, findings from the latest report from U-BIOPRED confirm a greater degree of sputum eosinophilia in two severe asthma groups compared to the mild and moderate groups, as well as higher blood neutrophil counts in patients with severe asthma. Sputum neutrophil counts were similar in the three asthma groups and were significantly higher than in the healthy control group. High neutrophil counts could be the result of using systemic GCs which have been shown to increase their survival (487).

Here we showed a positive correlation between increased IRF-1 expression and serum IgE levels before steroid treatment (**Figure 5.13**). This evidence leads us to speculate there is a potential mechanism involving IRF-1 in the recruitment of eosinophils via IgE and potentially CCL5 mechanisms, however this remains to be further confirmed. One other study showed that treatment with NOD1,2 ligand significantly enhanced the serum concentration of total IgE and the chemokine CCL5 which in turn recruited eosinophils and induced Th2 cells and the release of Th2 cytokines (i.e. IL-13) in BAL fluid of ovalbumin-sensitized allergic asthmatic mice (488). It is therefore possible that IRF-1 overexpression within the ASM could be linked to increased CCL5 production, leading to increased serum IgE and ultimately to

eosinophil recruitment. This is supported by another study performed in Han Chinese patients with allergic skin diseases in which two single nucleotide polymorphisms (SNPs) found in the promoter region of CCL5 were reported to be involved in the IgE expression and IgE-mediated allergic reactions (489). Our data is also supported by studies showing that polymorphisms in IRF-1 gene were associated with IgE regulation and atopy (461).

Apart from the correlation between IRF-1 expression and % sputum eosinophils pre-treatment with steroids, there was also a positive correlation between sputum eosinophils and ENo levels in these patients (**Figure 5.16 left**). This is very interesting, as ENo production was shown to orchestrate eosinophilic inflammation. ENo has also been used as a surrogate of steroid responsiveness (490). Following steroid treatment this relationship was lost and this could be explained by the inhibitory action of steroids on eosinophil numbers (**Table 5.1**).

Moreover, IRF-1 has been shown to induce the enzyme iNOS that produces nitric oxide (NO), important for both the clearance of pathogens and the differentiation of Th1 cells (491). NO is known to inhibit rhinovirus replication and virus-induced cytokine expression through inhibition of IRF-1 and NF-κB pathways in airway epithelial cells (454) (492). Very interestingly, another novel observation was the positive correlation between alveolar exhaled nitric oxide (aENo) and sputum eosinophils post-steroid treatment, which we did not observe in the biopsies prior to treatment (**Figure 5.16 right**). aENo fractions have been considered a potential biomarker of small airway inflammation (493). In recent years attempts have been made to develop techniques that calculate NO derived from large conducting airways and the small airways and alveoli using a simplified model called the 'two compartment model'(494). Although early papers have reported that aENo was elevated in severe

asthmatics (495, 496), this model failed to account for the NO diffusion between the two compartments. This suggests that steroid treatment can influence the relationship between the two fractions, although the evidence is conflicting. In an uncontrolled study, aENo was suppressed in severe asthmatics by oral prednisolone but not by inhaled fluticasone (495), suggesting that systemic treatment may be required to target the small airways adequately. This was in contrast with another study showing that aENo was insensitive to changes in dose and delivery of ICSs and was not suppressed by systemic corticosteroids (493).

Since we also noticed a correlation between CCL5 levels and aENo levels, it would be possible that the CCL5/IRF-1 pathway drives ENo release and the subsequent high eosinophilia in patients with severe asthma before treatment with steroids, and the release of aENo following therapy with steroids.

In this chapter we made a few very important observations which contribute to our knowledge of the mechanisms driving steroid insensitivity in asthma. This is the first report to show that IRF-1 is an insensitive transcription factor expressed within the ASM in biopsies from severe asthmatics. We propose that IRF-1 could be involved in the recruitment of eosinophils that drive inflammatory processes in severe asthma patients before treatment with oral steroids. This could involve IgE-mediated responses in linkage with mast cells and the production of CCL5 which is a chemoattractant for these inflammatory cells. More importantly, this is the first study to confirm a correlation of IRF-1 expression with serum IgE levels in adult biopsies (previously shown in children biopsies (461)).

We also observed a shift to a more neutrophilic pattern in biopsies of severe asthmatics post steroid treatment, although this could be explained purely by the effect

of steroids. The correlation of IRF-1, CCL5 and sputum eosinophil counts with aENo levels in biopsies of severe asthmatics post steroid treatment may be a reflection of alveolar inflammation. It would be interesting to determine whether the inability of these patients to respond to treatment lies in the fact that there are resistant pathways activated within the small airways.

CHAPTER 6

General discussion and conclusions

6.1   KEY FINDINGS AND SIGNIFICANCE

6.1.1   Evidence for the use of CpdA in steroid insensitive conditions

This study unravelled the unusual biological properties of CpdA, but also provided a greater understanding of the mechanisms underlying its anti-inflammatory effects. This is the first study to show that CpdA differentially suppressed the expression of chemokines in ASM cells in a model of GC insensitive state. This suggests that induction of GC insensitive genes by TNFα/IFNγ is complex and involves mechanisms with different sensitivity to CpdA.

The finding that the anti-inflammatory actions of CpdA were independent of the GRα is in contrast to current literature, mostly from De Bosscher's group which have described CpdA as a dissociated GRα compound. This was indirectly shown by assessing GRα nuclear translocation and/or using cells lacking GRα which prevented CpdA cellular actions (336, 360). However a few discrepancies between these studies and my present work must be emphasised. For example, although initial studies in A549 human alveolar cells and L929sA murine fibroblasts showed that CpdA induced a shift of GRα to a predominant nuclear localization (336), most of the latter studies reported a much weaker GRα nuclear import by CpdA when compared to other GCs (i.e. dexamethasone or flucinolone acetonide) in a variety of cells (i.e. rheumatoid arthritis fibroblast-like synoviocytes (341), (342), prostate cancer cells (359) and leukemia cell lines CEM and K562 (374)). In addition, the latest study by De Bosscher strongly supports our findings by similarly showing the inability of RU486 to prevent the CpdA inhibitory effects on cytokines secreted by PBMCs stimulated with Staphylococcus aureus–derived enterotoxin B protein. Moreover the authors even observed a more pronounced effect of combining RU486 with CpdA in the repression of IFNγ, IL-10, IL-17 and IL-1β (375). Their explanation was somewhat similar to ours, that CpdA may

be acting independent of the GRα. This possibility could reflect an effect of CpdA on receptor phosphorylation status. However, in the initial studies using A549 epithelial cells, CpdA was unable to phosphorylate the GRα at residue ser211 (336), supporting the emerging view that not all CpdA actions rely on GRα transactivation properties.

This study is not only the first to test the potential use of CpdA in ASM cells under steroid insensitive conditions but also the first to describe an effect of CpdA on the MAPK signalling pathway and on the transcription factor IRF-1 (to be discussed in detail in the following sections). Previous reports showed that CpdA suppressed the activity of other transcription factors such as: NF-κB, AP-1, Ets-1, Elk-1, SRF and NFATc (359, 361), although the underlying mechanisms were not investigated in detail. The relevance of our findings is further supported by the recent evidence for the use of CpdA in a Th2 mouse model of asthma. In this report, CpdA repressed IL-4-induced STAT6 translocation, as well as NF-κB translocation and exerted very strong anti-inflammatory actions by decreasing OVA-induced AHR, inhibiting the recruitment of inflammatory cells to the BAL fluid, blocking the infiltration of mast cells and inhibiting collagen deposition (349). In contrast to our study, the authors found that CpdA did not up-regulate MKP-1 when tested at 24 hours. However, as shown in our study, MKP-1 up-regulation was temporal and transient suggesting that CpdA may have upregulated MKP-1 at an earlier time point. Nonetheless, our study and results from current literature reveal that the anti-inflammatory effects of CpdA are cell type- and stimuli-specific, which could explain the discrepancies observed between the different reports with regard to GRα involvement. Moreover, as we showed in **Chapters 4** and **5**, CpdA effects are also gene specific.

6.1.2　A role of MAPK pathways in treating steroid-insensitive pathways

Targeting the MAPK cascade is an attractive strategy for reversing steroid insensitivity in asthma and a better understanding of how MAPKs regulate pro-asthmatic responses in ASM is of therapeutic importance. This thesis focused on two approaches: first, harnessing the effects of the endogenous MAPK deactivator MKP-1 and second, the use of soluble MAPK pharmacological inhibitors.

i)　　*MKP-1 up-regulation and modulation of the expression of the steroid-insensitive chemokine CXCL10*

This study provides a novel mechanism for the inhibition of fluticasone-resistant CXCL10 production in ASM cells stimulated with TNFα/IFNγ. Silencing experiments from **Chapter 4** convincingly showed that MKP-1 up-regulation by CpdA was required for the inhibition of CXCL10 induced by cytokines. Considering that the up-regulation of CXCL10 by exposure to TNFα/IFNγ was insensitive to fluticasone *in vitro*, this provides a possible pathway for its up-regulation in the asthmatics *in vivo* (97). Interestingly, our group discovered that from a panel of four fluticasone-resistant chemokines (CXCL10, CCL5, CX3CL1, and CCL11), CXCL10 was the only one not affected by targeting $KCa_{3.1}$, suggesting that its production is regulated via a distinct non-$KCa_{3.1}$ mechanism (332). In the present study, since the only kinase directly inhibited by CpdA in cells stimulated with TNFα/IFNγ was ERK, we questioned whether ERK inhibition by CpdA occurred via MKP-1 induction. In contrast to what we expected, directly inhibiting ERK using U0126 did not affect the production of cytokine-induced CXCL10 although ERK regulates CXCL10 production in human airway epithelial cells infected with HRV-16. In this model, ERK negatively regulated viral-induced CXCL10 production by acting at a transcriptional level, through increasing the interaction of the transcription factor Interferon Regulatory Factor-1

(IRF-1) with the ISRE site within the CXCL10 promoter (497). Our observation raises two possibilities: either the inhibitory effect of CpdA on ERK activation by TNFα/IFNγ occurs via MKP-1-independent mechanisms, or the inhibitory effect of CpdA on CXCL10 via MKP-1 involves ERK independent pathways. Little is known about MKP-1-independent mechanisms. As shown by this thesis and by other groups, there are challenges to targeting MKP-1. Our study demonstrated that MKP-1 up-regulation by CpdA occurred solely at 2 hours, while the TNFα/IFNγ-induced activation of most kinases occurred within 10 minutes. Another study similarly showed an early MKP-1 induction was effective in inhibiting IL-1β-induced CXCL1 and CXCL2, but not at later times (6 hours) (498). Also recent evidence has shed new light on the dynamic interaction of MKP-1 with its substrates, which is in contrast to the oversimplified view that MKP-1 only acts to switch-off the kinases. There are studies showing that ERK can phosphorylate and increase the stability of MKP-1 which could in turn inhibit p38MAPK activation (416, 422). Moreover, inhibition of p38MAPK was shown to enhance the activity of ERK independently of MKP-1 up-regulation, through phosphorylation of the upstream target MEK. All these events can alter the enzymatic activity of MKP-1, its interaction with downstream substrates, as well as the downstream interaction between the different kinases. Whether a similar mechanism occurs in ASM cells has not been investigated.

The fact that p38MAPK and JNK activation was not affected by CpdA suggests that other pathways could be involved in driving GC insensitivity by cytokines. For example, in ASM cells the JAK/STAT pathway was involved in synergistic production of GC insensitive CXCL10 by TNFα/IFNγ which was due to enhanced recruitment of the transcriptional coactivator CREB-binding protein (CBP) and RNA polymerase II (97). Since persistent ERK activation was shown to negatively regulate CREB activity

via increased association of CBP to pp90RSK, it is possible that CpdA may also have an effect via these ERK-sensitive pathways (499) however this remains to be investigated. In conclusion, this study provides evidence for the involvement of MAPK deactivator, MKP-1 in mediating the inhibition of fluticasone-resistant CXCL10. Since the effect of MKP-1 appears to be quite gene and stimuli specific, we cannot conclude that targeting this molecule could be of therapeutic benefit in treating other steroid-resistant pathways in ASM cells. Rather, these data support the implication for both MKP-1-dependent and -independent mechanisms in the inhibition of GC resistant chemokines in ASM cells which should be further investigated.

ii)   *Interaction between p38-JNK pathways and cytokine-induced CCL5 production*

This thesis shows that ASM is a potential source of GC resistant CCL5 in asthmatic patients and is the first to describe the modulation of fluticasone-resistant CCL5 by TNFα/IFNγ via p38MAPK and JNK inhibition. There are other reports in the literature supporting the implication of p38MAPK in driving steroid insensitive pathways, with most evidence derived from work in immune cells. For example the inhibition of p38MAPK in PBMCs prevented steroid insensitivity induced by IL-2 and IL-4 (410) or by IL-13 (409). p38 inhibition also reduced dexamethasone-resistant H3-Pser10 induction by LPS in PBMCs and alveolar macrophages (429). Our study extends these observations to structural cells. Only one other report describes a similar effect in ASM cells stimulated with TNFα alone (427). The role of JNK in driving the expression of the cytokine-induced fluticasone-resistant CCL5 is particularly interesting. An increase of bronchial mucosal c-jun and JNK phosphorylation was reported in severe asthma patients who were insensitive to high-dose therapy with prednisolone (433). Also in rhinovirus-infected epithelial cells, inhibition of both JNK

and IKK2 totally restored dexamethasone's ability to suppress IL-8 release, induce MKP-1 gene expression, and restore GRα nuclear translocation (432). The involvement of JNK in CCL5 expression has only been described in ASM under stimulation by TNFα and IL-1β (500) and it also regulated IL-8 and GM-SCF. Similar results were found in other cell types (i.e. macrophages, dendritic cells (501), and peripheral blood NK (natural killer) (502)). Finally, although this study has not assessed this aspect, a synergistic effect of p38MAPK with JNK in driving CCL5 production is possible and was previously demonstrated in ASM cells stimulated with PAF (414). Future studies should be designed to address this possibility in our cellular model.

iii)   *Interaction between p38-JNK pathways and cytokine-induced CX3CL1 production*

This study also described the role of p38MAPK and JNK inhibition in mediating the expression of CX3CL1, another chemokine with important chemoattractant functions, which could be targeted in steroid insensitive conditions. This has been previously reported although much higher concentrations of the inhibitors were used (25μM for SP610025 and 20μM for SB203580) (334). The authors also made an interesting finding that dexamethasone inhibited JNK activation by TNFα/IFNγ (334). It would be interesting to investigate whether this is relevant for fluticasone in our cellular model.

iv)   *The beneficial effect of combining MAPK inhibitors and fluticasone*

We made the observation that combining fluticasone and MAPK inhibitors had a greater effect on chemokine inhibition. SB203580 and fluticasone considerably reduced the production of TNFα/IFNγ-induced CCL5 and CXCL10, at much lower concentration (1μM) compared to the inhibitor alone. Also ERK inhibition with fluticasone further inhibited TNFα/IFNγ-induced CCL5 production. This enhancing

effect in ASM cells is novel and the mechanisms have not been completely elucidated. Since fluticasone on its own has no effect on cytokine-induced chemokines production, this suggests that there may be a mechanistic interaction between the two drugs leading to a greater effect. Most studies showing a similar effect of combining p38 inhibitor and GC were performed in alveolar macrophages and PBMCs from asthma and COPD patients although this effect was assessed using one concentration of each drug (503) (302). Another study in alveolar macrophages from COPD patients, showed that p38MAPK inhibitor BIRB-796 dose-dependently enhanced dexamethasone inhibitory effect on the production of IL-8, IL-6, TNFα, GM-CSF, IL-1Rα, IL-10, MDC, and CCL5 (504). This combined effect could be explained by the ability of p38 MAPK to prevent ligand-induced GRα phosphorylation, and the ability of dexamethasone to effectively bind to GRα (409). Additionally, p38 MAPK inhibition may also facilitate GRα transcriptional activity of various transcription factors (as seen for NF-κB (505)), by altering the chromatin structure via phosphorylation of histones (as shown previously for H3-Pser10 (429)). Although we did not look at the effect of fluticasone used in combination with the JNK inhibitor, there are a striking number reports showing the interactions between GRα activity and the JNK pathway. GRα can bind cytoplasmic JNK and prevent its association with upstream activators (i.e. MKK4 and MKK7) (506). Furthermore, GRα-ligated JNK can translocate to the nucleus, resulting in inactive JNK that may compete with active JNK for binding to c-Jun.

There is some evidence in the literature showing that CX3CL1 production by TNFα/IFNγ was enhanced by dexamethasone, while the ERK inhibitor had no effect (437). In our study we also noticed an enhancing effect of fluticasone on TNFα/IFNγ-induced CX3CL1 production. The mechanism involved is thought to be by increasing

CX3CL1 mRNA transcription via MAPK independent pathways as suggested by Sukkar and colleagues (437).

To conclude, these studies have shed some light on the gene specific effects of the MAPK inhibitors, however there is a need for full dissection of the molecular players involved in driving these pathways. Perhaps silencing of the kinases using siRNA or adenoviruses would have been a better option, as chemical protein kinase inhibitors can have non-specific effects. Future studies will have to employ multiple approaches, including gene silencing, gene transfer technology, expression of mutant proteins and possibly the assessment of the phenotype of transgenic and knockout mice.

6.1.3 *In vitro* role of IRF-1 in asthma

i) *IRF-1 mediates the expression of cytokine-induced steroid-insensitive chemokines CCL5 and CX3CL1*

Chapter 4 of this thesis already described one potential mechanism for reducing chemokine production in ASM cells, via inhibition of MAPK pathways. Here we provide evidence for a role of the transcription factor IRF-1. First, CpdA inhibited IRF-1 expression and nuclear translocation by TNFα/IFNγ, which prompted us to speculate an indirect effect of CpdA on IRF-1 in the inhibition of fluticasone-resistant genes. We were able to confirm through silencing experiments that IRF-1 regulates CCL5-induced chemokine expression. Limited evidence also reported a similar role of IRF-1 in vascular smooth muscle (473) and bronchial epithelial cells (455). However our study is the first to suggest a post-translational role of IRF-1 in the regulation of CX3CL1. One previous report from vascular endothelial cells showed that TNFα, in combination with IFNγ, stabilizes CX3CL1 mRNA via phosphorylation of p38MAPK and its downstream target MKK2 although the role of IRF-1 was not investigated (476).

6.1.4 *In vivo* evidence for the role of IRF-1 in severe asthma

The *in vitro* findings from this study suggest a role of IRF-1 in the pathogenesis of asthma via its ability to regulate pro-inflammatory responses in ASM cells. This is in line with indirect reports showing the implication of IRF-1 in atopy and its correlation with serum IgE levels in samples from asthmatic children (461). Whether IRF-1 is expressed *in vivo* in the airways in asthma has not been previously investigated.

i) *IRF-1 is up-regulated in ASM from severe asthma biopsies and its expression is insensitive to treatment*

Findings from **Chapter 5** clearly show the presence of activated IRF-1 (as judged by its nuclear presence) within the ASM in biopsies from severe asthmatics. Moreover, this activated state of IRF-1 in ASM appears to be GC insensitive as its expression was unaffected following a two week treatment with oral prednisolone. This confirms our previous *in vitro* studies showing that cytokine-induced IRF-1 was not affected by fluticasone in GC resistant conditions (255) and that its sustained activation in ASM impaired response to GCs (255, 362). The relevance of our observation is two-fold: **(1)** IRF-1 is constitutively activated in the ASM bundles of severe asthmatics despite GC therapy, and **(2)** IRF-1 could drive the expression of other steroid resistant molecules, as supported by our *in vitro* data (e.g. CCL5 and CX3CL1).

ii) *IRF-1 expression in ASM bundles correlates with serum IgE levels*

The present study shows that IRF-1 expression in ASM tissues correlated with serum IgE in our cohort of severe asthmatics, prior to treatment with oral GCs. Previous studies in children with asthma have also found a similar correlation (461). Furthermore, there is a lot of clinical evidence to support the importance of increased serum IgE levels as a marker for asthma severity. For example, cluster analysis studies identified an early-onset severe asthmatic group characterised by allergen sensitivity,

eosinophilia and high serum IgE levels (507). More importantly, targeting IgE using the monoclonal antibody omalizumab, proved to have clinical benefits in a subset of severe asthma patients ("Th2 high"), as discussed in the **Introduction** (**Section 1.5**) (157). Interestingly, studies looking at the effect of anti-IgE treatment on airway remodelling revealed a direct effect on the ASM, with a decrease in ASM and ECM proteins in severe asthma patients (508). Also, the recently reported increase in IFNγ levels in severe asthma (509) supports the migration of mast cells into the ASM bundle via increased expression of IRF-1 dependent CXC chemokines such as CXCL10 (as shown by Brightling *et al.* (91)). These studies imply that the role for mast cells in severe asthma may go beyond the presence of a Th-2 inflammatory process. We propose that ASM cells, in an IRF-1-dependent mechanism involving IgE, could participate in asthma pathogenesis via recruitment of mast cells and possibly eosinophils.

iii)   IRF-1 expression correlates with % sputum eosinophils

IRF-1 expression correlated with high sputum eosinophils pre-treatment with steroids and this was lost following treatment with prednisolone (**Figure 5.15**, **Table 5.3**). Higher sputum eosinophil counts are associated with more marked airways obstruction and reversibility, greater asthma severity and atopy (481). The most convincing evidence confirming the importance of eosinophils in severe asthma comes from trials using anti-IL-5 antibodies showing their beneficial effects in these patients as described previously in the **Introduction** (**Section 1.5, Table 1.10**). Our correlation results suggest a role of IRF-1 in the recruitment of eosinophils, possibly mediated via the production of CCL5, known to act as a potent chemoattractant for these inflammatory cells (485).

As previously mentioned in the **Introduction**, measures of eosinophilic airway inflammation are linked to a positive response to corticosteroid treatment (464, 482,

483), while a poor response to GCs has been associated with neutrophilic asthma (464), (484). Although we did not see a correlation between IRF-1 and neutrophils, the presence of neutrophil infiltrates in patients with severe asthma suggests that innate host defence pathways may be activated but the mechanisms involved are unknown. It would be interesting to determine whether IRF-1 plays any role in this possibly via the induction of cytokines, since this transcription factor is an important switch in driving Th1 innate immune pathways, in response to infections. It is known that during infection, neutrophils are recruited into the airways via activation of TLR4 and subsequently MyD88 by endotoxin (510). One possibility is that activation of the Th1/TLR4/MyD88 pathway may primarily be oriented toward limiting infection, leading to the activation of proinflammatory pathways in macrophages, recruitment of neutrophils and increased AHR. In asthmatics with superimposed infections, activation of this pathway on a background of aberrant Th2 cytokine-driven chronic inflammation may lead to acute exacerbations (186). A few other molecules have been shown to be involved in the recruitment of neutrophils, including reactive oxygen species (ROS) (511), the receptor for advanced glycation end-products (RAGE) (512) and ADAM8 (513). Most interestingly all of these have been shown to either be expressed or produced by ASM cells. It would be interesting to look at whether IRF-1 regulates any of these proteins.

iv)   *IRF-1 expression correlates with alveolar exhaled nitric oxide (aENo) in severe asthma biopsies post-treatment*

In addition to the correlation between IRF-1 expression and % sputum eosinophils pre-treatment with steroids, there was also a positive correlation between sputum eosinophils and ENo levels in these patients (**Figure 5.16 left**). ENo was described as a strong independent predictor of chronic systemic corticosteroid use in

patients with severe asthma (514) and as marker for monitoring asthma control (515), although a recent study showed no significant benefit for health-related quality of life, severe exacerbations or inhaled GC use (516). In addition, ENo has been used as a predictor for treatment efficacy to omalizumab treatment (517). Since its production orchestrates eosinophilic inflammation (490), it was not surprising that ENo correlation with sputum eosinophils was lost following treatment, providing one mechanisms by which steroids treat eosinophilia in asthma (**Table 5.1**).

Even more interesting was the positive correlation between alveolar exhaled nitric oxide (aENo) and sputum eosinophils and between IRF-1 and aENo post-steroid treatment, which we did not observe in the biopsies prior to treatment and the reason for this is to us unclear (**Figure 5.16 right**). Other studies have reported increased inflammatory cell counts in the small airways and alveolar tissue of severe asthmatics, compared to the larger airways (518). For example, mast cells were also found to be more prominent in the small airways and in the alveolar attachments of severe asthma patients than in the large or conducting airways (519). The area of research looking at inflammation within the small airways and its contribution to the functional impairment of severe asthma is still small. aENo fractions are being considered as a potential biomarker of small airway inflammation (493) and some reports suggest that the severity of asthma increases in proportion to the involvement of the small airways (520). Since the small airways are less accessible to the delivery routes for anti-inflammatory medications such as GCs, it is perhaps not surprising to observe the shift between ENo and aENo levels following treatment. Since we also noticed a correlation between CCL5 levels and aENo levels, it would be possible that the CCL5/IRF-1 pathway drives the release of ENo and the subsequent recruitment of eosinophils in patients with severe asthma before treatment with steroids, and the release of aENo

following therapy with steroids. The exact mechanism involved is however unknown. One supporting link between IRF-1 and ENo is that the enzyme that synthesizes NO, iNOS (521, 522) contains in its promoter region numerous consensus sequences for transcription factors, including one for IRF-1 (523). Since IRF-1 was proposed to drive the expression of iNOS in airway epithelial cells, via the constitutive activation of the STAT1 pathway (448), a similar pathway could be activated in ASM cells and this hypothesis should be further investigated.

6.2   FUTURE DIRECTIONS

This thesis performed a systematic investigation into the mechanisms by which TNFα/IFNγ influences GC responsiveness in ASM cells via the use of the natural compound CpdA. It is important to acknowledge one strong limitation for using CpdA for future studies, especially when considering its use as an alternative for GCs. Despite its interesting pharmacological profile, CpdA has a reduced stability and a relatively narrow therapeutic window. Used at high doses (~15mg/kg) for longer periods it leads to apoptosis via the release of GRα-independent metabolites (345). Also CpdA doses in most studies were approximately 10-fold higher compared to GCs, possibly due to a lower GRα affinity and the non-steroidal nature of the compound (336). This limits its potential use for future therapies. However, as shown in this study, its *in vitro* use is very powerful in helping to identify potential novel players in the pathogenesis of asthma, such as MKP-1, the MAPK kinases and the transcription factors IRF-1. Furthermore, prospective derivatives of this compound with a better pharmacological profile are very attractive candidates for clinical applications in the treatment of various inflammatory and autoimmune diseases such as rheumatoid arthritis, type I diabetes and asthma.

In addition, this study has shown that members of the MAPK pathway and the transcription factor IRF-1 contribute to the development of steroid insensitive pathways in ASM cells. ***The question is can we safely target these signalling pathways?*** The clinical relevance of using MAPK inhibitors is currently being tested in trials (e.g. dilmapimod used in the acute respiratory distress syndrome (524), and XG-102 used in intraocular inflammation (525)). However, despite their beneficial anti-inflammatory effects described *in vitro*, the initial *in vivo* studies also alerted to their potential cytotoxic effects in the liver (430) suggesting that safer approaches such as inhaled forms of p38 inhibitors are needed. If successful, future work should investigate the potential benefit of combinational therapy with GCs and safer options of p38 inhibition, as we attempted briefly in our study.

As for IRF-1, it is perhaps premature to speculate that directly targeting this transcription factor could be clinically beneficial, especially with the previous evidence that deficiencies in IRF-1 could result in autoimmune diseases (526). It would be important however to investigate the inflammatory profiles in IRF1$^{-/-}$ knockdown in mice in the context of asthma, which has not been done yet. But possibly a better approach would be to verify whether IRF-1 is up-regulated in other cell types in severe asthma, which could imply its usefulness as a marker for GC refractoriness.

Moreover, a more systems biology approach is required in delineating the exact mechanisms involved in driving steroid insensitive pathways in the ASM. For example, one question that remains unanswered from this Thesis is the exact mechanism by which CpdA inhibits the activation of IRF-1 by TNFα/IFNγ. To answer this, TF-ChIP-Seq could offer genome-wide information on the physical binding site of this transcription factor following treatment with the plant compound. Also it would be interesting to look at the epigenetic regulation of IRF-1 by ChIP-Seq, which would

reveal all the accessible genomic regions at the IRF-1 promoter. With regards to the MAPK signalling pathway and its involvement in steroid insensitivity, a better understanding of the relationship between the kinases and their substrates may be achieved using high-throughoutput RNAi screening. This could also help to identify novel transduction pathways. Finally, our study revealed the potential relevance of post-transcriptional and post-translational mechanisms in the regulation of chemokines. Proteomics-based approaches could provide much more detail into these mechanisms. We also await the results from larger studies that combine state of the art transcriptomic, proteomic, lipidomic and metabolomic technologies, which will help to identify multi-dimensional phenotypes of severe steroid insensitive asthma and potential new targets for treatment.

6.3   GENERAL CONCLUSIONS

Data presented within this thesis have demonstrated that steroid resistant pathways can be induced *in vitro* using cultured ASM cells and can be seen *in vivo* in the ASM bundles of severe asthmatics. These GC resistant pathways result in the production of different pro-asthmatic cytokines and chemokines that can have a significant impact in asthma pathogenesis via **1)** the recruitment of different inflammatory cells (eosinophils), **2)** regulation of the "Th2 responses" such as IgE production, and **3)** induction of transcription factors that can affect cells sensitivity to GC therapy (IRF-1) and the ability of ASM cells to produce immunomodulatory proteins. The present work suggests that targeting these transcription factors such as IRF-1 which regulates both airway inflammation and GC sensitivity could lead to better therapeutic options for asthma.

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