Airway Smooth Muscle and Mast Cell Interaction Modulates Corticosteroids Sensitivity

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fulfilment of the requirement of
Doctor of philosophy

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Abstact:

A proportion of patients affected by the severe form of asthma do not adequately respond to corticosteroids, and the underlying mechanisms are unknown. Because infiltration of mast cells in ASM bundle is a defining feature of asthma and as mast cells have been shown to modulate the function of ASM cells including to the anti-asthma therapy drug β2-agonists, I hypothesised that mast cells could regulate corticosteroids responsivenes to ASM.

The purpose of this PhD was to test whether healthy ASM cells pre-treated with conditioned media (CM) from non-activated or activated human lung mast cells for either 30 minutes (effect of preformed mediators) or 24 hours (effect of synthesised mediators) would affect the ability of fluticasone to **i)** repress TNF-α-induced expression of different chemokines including CCL5, CXCL10 and CXCL8 and **ii)** induce the expression of anti-inflammatory genes. The results show that fluticasone-dependent repression of CXCL10 and CCL5 induced by TNF-α was significantly impaired in cells that were first pre-treated with CM from 30-minute and 24-hour activated mast cells. The reduced expression of two fluticasone-inducible genes GILZ and MKP-1 by the CM from 24-hour activated mast cells suggested that transactivation activities were affected by mast cell mediators. The inhibitory effect of activated mast cell CM on fluticasone-induced transactivation was further confirmed using gene array analysis showing a profound reduction of a number of different steroid-inducible genes with anti-inflammatory and anti-asthma properties. The gene array data of changes in expression of four selected genes including GILZ, MKP-1, FKBP5 and PIK3R1 were validated using individual qPCR which demonstrated strong correlations between the two techniques with respect to gene modulation. Together, these studies show for the first time that CM from activated mast cells contains mediators that can regulate the responsiveness of ASM cells to corticosteroids by differentially inhibiting their transactivation properties.

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Abbreviations:

Abbreviation	Meaning
AA	Antibiotic antimycotic
AHR	Airway Hyperresponsiveness
AP-1	Activator protein -1
ASM cells	Airway smooth muscle cells
BSA	Bovine Serum Albumin
BT	Bronchial Thermoplasty
CCL11	Eotaxin
CCL5	Rantes
cDNA	complementary deoxyribonucleic acid
CER	Cytoplasm extrication reagent
CO2	Carbon dioxide
CSs	Corticosteroids
CTGF	Connective tissue growth factor
CX3CL1	Fractalkine
CXCL10	C-X-C motif chemokine 10
CXCL8	C-X-C motif chemokine 8
CM	Condition media
DMEM	Dulbecco's modified eagle medium
DUSP1	Dual specificity phosphatase 1
EDN1	Endothelin 1
ELISA	Enzyme Link Immunosorbent Assay
ERRFI1	ERBB receptor feedback inhibitor 1
FBS	Fetal bovine serum

FcεR1	High affinity IgE receptor 1
FEV-1	Forced Expiratory Volume in a second
FKBP5	FK506 binding protein 5
FP	Fluticasone propionate
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GDPD1	Glycerophosphodiester phosphodiesterase domain containing 1
GILZ	Glucocorticoid- Induced Leucine Zipper
GINA	Global Initiative for Asthma guideline
GLUL	Glutamate-ammonia ligase
GRE	Glucocorticoid response element
GRIP1	Glucocorticoid receptor interacting protein 1
GRα	Glucocorticoid receptor alpha
GRβ	Glucocorticoid receptor beta
HAT	Histone acetyltransferase
HDAC2	Histone deacetylase 2
HMC-1	Human mast cell line 1
HSP90	Heat shock protein 90
IFNγ	Interferon gamma
IgE	Immunoglobulin E
IkB-α	mitogen-activated protein kinase phosphatase 1
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
IRF-1	interferon regulatory factor
MAPK	Mitogen-activated protein kinase

MC	Mast cells
MKP-1	Mitogen-activated protein kinase phosphatase 1
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
NEAA	Non-essential amino acid
NER	Nuclear extrication reagent
NF-kB	Nuclear factor kb
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
nGRE	Negative glucocorticoid response element
NICE	National Institute for Health and Care Excellence
PBMCs	peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PER1	Period homolog 1 (Drosophila)
PIK3R1	Phosphoinositide-3-kinase, regulatory subunit 1 (alpha)
PP2A	protein phosphatase 2A
PP5	protein phosphatase 5
PTP	protein tyrosine phosphatases
qPCR	Quantitative Polymerase Chain reaction
RNA	ribonucleic acid
RT	Reverse transcription
SCF	Stem cell factor
SEM	Standard error of the mean
SLC19A2	Solute carrier family 19 (thiamine transporter), member 2
SN	Supernatant

SP	Sodium pyruvate
TGF-β	transforming growth factor beta
TNFAIP3	Tumour necrosis factor, alpha-induced protein 3
TNFα	Tumor necrosis factor alpha
TSC22D3	TSC22 domain family, member 3
T cells	T lymphocyte
iNOS	Nitric oxide synthases
TLSP	Thymic stromal lymphopoietin
B cells	B lymphocyte
CRTH2	Prostaglandin D2 receptor 2
HSP90	Heat Shock Protein 90
SRC	Steroid Receptor Coactivator
RT-PCR	Reverse transcription polymerase chain reaction
siRNA	Small interfering RNA (silencing RNA)
GM-CSF	Granulocyte-macrophage colony-stimulating factor
ADARB1	Adenosine deaminase, RNA-specific, B1
AFF1	AF4/FMR2 family, member 1
AK2	Adenylate kinase 2
AMPD3	Adenosine monophosphate deaminase 3
ANGPTL4	Angiopoietin-like 4
ANXA4	Annexin A4
AQP1	Aquaporin 1 (Colton blood group)
ARID5B	AT rich interactive domain 5B (MRF1-like)
ASPH	Aspartate beta-hydroxylase

ATF4	Activating transcription factor 4 (tax-responsive enhancer element B67)
BCL6	B-cell CLL/lymphoma 6
BMPER	BMP binding endothelial regulator
CALCR	CALCITONIN RECEPTOR
CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha
CEBPB	CCAAT/enhancer binding protein (C/EBP), beta
COL4A2	Collagen, type IV, alpha 2
CREB1	CAMP responsive element binding protein 1
CREB3	CAMP responsive element binding protein 3
CREB3L4	CAMP responsive element binding protein 3-like 4
CTGF	Connective tissue growth factor
CYB561	Cytochrome b-561
DDIT4	DNA-damage-inducible transcript 4
DIRAS2	DIRAS family, GTP-binding RAS-like 2
DUSP1	Dual specificity phosphatase 1
EDN1	Endothelin 1
EHD3	EH-domain containing 3
ERRFI1	ERBB receptor feedback inhibitor 1
FKBP5	FK506 binding protein 5
FOSL2	FOS-like antigen 2
GDPD1	Glycerophosphodiester phosphodiesterase domain containing 1
GHRHR	Growth hormone-releasing hormone receptor
GLUL	Glutamate-ammonia ligase

GOT1	Glutamic-oxaloacetic transaminase 1, soluble (aspartate aminotransferase 1)
H6PD	Hexose-6-phosphate dehydrogenase (glucose 1-dehydrogenase)
HAS2	Hyaluronan synthase 2
HNRNPLL	Heterogeneous nuclear ribonucleoprotein L-like
IL10	Interleukin 10
IL1RN	Interleukin 1 receptor antagonist
IL6	Interleukin 6 (interferon, beta 2)
IL6R	Interleukin 6 receptor
KLF13	Kruppel-like factor 13
KLF9	Kruppel-like factor 9
LOX	Lysyl oxidase
MERTK	C-mer proto-oncogene tyrosine kinase
MT1E	Metallothionein 1E
MT2A	Metallothionein 2A
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
NR3C1	Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)
PDGFRB	Platelet-derived growth factor receptor, beta polypeptide
PDP1	Pyruvate dehyrogenase phosphatase catalytic subunit 1
PDCD7	Programmed cell death 7
PER1	Period homolog 1 (Drosophila)
PER2	Period homolog 2 (Drosophila)

PIK3R1	Phosphoinositide-3-kinase, regulatory subunit 1 (alpha)
PLD1	Phospholipase D1, phosphatidylcholine-specific
PLEKHF1	Pleckstrin homology domain containing, family F (with FYVE domain) member 1
POU2F1	POU class 2 homeobox 1
POU2F2	POU class 2 homeobox 2
RASA3	RAS p21 protein activator 3
RGS2	Regulator of G-protein signalling 2, 24kDa
RHOB	Ras homolog gene family, member B
RHOJ	Ras homolog gene family, member J
SESN1	Sestrin 1
SGK1	Serum/glucocorticoid regulated kinase 1
SLC10A6	Solute carrier family 10 (sodium/bile acid cotransporter family), member 6
SLC19A2	Solute carrier family 19 (thiamine transporter), member 2
SLC22A5	Solute carrier family 22 (organic cation/carnitine transporter), member 5
SNTA1	Syntrophin, alpha 1 (dystrophin-associated protein A1, 59kDa, acidic component)
SPHK1	Sphingosine kinase 1
SPSB1	SplA/ryanodine receptor domain and SOCS box containing 1
STAT5A	Signal transducer and activator of transcription 5A
STAT5B	Signal transducer and activator of transcription 5B
TBL1XR1	Transducin (beta)-like 1 X-linked receptor 1

TNF	Tumour necrosis factor
TNFAIP3	Tumour necrosis factor, alpha-induced protein 3
TSC22D3	TSC22 domain family, member 3
USP2	Ubiquitin specific peptidase 2
USP54	Ubiquitin specific peptidase 54
VDR	Vitamin D (1,25- dihydroxyvitamin D3) receptor
VLDLR	Very low-density lipoprotein receptor
XDH	Xanthine dehydrogenase
ZFP36	Zinc finger protein 36, C3H type, homolog (mouse)
ZHX3	Zinc fingers and homeoboxes 3
ZNF281	Zinc finger protein 281
ACTB	Actin, beta
B2M	Beta-2-microglobulin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HPRT1	Hypoxanthine phosphoribosyltransferase 1
RPLP0	Ribosomal protein, large, P0
HGDC	Human Genomic DNA Contamination
RTC	Reverse Transcription Control
RTC	Reverse Transcription Control
RTC	Reverse Transcription Control
PPC	Positive PCR Control

1.Chapter one

Introduction

1.1. Asthma:

1.1.1. Overview and Definition:

Asthma is a heterogeneous disease characterised by chronic inflammation of the airways, which results in abnormal lung function and breathing problems. Asthma is a common condition with a prevalence of ~300 million people around the world that still kills 250,000 patients yearly worldwide [1], a number that keeps rising in developed countries [2]. Asthma can affect people of all ages. In the UK for example, one child out of 11 children suffers from asthma, with at least two children with asthma in each classroom [3]. Also, there are around 5.4 million people diagnosed with asthma only in the UK, including 1.1 million of children [3]. Asthma is associated with increased mortality and morbidity and it can impact the patient's social life causing absence from either school and work. In the UK only, an estimated 1.1 billion of pounds is spent on asthma care, a figure that include 666 million for prescriptions only.

Asthma is characterised by variable airway obstruction, airway hyper-responsiveness, inflammation and remodelling of the airways. This remodelled airway is characterised by an increase in airway smooth muscle mass, mucus hypersecretion, sub-epithelial membrane thickness, mucous gland hyperplasia and infiltration of various immune cells such as T cells, mast cells and eosinophil cells [4]. Patients with asthma often present with breathlessness, coughing, wheezing and chest tightness and this results in a significant limitation of people's quality of life. The definition of asthma according to GINA 2015 is "*Asthma is a heterogeneous disease, usually characterised by chronic airway inflammation. It is defined by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation*" [5].

1.1.2. Asthma pathophysiology:

The symptoms of asthma including airway obstruction and exacerbation results from a combination of different factors which involves airway inflammation, remodelling and hyperresponsiveness **(Figure 1.1)**.

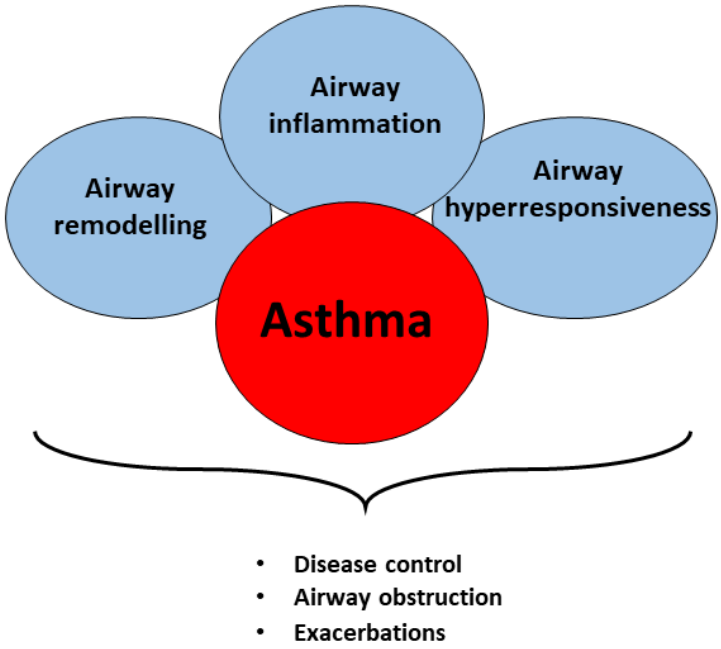

Figure 1.1: key asthma pathological features.

Asthma is believed to result from the intreaction of various factors occurring in the airway including chronic inflammatory procces which has associated with the structural changes (airway remodelling) and impaired lung function (airway hyperresponsiveness).

1.1.2.1. Airway inflammation:

There is no doubt that airway inflammation plays a central role in the pathophysiology of asthma by driving asthma's main characteristics including airflow obstruction, airway remodelling and bronchial hyper-responsiveness **(Figure 1.2)**. Airway inflammation can be triggered by several allergens, such as house dust mite, grass and tree pollens, when they are up-taken by dendritic cells. These activated dendritic cells initiate the typical TH2 response leading to the activation of different immune cells including eosinophils, neutrophils, mast cells and basophil cells which infiltrate in the airways and regulate pathological changes via the release of different pro-inflammatory cytokines (see below for details) [6]. The key Th2 cytokines that play a role in allergic asthma pathogenesis include IL-4, IL-5 and IL-13. IL-13 can stimulate the expression of various inflammatory mediators (iNOS, eotaxin, IL-33, TLSP) from lung structural cells such as the epithelium and airway smooth muscle, regulate eosinophil activation, and initiate IgE isotype switch by B cells **(Figure 1.2)** [6]. IL-5 is essential in the regulation of eosinophil proliferation, differentiation and survival while IL-4 participates in the proliferation and activation of Th2 cells and the stimulation of IgE isotype class-switching from B cells [7]. Th2 pathways lead to activation of both eosinophils or mast cells, which secrete a variety of pro-inflammatory mediators such as chemokines, cytokines and growth factor which initiate and/or perpetuate the inflammatory process in the lung [8-10]. Th2 inflammation can also be initiated as a consequence of alarmin production by the epithelium following exposure to different stimuli (allergens, viruses, helminths, environmental pollutants), known to exacerbate asthma. The best investigated alarmins in asthma pathogenesis using murine models include TLSP, IL-33 and IL-25 which have been all shown to activate the production of TH2 cytokines and stimulate key asthmatic features including allergic airway inflammation and airway

hyperresponsiveness [7,11]. This raises the possibility that alarmins may play a key role in driving asthma exacerbations by amplifying the TH2 inflammatory process in the lungs.

Conclusions from studies that have used either biased and unbiased analyses of several patient parameters including types of triggers, clinical characteristics and/or inflammatory markers have demonstrated that asthma is a syndrome composed different clinical phenotypes with at least two distinct TH2 profiles, called TH2 high and TH2 low. The Th2 high profile has been defined by the presence of high blood eosinophils of >220/mm3 and sputum eosinophil >3% while the TH2 low was characterized by the low level of Th2 cytokines and high level of Th1 cytokine including Interferon gamma (IFN-γ), Tumor necrosis factor alpha (TNF-α) and IL-2 [7,12]. Th2 low inflammation is mainly linked to obesity, post-infection and smoking and is less responsive to corticosteroid treatment [13]. Currently, there are a number of promising TH2-directed therapies being trialled in patients with asthma using monoclonal antibodies directed against TH2 regulators such as IL-13, IL-4, IL-5, CRTH2 and TLSP [7,14-16].

TH2 inflammation is a simplified concept that explains the development of airway inflammation in asthma; in fact, evidence shows that structural cells, by their ability to secrete various cytokines/chemokines, can also contribute to airway inflammation in asthma. Several *in vivo* and *in vitro* reports have shown that airway smooth muscle is an important player in asthma pathogenesis via the regulation of various feature including airway hyperresponsiveness, airway remodelling and inflammation [17-20].

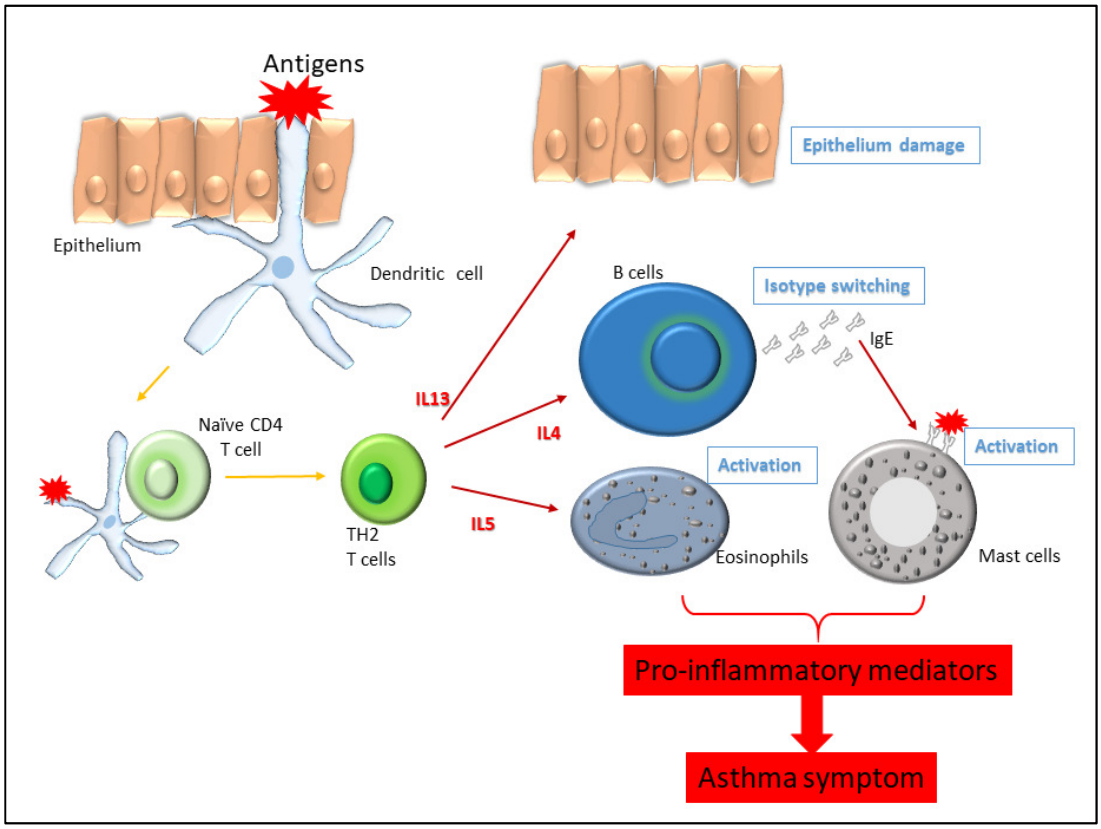

Figure 1.2: This figure represents the Th2 airway inflammation mechanism in asthma.

Adapted from [6]. The inflammation cascade starts when an antigen is recognised by the dendritic cell and results in T cell switching to Th 2 cells which mediate Th2 immune response via the production of various cytokines including IL-13, IL14 and IL5.
Various cell types can be affected by these cytokines including the epithelium by IL-13 to increase mucus secretion, B cells by IL-4 to initiate IgE production and eosinophils by IL-5 which increase their activation and recruitment in the lung. The allerge both mast cells and the eosinophils contribute to asthma pathogenesis.

1.1.2.2. Airway hyperresponsiveness

Airway hyperresponsiveness (AHR) is defined as an exaggerated response to various pharmacological stimuli such as methacholine and histamine (direct stimulus) or other non-specific stimuli such as exercise and cold air (indirect stimulus), leading to excessive narrowing of the airway in patients with asthma [21]. AHR is usually diagnosed by assessing the 20% fall in Forced Expiratory Volume (FEV1) induced by provocative doses of bronchoconstrictor agents [22]. The degree of airway hyperresponsiveness correlates with asthma severity [21] and has been extensively used for the diagnosis of asthma, especially in patients with little or no symptoms. AHR reflects the increased contraction of the bronchial smooth muscle, the primary effector tissue regulating the bronchomotor tone in the lungs. The mechanisms underlying AHR have not been elucidated although it is believed to be related to the phenotypic changes of the ASM including its increased mass (due to either ASM hyperplasia and/or hypertrophy) or resulting from the remodelling of the airways leading to an alteration of the factors opposing ASM contraction [23]. Indeed, there is a correlation between AHR and basement membrane thickness [24], extracellular matrix [25], airway wall thickness [26] and sputum eosinophil cell [27].

In addition to regulating airway remodelling, airway inflammation has been described as a critical player in AHR since a number of *in vitro* and *in vivo* studies clearly showed that different inflammatory stimuli (TNFα, IL-17, TGFß, IL-13, viruses, etc…), present in high levels in asthma, can experimentally reproduce AHR to contractile agonists [28-31]. The mechanisms responsible for cytokine-induced excessive ASM contraction are multiple and involved changes in contractile receptor expression, calcium signalling and rho pathways [30]. Recent studies have reported that mast cells could also play a detrimental role in AHR. In patients, mast cell infiltration was closely associated

with AHR, possibly via their ability to modulate the contractile function of ASM [27,32]. One proposed mechanism is the modulation of alpha-smooth muscle actin expression in ASM [33] as the number of mast cell infiltrated within the asthmatic ASM was found to correlate with alpha-smooth muscle actin content [34]. This finding was confirmed *in vitro* by co-culturing ASM and human lung mast cells [35]. Although the precise mechanisms by which mast cells modulate contraction of ASM have not been elucidated, mast cells can secrete a variety of cytokines which have been reported to induce excessive ASM contraction *in vitro* [34,35].

1.1.2.3. Airway remodelling

The advent of bronchoscopy techniques such as endobronchial biopsies has allowed doctors to demonstrate the presence of structural changes within the airway wall of asthmatics. These changes known as airway remodelling included increased ASM mass [36], epithelium dysfunction and thickening of basement membrane [37,38], subepithelial fibrosis and mucus hypersecretion [39] (**Figure 1.3**). All these features of airway remodelling have been shown to correlate with asthma severity, AHR and persistent airflow obstruction [40-42]. The inflammatory process and infiltration of the immune cells into the airway have been seen as the leading cause of the remodelling features in asthma [43]. For example, T cells [44], mast cells [32], and eosinophils [9,45] which infiltrate in asthmatic airways can influence the airway structure and function via the secretion and action of various cytokines including IL-4, IL-5, IL13, transforming growth factor beta (TGF-β), histamine and tryptase [46-49].

In asthma the airway epithelium is characterised by the loss of ciliated area [50], epithelium shedding [51], epithelium proliferation [52], increased mucus secretion [53] and increased number of goblet cells [54,55]. All of these features affecting the

epithelium behaviour are believed to be influenced by an imbalance of extracellular matrix (ECM) production and degradation creating a pro-fibrotic environment enhanced by the presence of growth factors such as TGFβ and fibroblast growth factor (FGF) among others [34,49,56]. Epithelium damage is associated with changes in expression of epidermal growth factor receptors (a marker of activation and injury) and CD44 (a marker of wound repair) which are both increased in asthmatics [57]. In addition, epithelium can also contribute to airway remodelling by producing various factors including growth factors [58]. Also, the thickness of reticular basement membrane located under the epithelium is another key marker of airway remodelling in asthma, and this has been linked to the increased deposition of collagens III and V [59].

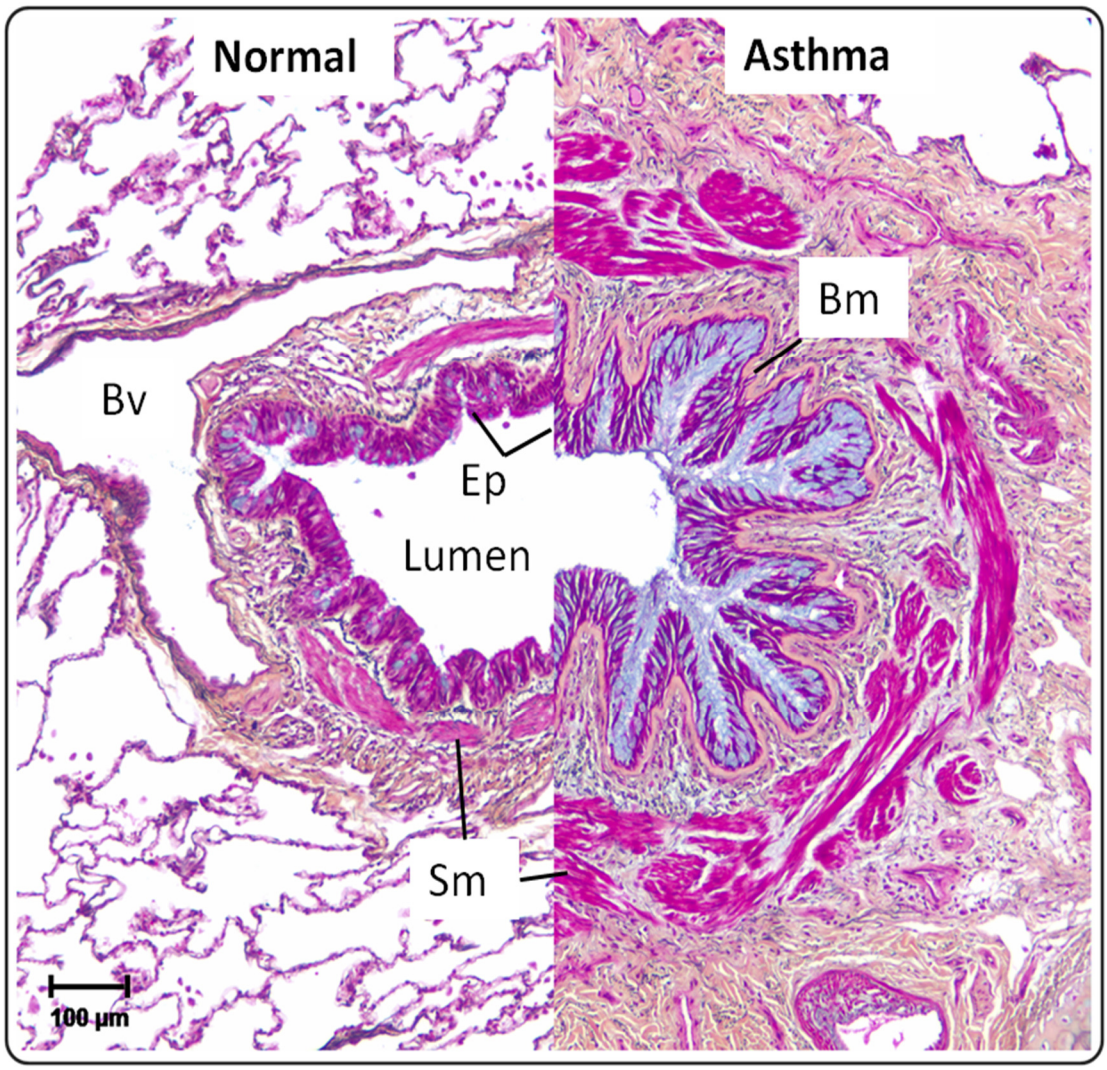

Figure 1.3: Airway structures in healthy and asthmatic airways

This graph represents the dramatic structural changes of the airways known as airway remodelling. The left panel represents a healthy airway, while right one shows an asthmatic airway. Epithelium lumen (Ep), smooth muscle (Sm), basement membrane (Bm) and blood vessel (Bv) (taken from [60])

1.1.3. Asthma phenotype or endotype

A number of different studies using biased and unbiased clustering methods have shown that asthma is a heterogeneous disease composed of different subgroups of clinical phenotypes [61,62]. Asthma phenotyping (observable characteristics) and endotyping (pathophysiological characteristics) are the common subgrouping methods that have been widely used to define asthma heterogeneity. Asthma phenotyping was traditionally based on whether asthma was either *intrinsic,* usually triggered by non-allergic factors and manifested by different clinical presentations (obesity, severe asthma, treatment-refractory asthma) and inflammatory markers (high eosinophils or neutrophils), and *extrinsic* asthma driven by allergens [13,63,64]. Recently, the inclusion of asthma endotype (pathobiology) by considering a set of different biological/inflammatory markers, allows to demonstrate the existence of Th2-high and Th2-low asthma where Th2-high asthma can be characterised by the presence of the Th2 inflammatory signature with IgE production, the Th2 cytokines IL-3,IL-4, IL-5, IL-9, IL-13 and high levels of eosinophils in blood and sputum [13,15,16,63,65]. The Th2-low type is characterised by the lack of Th2 signature and the presence of TNFα, IL-1, IL-8, IL-17 and IL-23 and neutrophilia [15,16]. A number of excellent reviews have discussed the different asthma phenotypes and their pathophysiological signatures and the challenge of finding better biomarkers to guide patients' response to the right therapies [13,15,65]. It is clear that these phenotype-specific therapies will revolutionise the treatment options in asthma, especially for patients with Th2 asthma where different specific monoclonal antibodies are currently being trialled in patients [13,15,65].

1.1.4. Asthma treatment:

The goals of current asthma treatment and guideline management are to control asthma symptoms and improve the quality of life of asthmatic patients. The most used medicines to treat asthma are the anti-inflammatory drugs called corticosteroids, the bronchodilatory therapy using β-2 agonists and leukotriene modifiers. More recent drugs, used in TH2 asthma, include monoclonal antibodies (see below) which have proven to be effective in treating 5-10% of patients where the disease is difficult to control. These patients fail to gain any clinical benefit from the traditional therapies even at high doses or with the use of oral corticosteroids, and the cause and mechanism of severe asthma remain unknown [9,66,67]. These severe asthma patients have a poor disease control, suffer from several exacerbations with a greater risk of dying and have a high percentage of hospital admission and treatment [67]. It is therefore essential to design better drugs with improved clinical efficacy.

Phenotyping asthma patients has led to the design of better personalized medicine. Numerous clinical trials are currently investigating different anti-Th2 therapies by targeting different key TH2 cytokines such as anti-IL5 (mepolizumab), anti-IL5 receptor (benralizumab), anti-IL13 (lebrikizumab), anti-IgE (omalizumab) and anti-IL4 receptor (dupilmab). Omalizumab and mepolizumab are the only therapies currently approved for the treatment of patients. Because of their role in promoting TH2 inflammation, alarmins such as TSLP (AMG157) (and possibly IL-25/IL-33 axis) are also being studied in asthmatic patients [13,15]. Alternatively, bronchial thermoplasty (BT) has shown promising results in the treatment of severe patients. BT is an endoscopic procedure approved in 2010 by the FDA which is based on the application of 65°C radiofrequency energy in the large airways. It is recommended for severe asthma patients and was originally designed to reduce the amount of airway smooth muscle that is responsible for

the abnormal airway obstruction [68]. BT can be used in both TH2 high and TH2 low patients who do not respond to currently available therapies including monoclonal antibodies. BT therapy has shown to reduce severe exacerbations and improve the quality of life [69].

1.2. Corticosteroids

Overview:

Glucocorticoids are the most potent anti-inflammatory medicine currently available due to their pleiotropic effects on various immune cells [70,71]. CSs have been used widely for asthma treatment with undeniable effects including the improvement of asthma control, lung function, and bronchial hyper-responsiveness. According to Global Initiative for Asthma guideline (GINA) [5], CSs are prescribed for patients with a mild, moderate, or severe disease with different dosage and efficacy. However, a minority of asthmatic patients (around 5-10%) poorly respond to CS who often require high doses of inhaled or oral CSs [9].

1.2.1. Molecular Mechanisms of Action of Corticosteroids

The anti-inflammatory actions of CSs are mediated via two distinct mechanisms called trans-activation and trans-repression which regulate the inflammatory process via the inhibition of the transcription and/or translation of several pro-inflammatory genes.

1.2.1.1. Transactivation

CSs signalling pathway begins with the passive diffusion of CSs into the cytoplasm and the binding to the glucocorticoid receptor alpha (GRα). Ligand binding to the receptor results in a conformational change of GRα that causes a disociation from other molecular chaperones such as heat shock protein 90 (HSP90) and immunophilins

[72,73]. As a consequence, the bound CS-GRα translocates to the nucleus and activates the expression of mitogen-activated protein kinase phosphatase 1 (MKP-1), Glucocorticoid-induced leucine zipper (GILZ), FK506 binding protein 5 (FKBP5), or inhibitory protein of NF-kB (IκBα) [74,75]. The transactivation signalling pathway depicted in **(Figure 1.4)** is initiated when two of the ligand CS-GRα (to form a homodimer) bind to the palindromic Glucocorticoid Response Elements (GREs) located at the promoter site of the different target genes. Maximal transcription of the gene by the homodimer CS-GRα is ensured by the recruitment of different key transcriptional coactivators such as glucocorticoid receptor interacting protein 1 (GRIP1) from the SRC/p160 family members which have histone acetyltransferase (HAT) activation [76]. HAT activity controls the acetylation of the core histone proteins on the chromosome and results in uncoiling of the DNA [77], thus facilitating the binding of homodimer CS-GRα to GREs in target genes and the recruitment of RNA polymerase. Examples of anti-inflammatory action of CS-inducible proteins include MKP-1 that inhibits mitogen-activated protein kinase (MAPK) pathways and GILZ or IκBα which inhibit the Nuclear Factor-κB (NF-κB) [78].

Another CS anti-inflammatory mechanism occurs when the homodimer CS-GRα binds to negative GREs (nGREs) that are located in the start region of pro-inflammatory genes such as IL-6 and block their transcription activities. Binding of CS-GRα to nGREs also inhibits the coiling of the chromosome [73,79]. Lastly, CSs also induce the expression of ribonuclease or other mRNA destabilising proteins as an additional inhibitory mechanism by which trans-activation leads to the destabilisation of pro-inflammatory mRNAs and reduces their expression levels [79].

1.2.1.2. Trans-repression

Another classical anti-inflammatory mechanism of CSs is called *trans-repression* that is also initiated via the identical process of GRα activation in the cytoplasm by CS, with the exception that the ligand-receptor (CS-GRα) complex does not act as a homodimer. The bound CS-GRα can inhibit gene expression via the uncoiling of the DNA following the recruitment of histone deacetylase 2 (HDAC2) [74]. However, the main anti-inflammatory mechanism occurs when CS-GRα complex binds directly to key pro-inflammatory transcription factors such as NF-κB and activator protein -1 (AP-1) in the nucleus and inhibits their pro-inflammatory activity [80]. In addition, CS-GRα complex can block the phosphorylation of JNK or p38 MAPK, thus inhibiting their activation cascade or nuclear translocation [74,78]. Recently, Amrani lab has discovered that the pro-inflammatory transcriptional factors IRF-1 bind to a transcriptional coactivator called GRIP-1 that is required for the induction of pro-inflammatory genes [81]. Because GRα also requires GRIP-1 for its transcriptional activities, competing for GRIP-1 by both GRα and IRF-1 has been shown as a new mechanism for inhibiting IRF-1 dependent genes [17].

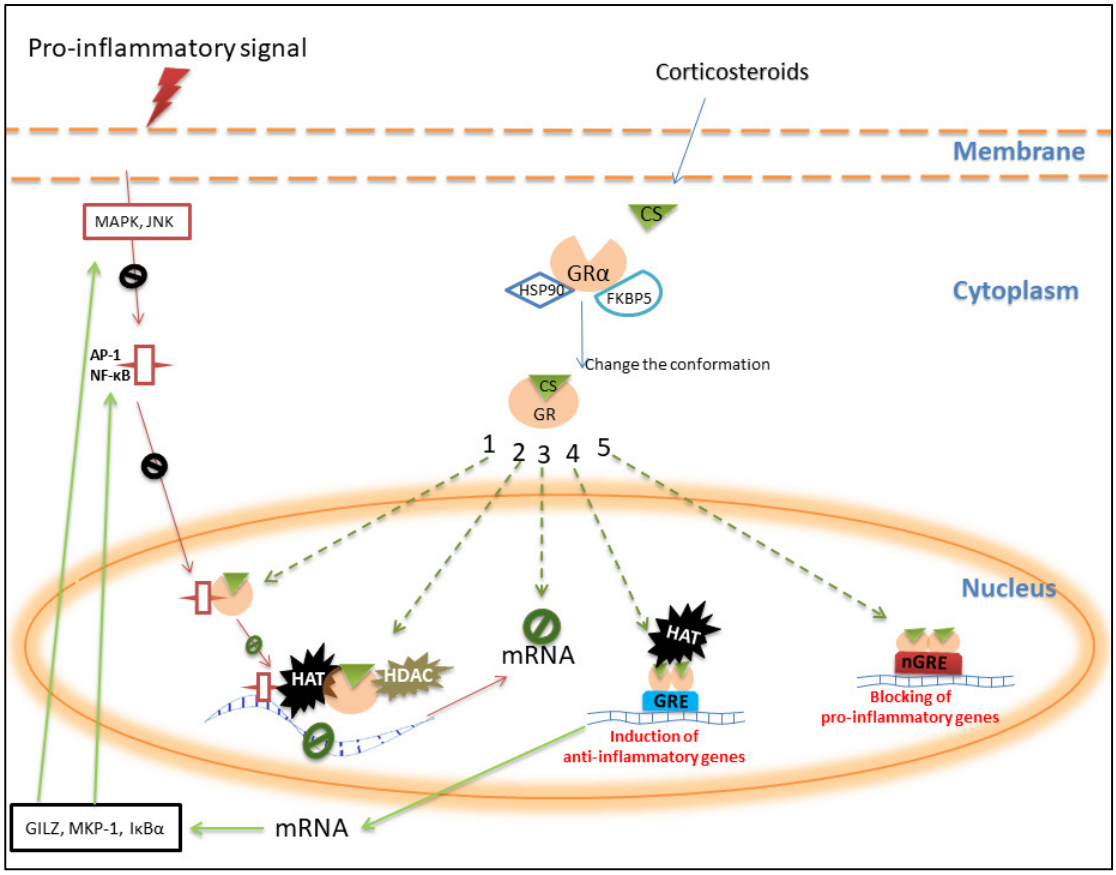

Figure 1.4: Molecular mechanisms mediating the anti-inflammatory actions of CSs.

After the diffusion of CS into the cell and binding to GRα, the formed CS-GRα complex can interacts directly to key pro-inflammatory transcription factors in the nucleus and inhibits their ability of induce the transcription of the target genes (1). Alternatively, CS-GRα complex can inhibit their action by recruiting histone deacetylase 2 (HDAC2) by causing uncoiling of the chromosome (2). CS-GRα can destabilise the mRNA level of pro-inflammatory proteins (3). The dimer CS-GRα interacts with GRE and induces the expression of anti-inflammatory genes (4). CS-GRα also binds to nGREs and negatively inhibits the expression of target genes (5)(adapted from [79].

1.2.2. Corticosteroid Insensitivity

1.2.2.1. Clinical Definition:

Patients who failed to adequately respond to CSs therapy have been labelled as either corticosteroid-resistant or corticosteroid-insensitive. Leung and colleagues have defined corticosteroid resistant patients as those who do not improve their lung function assessed by the Forced Expiratory Volume in one second (FEV_1) by more than 15% following a daily course of 40 mg prednisolone for 1 week or 20 mg for two weeks [82]. The issue with this definition is that these steroid-resistant patients only represent a small group of all asthmatics. Rather, it is important to use the term "corticosteroid-insensitive patients" to reflect better the clinical heterogeneity of patients who remain difficult to manage despite their daily high doses of inhaled or oral CS use.

1.2.2.2. Clinical Importance of CSs Insensitivity:

CS insensitivity is the primary issue in severe asthma patients who are more likely to die because of the lack of disease control. The reduced sensitivity of these severe asthma patients to CSs is characterised by the need of a high dosage of inhaled and/or oral CSs often requiring alternative therapies such as monoclonal antibodies (omalizumab or mepolizumab) or bronchial thermoplasty (GINA and NICE guideline). Although severe asthma patients only represent around 5-10% of the asthma population [67,83], they account for more than the half of the total cost of asthma treatment due to their frequent hospital admissions, emergency room visits, high rate of severe exacerbations and high dosages of medications [67]. Severe asthma is therefore defined clinically as a group of asthma patients whose disease cannot be adequately controlled despite a high dose of inhaled and systemic CS. According to NICE (guideline TA278, 2013), '*severe persistent allergic asthma is defined as poor control despite eliminating*

environmental allergens and correctly optimising standard care'. The cause of this uncontrolled asthma is unknown [9], and different studies have tried to investigate the potential mechanisms causing the reduced sensitivity of severe asthma patients to CS therapy.

1.2.3. Potential Mechanisms of CS insensitivity:

1.2.3.1. Mechanisms from studies performed in immune cells:

The mechanism of corticosteroid insensitivity in asthma was first investigated using different cellular models derived from immune cells such as alveolar macrophages, T cells and peripheral blood mononuclear cells (PBMCs). For instance, it has been shown that the reduced CS sensitivity in T cells from severe asthma was associated with a high expression level of GRβ (n=6), an isoform of GRα, that acts as a dominant negative inhibitor of GRα and interferes with its transcription activities [84]. Other studies showed that GRβ can interfere with CS pathways in severe asthma via multiple mechanisms including the inhibition of GRα nuclear translocation or GRα interaction with GRE on the target genes as well as GRα transcription activity [74,79]. Although increased GRβ gene expression has been reported in both infiltrating blood cells and in airway epithelial cells of asthmatic patients (n=7) [85,86], the implication of GRβ in asthma still remains controversial. CS insensitivity could also result from an impaired nuclear ligand binding affinity of GRα as reported in PBMCs and T cells from severe asthma patients compared to healthy donors (n=19) [87]. Altered GRα phosphorylation due to a defect of the protein phosphatase 2A (PP2A) expression and/or activity and protein tyrosine phosphatases PTP-RR has been reported in the PBMCs of severe asthma patients (n=7) [88,89]. PP2A and PTP-RR are essential for regulating GRα function by affecting its phosphorylation of GRα at ser^{226} and its nuclear translocation [88,89].

Furthermore, abnormal phosphorylation of GRα is another mechanism that has been associated with CSs insensitivity in severe asthma. GRα phosphorylation at ser^{211}, ser^{203} and ser^{226} residues can affect the recruitment of indispensable transcriptional co-factors to GRα, its nuclear translocation and binding to the GREs on the target genes [73,78,90].

The different studies in immune cells show that no firm conclusion can be made regarding the mechanisms underlying CS insensitivity in severe asthma as different mechanisms have been reported in various cell types and in a limited number of patients.

1.2.3.2. Mechanisms from studies performed in ASM cells

Structural cells such as ASM cells also could also be involved in regulating CSs insensitive feature in severe asthma. *In vivo* and *in vitro* studies have shown that ASM is a source of various pro-asthmatic mediators that are capable of regulating various aspects of asthma including airway inflammation, airway hyperresponsiveness and airway remodelling [18,31,91]. The concept that ASM cells have a reduced *in vitro* response to corticosteroid therapy in severe asthma was first introduced by Chung's lab from Imperial College back in 2012 [20] and confirmed by Amrani's Lab. The authors found that the anti-inflammatory response of dexamethasone to suppress TNF-α-induced chemokine production including CXCL-8, CX3CL1, CCL-11 and CCL-5 was reduced in ASM cells derived from severe asthmatics compared to ASM cells from non-severe asthmatics or healthy subjects [20,92]. The proposed mechanisms to explain such impaired CSs sensitivity observed in cells from severe asthmatics include a reduced level of either cytosol level the GRα or its nuclear translocation [92,93]. In addition, ligand-induced GRα phosphorylation at serine 211 was also found to be reduced in cells from severe asthmatics [92]. This is an important observation as GRα phosphorylation at serine 211

was shown to be essential for both transactivation properties [94]. Amrani and colleagues, therefore, suggested that impaired GRα nuclear translocation in ASM cells from severe asthmatic resulted in the suppression of GRα transcription activity [94,95], as confirmed by measuring the reduced expression of the anti-inflammatory gene expression of GILZ seen in ASM cells from severe asthmatic compared to ASM cells from control [92].

Furthermore, ASM cells from severe asthmatic also expressed a high level of the protein phosphatase 5 (PP5) compared to ASM cells from control confirmed both by quantitative PCR and flow cytometry [92]. Interestingly, transfected ASM cells from severe asthmatic with siRNA for PP5 restored the nuclear translocation and enhanced GILZ gene expression and fluticasone's ability to inhibit TNFα-induced production of CCL11 and CCL5 compared to control [92].

Other mechanisms susceptible of altering CSs sensitivity involved the different signalling pathways p38 MAPK and NF-κB which have been reported to affect GRα trans-repression and trans-activation activities [20,93]. Interestingly, increased activation of p38 MAPK has been reported in ASM cells from severe asthmatics [20,93]. The addition of p38 inhibitor (SB203580) to either dexamethasone or fluticasone propionate restores the CS-induced GRα function measured by its nuclear translocation and expression of anti-inflammatory protein leucine zipper as well as reduction of chemokine expression of CCL11 and CXCL8 [20,90]. p38 MAPK attenuates GRα via phosphorylation of serine 203 and 211 residues.

However, most of the information regarding the mechanisms associated with CS insensitivity comes from the studies that have used an inducible model of CS insensitivity (i.e., healthy ASM cells treated with both TNF-α and IFN-γ [94,95]). This inducible model developed by Amrani's lab has been extensively used to dissect the mechanisms of CSs insensitivity in ASM cells [17] **(Figure 1.5)**. As reported in ASM cells from

severe asthmatics [92,94], CSs insensitivity of ASM cells induced by these cytokines was found to involve an increased expression of PP5 which inhibits CS-induced GRα function by reducing its phosphorylation at ser^{211} residues and transcriptional activities [92]. Furthermore, health ASM cells stimulated with TNF-α and IFN-γ resulted in high expression of GR-β [74,95], which was shown to attenuate cellular responses to CSs via the interference with GRα transcriptional activities [74]. Interestingly, short term exposure to TNF-α and IFN-γ also led to an impaired response to CSs, in a manner that was PP5 and GR-β independent but involving the expression of interferon regulatory factor-1 (IRF-1) [92,96]. IRF-1 was found to be functionally interacting with GRα by limiting GRα access to its transcriptional coactivator GRIP-1, thus reducing its transcription activities [81].

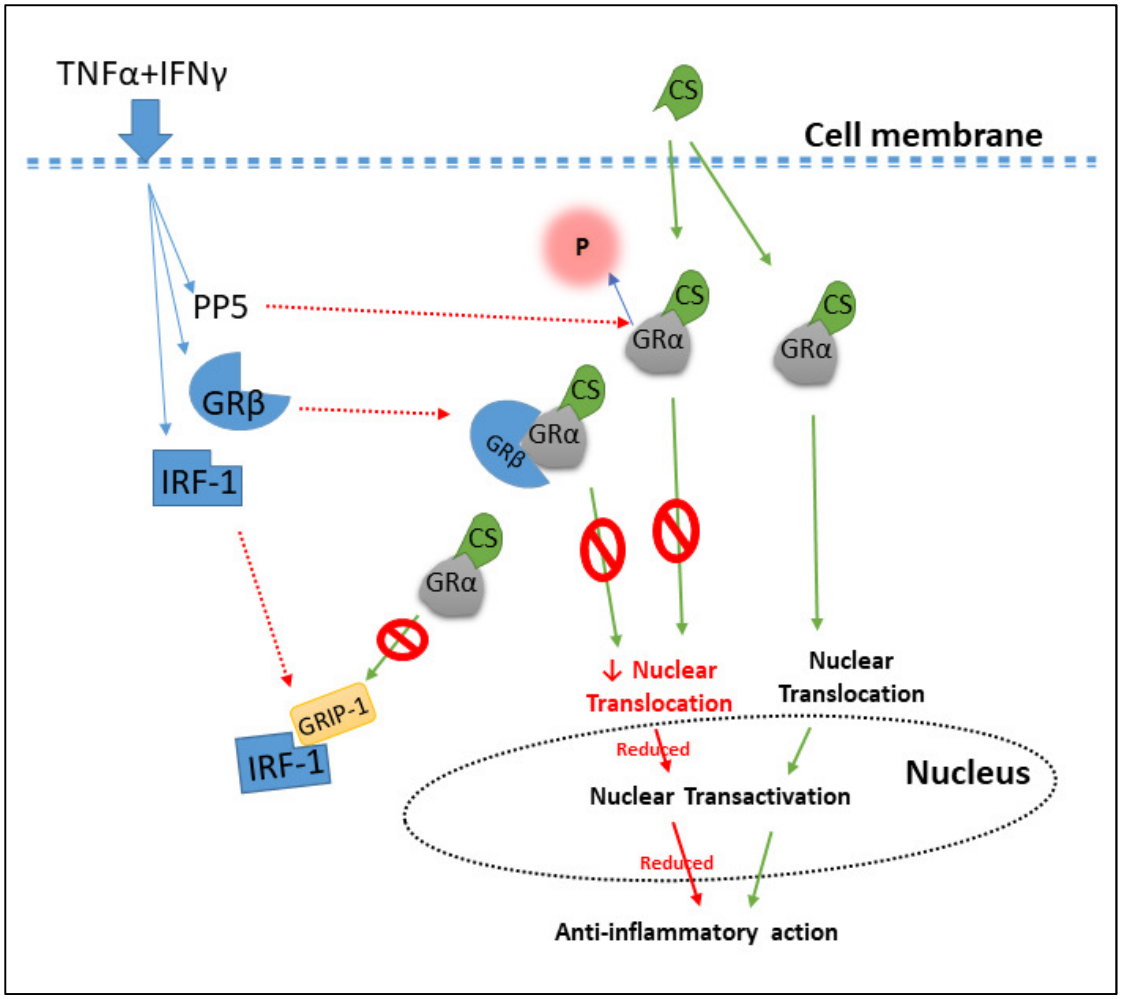

Figure 1.5: Summary of the molecular mechanisms responsible for CS insensitivity induced by TNF-α/IFN-γ in healthy ASM cell.

CS insensitivity can occur by increasing the level of PP5 which phosphorylates GRα on ser^{211} residues. Also, GRβ can bind to GRα-CS complex and block its nuclear translocation. Lastly, IRF-1 competes with GRα for the recruitment of GRIP-1. All of these mechanisms lead to a reduced GRα nuclear translocation and/or gene transcription. Adapted from [17]

1.3. Evidence of airway smooth muscle and mast cell interaction

1.3.1. Role of airway smooth muscle in asthma

Overview:

Airway smooth muscle (ASM) plays a key role in regulating airway narrowing in response to various pharmacological or natural agents such as histamine, methacholine or serotonin [91]. Accumulating *in vitro* and *in vivo* evidence from different groups has demonstrated that ASM could play a pivotal role in asthma pathogenesis by modulating various pro-asthmatic responses including i) airway hyper-responsiveness, ii) remodelling of the airway wall and iii) the local inflammatory process (reviewed in [17,97,98]).

1.3.1.1. ASM and airway inflammation:

Airway inflammation is characterised by the infiltration of activated inflammatory cells such as mast cells, eosinophils, T cells, neutrophils which can participate in the local inflammatory process [99]. ASM cells have been shown to produce a variety of chemokines that could participate in the Th2 and non-Th2 inflammation via the recruitment of these different inflammatory cells. For instance, ASM cells exposed to different inflammatory stimuli such as cytokines produce CXCL10, CXCL8, CXCL1 [100,101] CCL11 [102] and CCL5 [103] that promote inflammatory cell infiltration into asthmatic ASM bundle including mast cells, neutrophils, and eosinophils [101,103,104]. It is interesting to note that the production of CXCL1 by ASM cells, which has been shown to inhibit human lung mast cells chemotaxis in response to Th1 or Th2 cytokines, was dramatically reduced in cells from asthmatics [101]. This finding suggests that the increased of human lung mast cell

infiltration present within asthmatic ASM could also be due to the decreased production of inhibitory signals.

Also, ASM cells were also reported to produce cytokines that are important in initiating inflammation cascade. For instance, ASM cells can be involved in the allergic inflammation in asthma by their ability to secrete different alarmins such as IL-33 and Thymic stromal lymphopoietin (TSLP) [105,106] known to activate dendritic cells and other key inflammatory cells such as eosinophils, T cells and mast cells [106,107]. Interestingly, the levels of IL-33 and TSLP were found to correlate with asthma severity [105,106,108]. Because of their potential role in the Th2 response, both IL-33 and TSLP represent novel targets in the treatment of allergic asthma [17].

Lastly, ASM cells can produce survival factor for different inflammatory cells. ASM produces stem cell factor (SCF) and expresses adhesion molecules, such as CADM-1, which have been involved in enhancing the survival of mast cells, thus providing one mechanism explaining the persistent infiltration of mast cells within the ASM seen in asthmatics [109]. ASM can also produce GM-CSF, which is known to enhance eosinophil survival, thus also contributing to eosinophilic inflammation seen in Th2 asthma [19].

1.3.1.2. ASM cells and airway remodelling:

Structural changes of the airway wall in asthma affect different lung cells and are characterised by epithelium alterations, sub-epithelial fibrosis, mucus gland enlargement, sub-basement membrane thickening, ASM hyperplasia and hypertrophy and infiltration of various immune cells [43]. The impact of airway remodelling, and more specifically of the ASM changes, in asthma is likely due to the reduction of the airway lumen causing airway narrowing. Indeed, increased ASM mass was shown to correlate with impaired lung function (low FEV1) and asthma severity [110]. In fatal asthma, post-mortem

analyses showed SM hyperplasia and hypertrophy were also increased compared to healthy [111] (**Figure 1.6**). One key mechanism of airway remodelling is the migration of ASM cells which can be induced by various cytokines [112].

ASM cells could contribute to airway remodelling process via the release of various growth factors such as TGFβ which has been reported to act in an autocrine and paracrine manner to cause the differentiation and proliferation of ASM cells, regulate epithelial-to-mesenchymal transition (EMT) process and regulates mucus hypersecretion [34]. TGFβ also stimulates a pro-contractile phenotype in ASM by increasing levels of smooth muscle alpha actin, a feature that could contribute to fixed airflow obstruction [34]. The proliferation of lung fibroblasts and myofibroblasts as well as the production of extracellular matrix proteins and TGFβ are all responses that could participate in the remodelling process in asthma [113,114]. Also, production of the extracellular matrix such as collagen I, III and V and fibronectin by ASM cells in asthma can be involved in promoting airway wall thickening [98]. For instance, the presence of collagen I increases the proliferation of ASM cells in response to mitogens [98,115]. It is clear that production of TGFβ by ASM is a central player in airway remodelling, although these structural changes also could occur via the activity of infiltrated inflammatory cells in response to the different chemokines [101]. Biopsies studies from severe asthmatics showed that *in vivo* ASM produce a variety of chemokines and survival factors for key inflammatory cells [110].

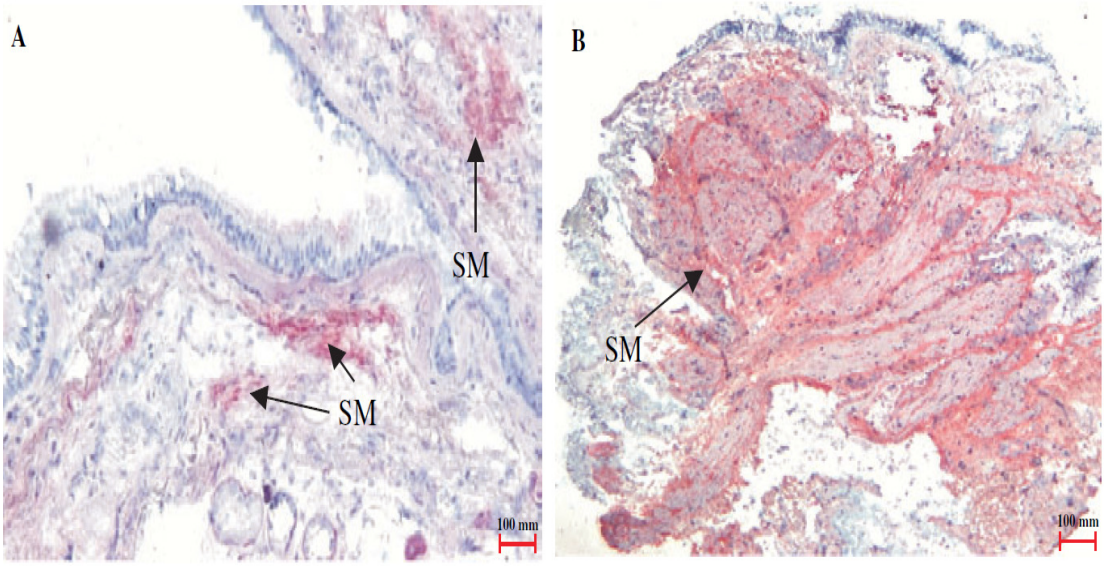

Figure 1.6: ASM remodelling in biopsies from healthy and asthmatic.

The proportion of ASM (SM) is significantly increased in asthma (B) compared to that seen in healthy control (A). This ASM proliferation contributes to the airway narrowing via the reduction of airway lumen (X40 magnification, taken from [116]).

1.3.1.3. ASM cells and airway hyperresponsiveness:

Airway hyperresponsiveness (AHR) can be defined as an exaggerated bronchoconstrictor response to a variety of stimulus that results in an excessive narrowing of the airways [117]. AHR can be diagnosed using direct pharmacological stimuli such as histamine or methacholine [118,119], and indirect stimuli such as exercise, cold air and house dust [120,121]. The mechanisms causing AHR are multiple and have included a contractile defect of ASM itself as suggested by several studies using tissues from asthmatic patients [36,98]. AHR can also be caused as a result of increased ASM mass (hyperplasia or hypertrophy), a central feature of the remodelling process occurring in the airway wall of severe asthmatics [116,122]. This increased ASM mass often correlates with disease severity and impaired lung function [110] **(Figure 1.6B)** and could participate in the excessive narrowing of the airways by either enhancing its increased contractile function or via thickening of the airway wall [123,124].

Changes in ASM function that could contribute to AHR include an increased expression of certain contractile proteins like-smooth muscle alpha actin [33], an observation that did correlate *in vivo* with the number of mast cells within the asthmatics ASM [34]. Co-culturing ASM and human lung mast cells did support the role played by mast cells in the regulation of AHR via a direct action on ASM [35]. Also, TNFα and matrix metalloproteinase-1 (MMP-1) have also been reported to regulate ASM contraction [125,126]. Several reviews have confirmed that various cytokines such as TNFα, IL-13, IL-1 and IL-17 can participate in AHR by enhancing the contractile function of ASM [28-31]. Different mechanisms have been associated although the main mechanism involved a change in calcium regulatory mechanisms.

1.3.2. Role of mast cells in asthma

Overview:

Mast cells are derived from myeloid progenitor cells which come from the bone marrow [127]. Unlike other immune cells which differentiate and mature in the bone marrow or the blood, myeloid progenitor cells which express $DC34^+$, $CD117^+$ (c-kit) and $CD13^+$ stay in the bloodstream as undifferentiated mononuclear cells [128,129]. These cells ($DC34^+$, $CD117^+$ (c-kit) and $CD13^+$) can differentiate into mature mast cells by IL3, SCF [128] and Lysophosphatidic acid [130]. Also, SCF, IL6 and IL10 are important for mast cell proliferation and survival [109].

Based on the inflammatory signals, immature circulating mast cells infiltrate into various tissues such as skin, gut, lung and to become mature mast cells [131,132]. There are two types of mature mast cells called mast cell positive for both tryptase and chymase (MC_{TC}) and mast cell positive for only tryptase (MC_T) and both can be detected in the human lung [133] and skin [134]. All mature mast cells, can produce a range of mediators can be release after activation known as preformed or synthesised mediators **(Table 1.1)**.

Table 1.1: Mast cell preformed and synthesised mediators:

Type of mast cell mediators	Mediators	Reference
Preformed	Histamine, Tryptase-α, -γ, -βI, Serotonin (5-HT), Dopamine, Polyamines, Chymase, β-hexosaminidase, β-glucuronidase, β-D-galactosidase, Arylsulphatase A, Cathepsins C, B, L, D, and E, Carboxypeptidase A, Cathepsin G, Granzyme B, TNF-α, IL-4, IL-15, Matrix metalloproteinases, Heparanase, Angiogenin, Active Caspase-3, Heparin, Chondroitin sulphate, MCP-1 (CCL2), RANTES (CCL5), MCP-3 (CCL7), eotaxin (CCL11), MCP-4 (CCL13), IL-8 (CXCL8), TGF-β, bFGF-2, VEGF, NGF, SCF, LTB4, Corticotropin-Releasing Hormone, Endorphin, Prostaglandin D2, E2, Endothelin-1, LL-37/Cathelicidin, Arylsulfatase, Renin, Substance P, Vasoactive Intestinal Peptide, Eosinophil Major Basic Protein (MBP), Leukotrienes B4, C4, Platelet Activating Factor, Kinogenases.	[135-138]
synthesised	IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-12, IL-13, IL-17, IL-33, IFN I and II, TNF-α, MIP-2β, Nitric Oxide, Complement Factor C3 and C5, SCF, GM-CSF, β-FGF, NGF, PDGF, TGF-β, VEGF.	[135,136,138]

There is no doubt that mast cells have a potent role in asthma through their ability to initiate and regulate the Th2 allergic inflammation in the lungs [4,139]. A number of different groups using immunohistochemistry applied to endobronchial biopsies have therefore investigated whether mast cell number and/or activation state were altered in the asthmatic airways (see **Table 1.2**). The conclusions of these studies were that mast cell number (stained with tryptase and/or chymase) were indeed increased in different lung tissues including the epithelium, lamina propria, and more interestingly in the ASM bundles [32,133,140]. Increased number of mast cell within these tissues correlated with disease severity [32], impaired lung function [32], and was not present in other conditions such as eosinophilic bronchitis [32]. Only one study found that the number of mast cells that were positive for both chymase and tryptase was increased in epithelium and submucosa of severe asthma and correlated with disease severity [133].

Table 1.2: Studies which investigated the presence of mast cells within the airways in asthma.

Studies	Finding
Pesci, Foresi et al. 1993 [141]	• Present in the epithelium • Higher in asthma.
Caroll, Mutavdzi et at. 2002 [142]	• Present in ASM bundle in fatal asthma • Degranulation of MC in ASM bundle correlates with disease severity.
Brightling, Bradding et al. 2002 [32]	• Present in ASM bundle in asthma • Number correlated with impaired lung function
Berger, Girodet et al. 2003 [143]	• Present in ASM bundle in asthma • Number correlated with TGFβ expression in ASM
Amin, Janson et al. 2005 [116]	• Present in the epithelium, lamina propria and ASM bundle in asthma. • Number higher in ASM bundle of allergic vs non-allergic asthma
Brightling, Ammit et al. 2005 [100]	• Present in ASM bundle in asthma • Number correlated with CXCL10 expression in ASM
El-shazly, Berger et al. 2006 [103]	• Higher number in ASM bundle in asthma. • Number correlated with neuropeptide vasoactive intestinal peptide expression in ASM
Begueret, Berger et al. 2007 [122]	• Present in ASM bundle in asthma
Woodman, Siddiqui et al. 2008 [34]	• Present in ASM bundle in asthma
Balzar, Fajt et al. 2011 [133]	• Higher MC_{TC} in epithelium and submucosa layer in severe asthma
Singhania, Rupani et al. 2017 [144]	• Gene expression of tryptase α/β1/β2 and carboxypeptidase A3 was higher in peripheral airways in severe asthma compared to healthy

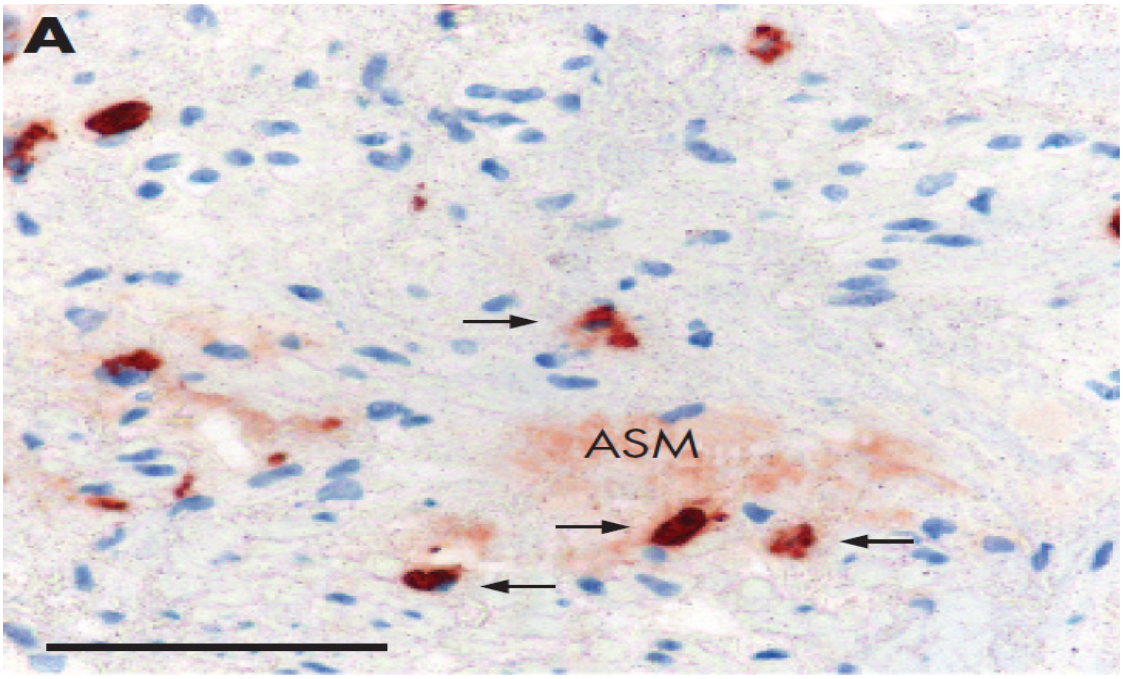

Figure 1.7: Asthmatic biopsy showing close association of ASM and tryptase-positive MC.

The number of mast cells within the ASM bundle was shown to correlate with impaired lung function (PC20) and was absent in eosinophilic bronchitis biosy. Immunochemistry assay. (magnification X400) [32]

1.3.2.1. Importance of mast cells mediators in asthma pathogenesis:

A number of mast cell products have been strongly implicated in the pathogenesis of asthma by being involved in the regulation of AHR and inflammation **(Figure 1.8)** [10,145]. Mast cell mediators such as IL-4 and IL-13 [146] have been shown to induce AHR in mice [147,148] and the Th2 inflammation via the activation of T cell proliferation, and B cell production of IgE while other mediators such as IL-5 can promote eosinophils infiltration and TNFα, another key pro-asthmatic cytokine produced by mast cells, is highly increased in biopsies from asthmatics [47] and has been linked to the regulation of airway inflammation, hyperresponsiveness and airway remodelling [149]. For instance, inhaled nebulized TNF-α causes AHR and increases neutrophils in sputum in healthy subjects [150]. Moreover, tryptase (mainly produced by mast cells) can contribute to airway remodelling by regulating the proliferation and differentiation of various structural cells in the lung including ASM cells [34,151]. Also, histamine, prostaglandins (PGD2), alarmins (TSLP, IL-33) and leukotrienes are produced by human lung mast cells can mediate pro-inflammatory cascade in asthma [152], (see previous sections of ASM and airway inflammation). The fact that the number of mast cells is higher in the epithelium and ASM bundle of asthmatic patients compared to healthy subjects suggests that interaction of mast cell-with lung structural tissues is vital in the disease [32,141]. For example, mast cell number or/ and degranulated mast cell was increased in asthmatic ASM bundle and correlated with AHR [32] and fatal asthma [142]. Also, mast cell number and degranulated mast cell were correlated with mucus in fatal and non-fatal asthmatic patients [153].

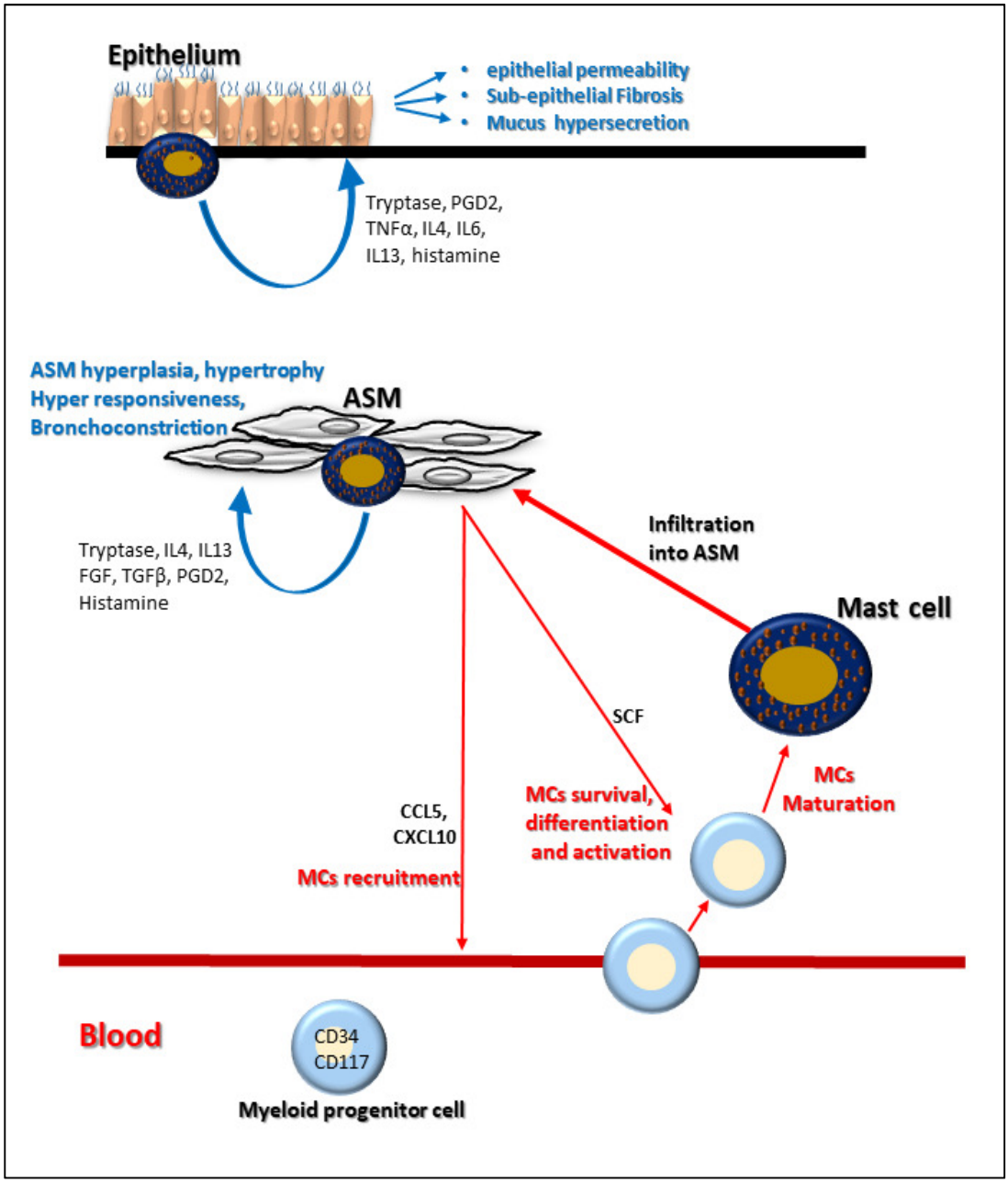

Figure 1.8: Implications of mast cell interaction with lung structural tissues in the pathogenesis of asthma.

Circulating progenitor cells infiltrate into the lung mature and interact with ASM or epithelium via the release a variety of mediators which can cause ASM hyperplasia and hypetrophy, change epithelial permeability, sub-epithelial fibrosis and mucus hypersecretion. Adapted from [10].

1.3.2.2. Therapeutic benefit of targeting mast cells in asthma:

As discussed before, human lung mast cell contains a variety of cytokines, chemokines and growth factors that are capable of initiating and/or perpetuating asthma pathogenesis by regulating the function of different lung structural tissues **(Figure 1.8)**. Different studies have therefore examined whether inhibiting mast cells mediators or mast cells degranulation can provide better anti-asthma treatment. One recent target currently investigated is the spleen tyrosine kinase (SYK) since it has been reported to be essential for mediating FcεR1-dependent mast cell degranulation and the early asthmatic responses [154]. Pre-clinical studies confirmed that SYK inhibition can abrogate ovalbumin-induced early asthma response in the Brown Norway rat model of allergic asthma and another model in mice [155,156], and suppress IgE-dependent airway constriction in an in ex vivo model of bronchoconstriction (human precision lung cut slices) [155]. However, the strongest evidence supporting a role of mast cells in asthma pathogenesis comes to the clinical benefits provided by the anti-IgE therapy called Omalizumab which was the first drug approved for the treatment of allergic asthma in 2003. Omalizumab is a humanised monoclonal antibody that binds to circulating free IgE and prevents mast cell activation. Treatment with a humanised monoclonal antibody is not without risk due to remaining mouse sequences and interference in immune regulation [157]. However, omalizumab therapy improved asthma symptoms, decreased the rate of exacerbations and reduced markers of inflammation and airway remodelling [158-160] and improve pulmonary function by assessing FEV1 and morning peak expiratory flow [158,159,161]. ***More importantly, omalizumab treatment was associated with a reduced usage of corticosteroid (both oral and inhaled) for patients with severe asthma [158,161,162]. This strongly suggests that mast cells play a central role in the reduced therapeutic response of patients to corticosteroid therapy.***

Mast cells produced cytokines, such as IL-5 [163], IL-4 [164], TLSP [165] and IL-13 [166], represent potential new therapeutic targets for their role in asthmatics with TH2 inflammation [138,146,167].

1.3.2.3. Mast cells and corticosteroid insensitivity:

A **direct** role of the mast cell in the regulation of CSs sensitivity in asthma has been suggested by studies that have shown that Omalizumab treatment was associated with a reduced dosage of oral CS in patients with severe disease [158,161,162]. However, studies have shown that various features involving mast cells are insensitive to CS therapy including mast cell infiltration in asthmatics ASM, their increased number in ASM of fatal asthma [168] which was found to correlate positively with AHR [32] as well as asthma severity [133]. There is, however, **indirect** evidence to link mast cells and CS responsiveness in asthma. *In vitro* studies have shown that a large number of mast cell mediators can, in fact, blunt CS sensitivity in various cell types **(Table 1.3) (Figure 1.9)**. This assumption comes from the fact that some mast cell mediators such as IL-2, IL-4 [167,169-171] or IL-13 [172], can be found highly expressed in the airways of asthma patients who are corticosteroid insensitive [173] even through other cell types such as T cells and eosinophils can produce these mediators.

TGFβ, a well-known mast cell product [46], has been recently reported to inhibit the anti-inflammatory action of CSs in human bronchial epithelial cells, A549 cells and BEAS-2B cells [174,175]. The study showed that TGFβ impaired dexamethasone-induced GRα transactivation as shown by the reduced induction of the anti-inflammatory genes such as IκB-α and GILZ [174]. Interestingly, TGFβ effect was complex and did not involve the non-canonical pathways and Smad4-dependent pathways but did require the TGFβ-receptor ALK-5 [176,177].

Another pro-asthmatic cytokine produced by mast cells recently described to interfere with CSs sensitivity is interleukin 17 (IL-17) [172]. IL-17, whose levels are significantly increased in the induced sputum and bronchial biopsies in severe asthmatics, participates in the pathogenesis of asthma by acting on the epithelium or airway smooth muscle and driving neutrophilic inflammation which is poorly responsive to CS therapy [178]. When exposed to human bronchial epithelial cells, IL-17 markedly suppressed the ability of budesonide to repress TNFα-induced IL-8 production [179]. The IL-17 effect was mediated via the phosphoinositide-3-kinase (PI3K), and decrease histone deacetylase (HDAC) function [179]. Indeed, the inhibitory effect of IL-17 on HDAC2 was also demonstrated in the 16HBE cells, PBMC and alveolar macrophages [180,181]. Another mechanism by which IL-17 induced CS insensitivity reported in PBMCs and airway epithelial cells was the upregulation of GR-β which is known to inhibit GRα function [17,173].

Amrani's lab has provided extensive evidence for a role of two other mast cell cytokines (i.e., TNFα and IFNγ) in promoting CS insensitivity in ASM cells (reviewed in [17]). Briefly, the mechanisms by which these cytokines blunted CS response in healthy ASM cells were multiple and involved an inhibition of GRα by different inhibitory proteins including the transcription factor IRF-1 [96], the dominant isoform GRβ [95] which both repressed GRα transactivation or the protein phosphatase 5 (PP5) [92] which dephosphorylated GRα on ser^{211} residues that are essential for GRα transcriptional activities.

Macrophage migration inhibitory factor (MIF), a cytokine normally produced by eosinophils [182] and macrophages [183] was recently shown to be also released by the activated human lung mast cells [184]. It is interesting to note that MIF levels are increased in human diseases characterised by poor response to CS therapies including in

patients with asthma and ulcerative colitis [182,185]. *In vitro* studies have reported that MIF can blunt the anti-inflammatory action of CS in various cell types. Thus, MIF can block the capacity of dexamethasone to repress NF-κB activation and IL-8 and TNFα production by RAW 264.7 macrophages and U-937 cells stimulated with LPS alone or with phorbol myristate acetate (PMA), respectively [186]. In addition, production of TNFα, IL-1β, IL-8 and IL-6 can be inhibited by corticosteroids in LPS-stimulated PBMC, but the addition of MIF markedly blocked CSs inhibitory action [187]. The inhibitory effect of MIF on CS responsiveness appears to involved a decrease in GRα transactivation properties because MIF inhibited the induction of MKP-1 [186] and GILZ [188] by dexamethasone. Interestingly, MIF can induce the PI3K and NF-kB [189] which are known to degrade the anti-inflammatory transcriptional factors such as IκB-α [190].

IL-2 and IL-4 are also cytokines produced by mast cells [167,169-171] that can suppress the anti-inflammatory action of CSs in many cells such as T cells [191], macrophages [88], eosinophils [192] and PBMCs [87]. Multiple mechanisms have been suggested. Dexamethasone affinity for GRα, the nuclear translocation of GRα and induction of MKP-1 seen in T cells and PBMCs were reduced by IL-2 and IL-4 [87,191,193]. IL-2 and IL-4 also modulated GRα phosphorylation and the induction of GILZ and MKP-1 in dexamethasone-treated eosinophil [192]. PBMCs from severe asthma shows a reduction of PP2A expression and activity which was reported to be essential for the nuclear translocation of GRα seen in healthy PBMCs. Interestingly, PP2A expression/activity can be impaired by IL-2 and IL-4 in U937 monocyte cell line [88,89].

IL-13 which is also produced by mast cell [194] has been shown to associate not only with AHR, markers of allergic inflammation (IgE levels) and mucus hypersecretion [195,196] but also with the development of CS insensitivity [197,198]. IL-13 was shown

to reduce the binding affinity of GRα in the nucleus of healthy PBMCs [87,199], or increase the activity of p38MAPK pathways [87] which has been reported to phosphorylate GRα on inhibitory residues [200]. An *in vivo* study by Townley and colleagues demonstrated that inhaled IL-13 reduces fluticasone action in repressing ovalbumin-induced AHR in a murine model of allergic asthma [201].

Table 1.3: Different mast cell mediators that have been reported to interfere with corticosteroid sensitivity in various cell types relevant to asthma pathogenesis.

Mediators	Cell models	Authors
TNF-α + IFN-γ	Human ASMs	Tliba et al. 2008 [96] Chachi et al. 2015 [17]
IL-17	Bronchial Epithelium PBMCs	Irusen et al. 2002 [87] Zijlstra et al. 2012 [179]
IL-4 + IL-2	PBMCS T cells Macrophages Eosinophils	Irusen et al. 2002 [87] Goleva, Li et al. 2008 [191] Kobayashi, Mercado et al. 2011 [88] Pazdrak et al. 2016 [192]
TGFβ	Bronchial Epithelium	Salem et al.2012 [175] Keenan et al. 2014 [174]
IL-13	PBMCs	Goleva, Li et al. 2008 [191]
MIF	PBMC Macrophages Monocytes Fibroblasts	Calandra et al. 1995[187] Roger et al. 2005 [186] Roger et al. 2005 [186] Fan, H et al. 2014 [188]

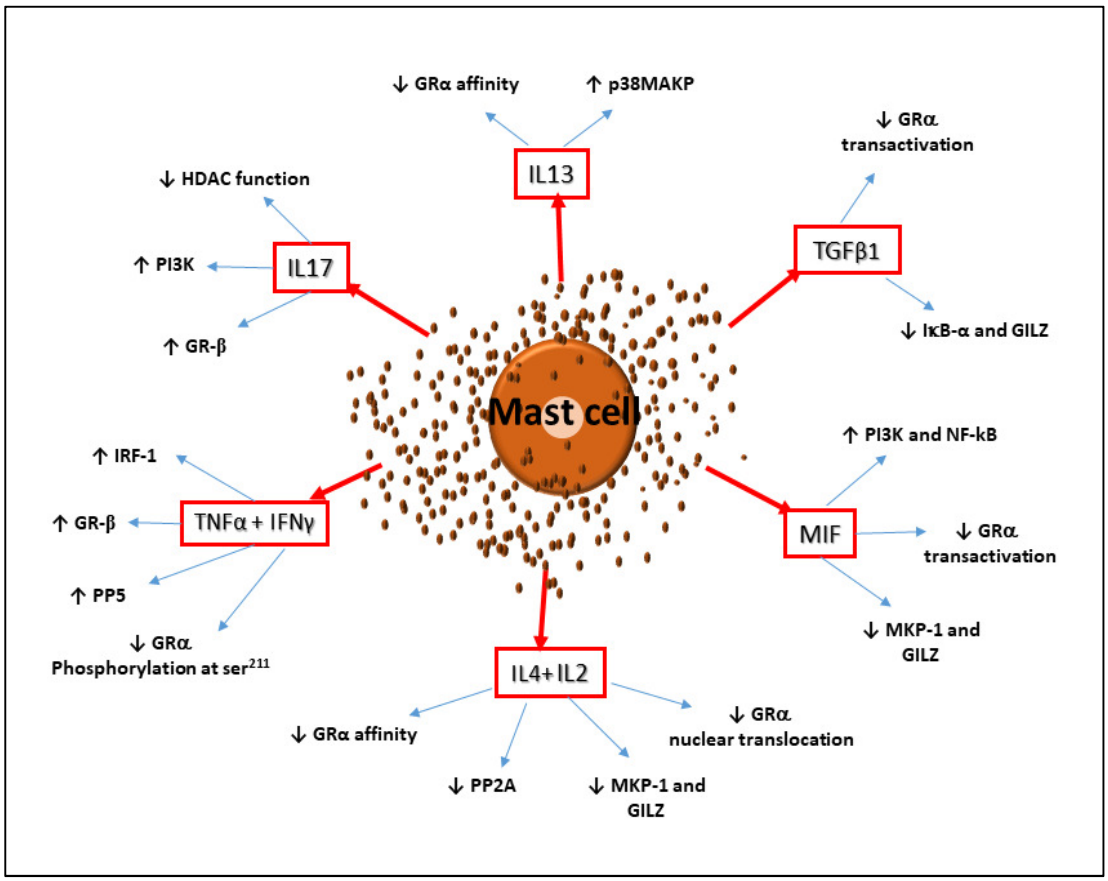

Figure 1.9: Molecular mechanisms of impaired corticosteroid signalling induced by mast cell mediators.

1.3.3. Bidirectional interactions between mast cells and ASM cells

1.3.3.1. ASM cells regulate mast cell function

Multiple *in vitro* studies showed that ASM cells can regulate various key functions of mast cells including their migration, proliferation and survival. For example, the infiltration of mast cells within the ASM tissues seen in asthmatic patients could be mediated by the various chemokines produced by ASM cells such as CXCL10, CXCL8 [100,101] and CCL11 [103], all shown to promote mast cell chemotaxis *in vitro*. The CXCL10 effect could be mediated by activation of CXCR3 shown to be highly expressed in human lung mast cells infiltrated within the asthmatic ASM tissues. More importantly, expression of CXCL10 was found to be higher in asthmatic ASM tissue [100]. ASM cells also produce TGF-β which acts as a chemotactic factor for mast cells [34,143]. Notably, it has been found that the expression of TGF-β in ASM layer correlates with the number of the mast cells within the ASM bundle and disease severity [143]. Furthermore, ASM cells can also produce factors that negatively regulate mast cell migration. For instance, ASM cells from severe asthmatic do not produce CXCL1 in response Th1 or Th2 cytokines compared to ASM cells from control [101]. The observation that CXCL1 inhibits mast cells migration [101] could explain the increased migration of mast cells toward asthmatic ASM cells.

Moreover, ASM cell and mast cell interaction promotes mast cell proliferation and survival. For example, co-cultured ASM cells and mast cell results in physical cell-cell contact involving CADM1 (ASM receptor) and CD117 (mast cell receptor) which resulted in the production of stem cell factor and IL-6 by ASM cells [109]. This cell-cell interaction and cytokine production by ASM cells caused mast cell survival and proliferation [109].

One study in 2007 by Begueret demonstrated how the infiltrated mast cells in ASM bundle in asthma exhibits a close cell-cell interaction with ASM cells which was associated with mast cells degranulation compared to control subjects **(Figure 1.10)** [122]. The observation that ASM cells secrete SCF [143,202] known to activate mast cells [10] suggests one mechanism of mast cell activation in ASM. Moreover, the infiltrated mast cells in ASM and mucosal mast cells in asthma have same activation feature which include smaller size and few granules and cytoplasmic pseudopods **(Figure 1.10)** [122]. Usually, mast cells are being activated by IgE cross-linking, and recently our group has reported that co-culturing ASM cells and mast cells can also cause mast cell activation in an IgE-independent manner [35,109].

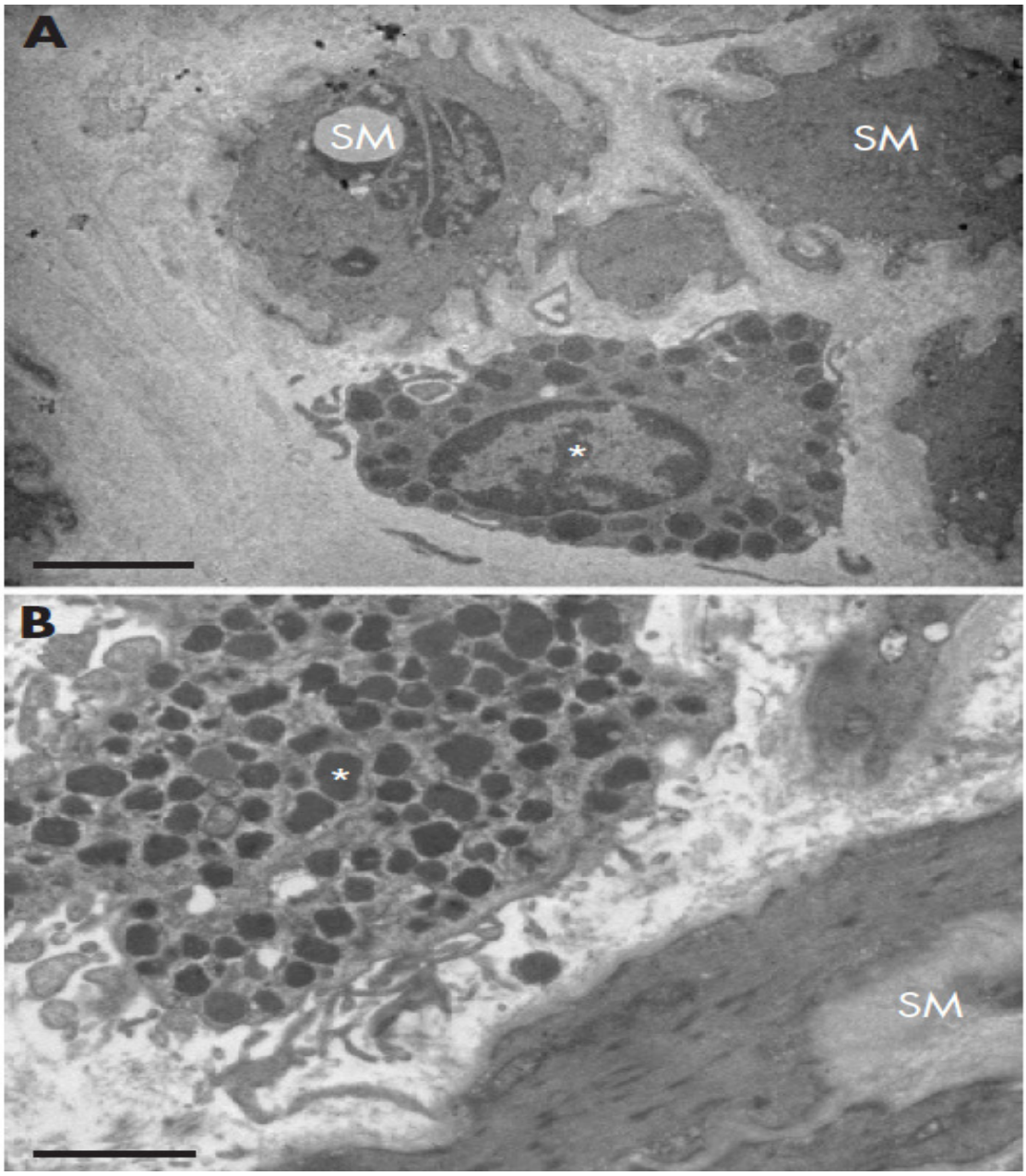

Figure 1.10: Mast cell (*) and airway smooth muscle (SM) cell-cell interaction taken by electron microscopy from a biopsy of asthmatic (A) and control (B) subjects.

(A) Asthmatic mast cell (*) in contact with ASM has a small size with a few granules. (B) mast cell (*) in control ASM has bigger size and more granules. (5µm) [122]

1.3.3.2. Mast cells regulate ASM cell function

The activation of mast cell causes a degranulation of either stored or synthesised mediators that have the capacity to regulate cytokine production in ASM cells. For example, conditioned media (CM) from activated mast cells (using IgE- anti-IgE mothed to activated mast cell for 2 hours) can reduce CCL11 and increase CXCL8 expression in both asthmatic and non-asthmatic ASM [203]. Also, the CM of mast cells can inhibit the combined effect of the pro-inflammatory cytokines IL1β, TNFα or IFNγ on CXCL10 production from both asthmatic and non-asthmatic ASM cells via tryptase activity [104]. Moreover, it has been shown that mast cells alter ASM cell contractility. For instance, β-tryptase or histamine, a major mast cell product, stimulated the production of TGFβ in ASM cells which then acted in an autocrine manner to induce a high level of alpha-smooth muscle actin [34,48,143,151]. Human lung mast cells also regulated the degree of bronchoconstriction via renin-Angiotensin II in mild asthma patients [204,205].

On the other hand, CM from 24-hour activated mast cells increased CXCL10 production from asthmatic and non-asthmatic ASM cells induced IFNγ via a mechanism involving TNFα [104]. Moreover, CM from 24-hour activated mast cell had a similar suppressive effect on CCL-11 production in both asthmatic and non-asthmatic ASM cells while CXCL8 expression was increased in healthy ASM cells only [203]. Interestingly, CM from 24-hour activated mast cells inhibits only the asthmatic ASM cells DNA synthesis, and this effect is abolished by anti-IL-4 and IL-3 [203].

Recently, a co-culturing system between human lung mast cells and ASM cells revealed that mast cells modulate β2-agonist responsiveness in ASM cells [35] showing that modulation of ASM function by mast cells can result from either physical cell-cell interaction and/or indirect mechanisms via the action of different mast cells mediators. Also, another study showed that co-cultured MCs and ASM cells results in degradation

of ASM cell-derived CCL11 and inhibition of ASM cell migration toward CCL11 by CCR3 [206]. Primary human mast cells also can mediate ASM cell contraction in collagen gel assay [207] and ASM cell proliferation [208] via the activation of matrix metalloproteinase (MMP 1) which are produced by mast cells.

1.4. Hypothesis:

Understanding the reasons why patients with severe asthma have a decreased response to corticosteroid therapy is an unmet clinical need. In 2002, two studies have introduced compelling evidence showing the clinical importance of interaction between ASM cells and mast cells in the pathogenesis of severe asthma. Brightling and colleagues demonstrated that mast cell infiltration within ASM tissue was associated with impaired lung function [32,133], while Carroll and colleagues reported the higher number of the degranulated mast cell in ASM bundle correlated with disease severity [142]. More recent *in vitro* studies have confirmed the existence of bidirectional interactions between mast cells and ASM that could impact the degree of airway inflammation, remodelling and hyper-responsiveness.

In this PhD thesis, I hypothesized that human lung mast cells regulate corticosteroid sensitivity in human ASM cells. This hypothesis was based on several lines of evidence showing that mast cell mediators can affect various ASM function, including chemokine production, cell proliferation, cell contractility [147], and interestingly response to β2 agonists [35]. More importantly, I have discussed in previous sections **(1.3.2.3. Mast cells and corticosteroid insensitivity)** indirect evidence that many mediators secreted by mast cells have the potential to blunt the anti-inflammatory actions of corticosteroids in various cell types such as ASM cell, epithelium and immune cells **(Table 1.3)**.

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Therefore, I hypothesised that sensitivity of ASM cells to corticosteroids would be affected by incubating cells with conditioned media from IgE-activated mast cells (**Figure 1.11**).

I will determine whether the ability of corticosteroids to inhibit TNFα-induced production of different inflammatory chemokines is affected by:

(a) ***Short-term activation*** of human lung mast cells (30-minute) to determine the effect of stored mediators.

(b) ***Long-term activation*** of mast cell (24-hour) to determine the effect of newly synthesised mediators.

(c) Dissect the underlying mechanisms driving corticosteroid insensitivity by human lung mast cells ***by focusing on their transactivation properties***.

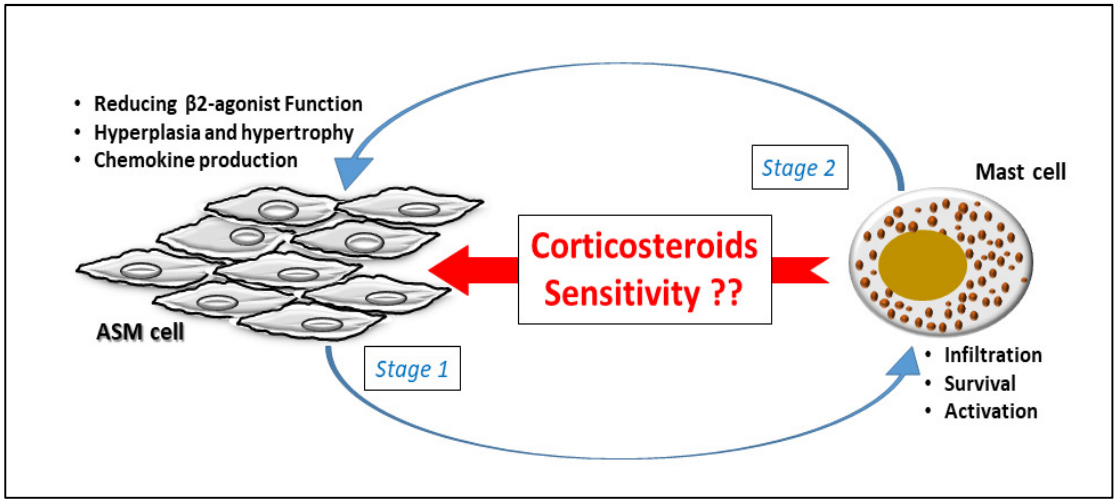


Figure 1.11: Bidirectional interactions between ASM cells and mast cells.

This graph summarises the main hypothesis of this thesis suggesting that multiple cross talks exist between ASM cells and mast cells leading to changes in infiltration, survival and most importantly activation of mast cells. This crosstalk has been shown to affect different ASM functions. Here, I will investigate whether mast cell can also impair the ASM response to corticosteroids by looking at their anti-inflammatory actions.

2.Chapter two

Methodology

A. Materials:

All the of the products (reagents and equipment) that have been used in the experiments are listed with the provider and catalogue number.

2.1. Reagents used for ASM cell culture:

Table 2-1: ASM cells culturing media:

Reagent	*Catalogue Number*	*Provider*
DMEM with Glutamax-1	1563895	Gibco
IMDM with Glutamax-1	31980-022	Invitrogen Ltd
Fetal bovine serum (FBS)	F9665	Sigma
Non-Essential Amino Acids (NEAA)	1140-035	Gibco
Antibiotic-Antimycotic (AA)	15240-062	Gibco
Sodium pyruvate (SP)	S8636	Sigma
Insulin-transferrin-selenium (ITS)	41400-045	Gibco
Trypsin	25200-072	Gibco
Trypan blue	T8154-100ml	Gibco

2.2. Reagents used for ASM cell stimulation and culture:

Table 2-2: Reagents used for stimulation or activation

Reagent	*Catalogue Number*	*Lot number*	*Provider*
TNF-α	210-TA	DDHB0115121	R&D system
Fluticasone	F9428-5mg	034M4722V	Sigma
IFN-γ	285-1F	RAX1915031	R&D system
IgE	AP175	2328316	Millipore
Anti IgE	AG301	2474843	Millipore
FcεR1	MAB6678	CGXM0115071 Colone:773704	R&D system
IL-6	206-IL-050	-	R&D system
IL-10	217-IL-005	-	R&D system
SCF	255-SCF-050	-	R&D system

2.3. Reagents used to assess mast cell activation (Beta-hexosaminidase):

Table 2-3: Beta-hexosaminidase reagents

Reagent	*Catalogue Number*	*Provider*
Citric acid	C-7129	Sigma
Trisodium citrate	S/P500/54	Fisher
4-Nitrophenyl N-acetyl-β-D-glucosaminide (pNAG)	N9376	Sigma
Sodium carbonate Na2CO3	L13098	Alfa-Aesar
Sodium bicarbonate NaHCO3	S5761	Sigma

2.4. Materials and reagents used in ELISA:

Table 2-4: ELISA material and reagents

Material	*Catalogue Number*	*Company*
ELISA plates	675061	Greiner Bio-one
PBS		
NaCl	5/3120/60	Fisher
KCl	P9333-500g	Sigma
Na2HPO4	S0870-100g	Sigma
KH2PO4	P3579-500g	Sigma
TBS		
NaCl	5/3120/60	Fisher
Tris Base	T-6066	Sigma
BSA	A7030-100G	Sigma
Tween® 20	PB337-500	Fisher
Substrate solution	T4444-100ml	Sigma
Stop solution	J/842917	Fisher
Human CCL5 duoset kit	DY278	R&D
Human CCL11 duoset kit	DY320	R&D
Human IP10 duoset kit	DY266	R&D
Human CXCL8 duoset kit	DY208	R&D
Human TNFα duoset kit	DY209	R&D

2.5. *Products used for mast cell isolation and culture:*

Table 2-5: Mast cell material

Product	*Supplier*	*Catalogue Number*
CD117 antibody	BD Biosciences	555713
Specimen containers	Elkay Lab Products	500-3000-12s
DynaBeads (Sheep anti-mouse IgG)	Fisher Scientific UK	11031
100µm nylon gauze		10467752
Distel disinfectant		HYG-700-041R
70µm Cell Strainer		11597522
DMEM GlutaMAX Hepes [+]	Invitrogen Ltd	32430100
DPBS (Mg [-] Ca^{2+} [-])		14190-094
Horse Serum		26050-070
Fetal Bovine Serum		10500064
Disposable forceps	Scientific Laboratory Supplies	INS4424
Hyaluronidase	Sigma-Aldrich Company Ltd.	C2674
Collagenase Type 1A (1g)		H3506
HBSS (Modified)		H9394-6X 500ML
50µm nylon gauze	Scottex Precision Textiles Ltd www.scottex-filters.com	Order by name and sizes

2.6. Reagents used for cell viability:

Table 2-6: List of MTT assay reagents

Reagent	*Catalogue Number*	*Provider*
MTT	475989	CALBIOCHEM
NP-40	492016	CALBIOCHEM
HCL	258148	SIGMA
Isopropanol	I-9516	SIGMA

2.7. Materials used for RNA isolation:

Table 2-7: List of products for RNA isolation

Reagent	*Catalogue Number*	*Provider*
Methanol	154903-2L	Sigma
Ethanol	E/8600/17	Fisher
PureLink® RNA Mini Kit	12183018A	Thermo Fisher Scientific
PureLink® DNase	12185-010	Thermo Fisher Scientific
2-Mercaptoethanol	21985-023	Gibco

2.8. *Materials used for molecular biology:*

Table 2-8: List of kits and primers used for RT-PCR and qPCR

Reagent	*Catalogue Number*	*Provider*
RevertAid first strand cDNA synthesis kit	K1613	Fermentas UK
GILZ forward primer	22326984	Eurofins Genomics
GILZ reverse primer	22326985	Eurofins Genomics
MAP-1 forward primer	21577764	Eurofins Genomics
MAP-1 reverse primer	21577765	Eurofins Genomics
IRF-1 forward primer	23005166	Eurofins Genomics
IRF-1 reverse primer	23005167	Eurofins Genomics
β-actin forward primer	21577766	Eurofins Genomics
β-actin reverse primer	21577767	Eurofins Genomics
GAPDH forward primer	21577770	Eurofins Genomics
GAPDH reverse primer	21577771	Eurofins Genomics
CCL5 forward primer	22663184	Eurofins Genomics
CCL5 reverse primer	22663185	Eurofins Genomics
CXCL8 forward primer	22663188	Eurofins Genomics
CXCL8 reverse primer	22663189	Eurofins Genomics
CXCL10 forward primer	21577768	Eurofins Genomics
CXCL10 reverse primer	21577769	Eurofins Genomics
PP5 primer	sc-44602-PR	Santa Cruz
RT² First Strand Kit	330401	QIAGEN
RT² SYBR Green/ROX PCR Master mix	330521	QIAGEN
FKBP5 primer	PPH02277A-200	QIAGEN
PIK3R1 primer	PPH00713F-200	QIAGEN
TNFAIP3 primer	PPH00063A-200	QIAGEN
Fast SYBR Green Master mix	4472908	Fisher Scientific

2.9. Materials used for RT2 Profiler PCR Arrays:

Table 2-9: List of kits used for PCR array.

Reagent	*Catalogue Number*	*Provider*
Human glucocorticoid signalling pathways	PAHS-154Z	QIAGEN
RT² First Strand Kit	330401	QIAGEN
RT² SYBR Green/ROX PCR Master mix	330521	QIAGEN

B. Methods:

2.10. Human lung Mast cells

2.10.1.Human lung Mast cells (HLMCs) Isolation

Mast cells (MCs) were obtained from human lung tissues (biopsy tissues and lung resection tissues) from 93 donors as described previously [109,209]. The lung tissues were cut into small fragments (around 1 cm^3) within one hour of obtaining the sample and placed into a beaker containing a funnel covered with 100 µm nylon gauze before being washed twice with Dulbecco's Modified Eagle Medium (DMEM) with GlutaMAX™ and 2% of FBS. The lung tissue fragments was then stored overnight in 4°C with mast cell media (DMEM media with 10% of Fetal bovine serum (FBS), 1% Antibiotic-Antimycotic (AA), 1% non-essential amino acid (NEAA), 100 µg/ml stem cell factor (SCF), 10µg/ml IL-10 and 50 µg/ml IL-6).

Next day, the cell dispersion procedure was ensured by enzymatic digestion by exposing the lung fragments to a mixture of collagenase (75mg to each 10g tissue) and hyaluronidase (37.5mg to each 10g tissue) for 75 minutes at 37 °C on a magnetic stirrer. Then, the digested lung tissues were filtered through 100µm nylon gauze and rinsed twice with 2% of FBS-DMED before discarding the lung fragments. The cell suspension then was filtered by using 50µm nylon gauze and centrifugated at 230g for 8 minutes. Then, the supernatants were discarded and pellets were suspended with Hanks' Balanced Salt Solution (HBSS), 2% fetal bovine serum (FBS), 10% horse serum and 1% bovine serum albumin (BSA) and incubated at 4 °C for 30 minutes.

After the incubation, a solution containing HBSS with 2% FBS was added to the cells, and the mixture was passed through a 70µm cell strainer and centrifuged again at 4 °C for 30 minutes. The supernatants were then discarded, and the pellets were re-

suspended with HBSS/ 2% FBS containing sheep anti-mouse IgG Dynabeads which were conjugated with mouse α-human CD117 (4 beads for each mast cells) and incubated for 1.5 hours at 4°C on the roller rocker. Next, the suspension was passed through 70µm cell strainer into new falcon tube and placed in Dynabeads MPC-1 (Magnetic Particle Concentrator) magnet for 5 minutes. This was followed by purifying the positive beaded mast cells by MPC-1 and discarding the supernatants. Next, the falcon tube was removed from MPC-1 and cells were re-suspended with HBSS/ 2% FBS. These purifying steps were repeated 3 times and mast cells were incubated as followed.

2.10.2.Human lung mast cell culture

Human lung MCs were cultured in 6 wells plate with each well containing 1 million cells per 1 ml of MC media consisting of DMEM supplemented with 10% of FBS, 1 AA, 1% of NEAA, 100 µg/ml of SCF, 10 µg/ml of IL-10 and 50 µg/ml of IL-6 and grown in the incubator at 37 °C and 5% CO_2.

2.10.3.HLMCs Activation

2.10.3.1. Mast cell activation using IgE and anti-IgE method:

MCs were seeded with 1 million cells per 1 ml of DMEM (supplemented with 10% of FBS, 1 AA, 1% NEAA) in each well of 6 wells plates. Then, MCs were sensitized with 1µg/ml of IgE and incubated at 37 °C and 5% CO_2 for 24 hours. Next, MCs were activated by the addition of anti-IgE (1µg/ml) or (or sham control) and incubated at 37 °C and 5% CO_2 for 30 minutes [35]. Next, media and the cells were transferred to a universal tube and centrifuged at 1300 rpm for 10 minutes. Finally, the conditioned media (CM) were collected and stored at -80 °C until the day of the experiments.

2.10.3.2. Mast cell activation using the anti-FcεR1 method:

MCs were also activated using another method using an activating antibody against the FcεR1 on MC which induces degranulation of MCs [210]. For this experiment, 1 million of mast cells in 1 ml of MC media (DMEM supplemented with 10% of FBS, 1 AA, 1% NEAA) were seeded in 6-well plates and incubated at 37 °C and 5% CO_2 for 30 minutes (or 24 hours in the presence or the absence of 2X of anti- FcεRI (1:150) antibody (to achieve a final concentration with MCs of 1:300). Finally, MCs were centrifuged at 243 g at 4 °C for 8 minutes in a pre-chilled centrifuge before the supernatants were collected and stored at -80°C until the day of the experiments. The pellets of cells were also frozen to be used to determine MC degranulation by assessing levels of Beta-hexosaminidase or TNFα.

2.10.4.Mast cell activation markers

2.10.4.1. Assessing beta-hexosaminidase activity in MC supernatants

MC degranulation was measured by assessing beta-hexosaminidase activity in the collected supernatants as described before [211]. Firstly, 40 µl of MC media as a baseline control (DMEM containing FBS 10%, AA 1%, and NEAA 1%) and supernatants from activated and non-activated MCs were mixed with the substrate solution consisting of 20 ml of citric buffer, 13.6 mg of p-nitrophenyl N-acetyl beat-D-glucosamine) (**Table 2-10**) and incubated for 75 minutes in 37 °C in the incubator. The enzymatic reaction was stopped by adding sodium carbonate buffer (**Table 2-11**), and the plate was read at 405 nm in a microplate reader.

Table 2-10: substrate solution preparation

Citric acid (0.05M) 1.9 g of citric acid + 500 ml dH_2o Stored at 4°C	**Citrate Buffer** (pH4.5) 45.5 ml of Citric acid (0.05M) + 50.5 ml of Trisodium citrate (0.05M) Stored at 4°C	**Substrate solution** (2mM): 13.6 mg of pNAG + 20 ml of Citrate Buffer pNAG is (4-Nitrophenyl N-acetyl-β-D-glucosaminide) Stored at -20°C
Trisodium citrate (0.05M) 2.9 g of citric acid + 500 ml dH_2o Stored at 4°C		

Table 2-11 : Sodium carbonate buffer preparation

Sodium carbonate Na^2CO_3 (0.05M) 1.06 g of Sodium carbonate + 200 ml dH_2o Stored at room temp	**Sodium Carbonate Buffer** (pH 10) 60ml of Sodium carbonate (0.05M) + 40ml of Sodium bicarbonate (0.05M) Stored at room temp
Sodium bicarbonate $NaHCO_3$ (0.05M) 0.84 g of Sodium bicarbonate +200 ml dH_2o Stored at room temp	

2.10.4.2. Assessing levels of TNFα in the MC supernatants

MC degranulation was also confirmed by assessing the levels of TNF-α [211,212] by ELISA. Briefly, ELISA plate was loaded with anti-TNFα capture antibody **(Table 2-12**) and left overnight at room temperature. The following day, the plate was washed three times with washing buffer (**Table 2-12**), blocked using a blocking buffer (**Table 2-12**) for 1 hour and followed by three washes. Then, the samples of interest or standard (from 15 pg/ml to 1000 pg/ml) were loaded and incubated for 2 hours at room temperature. Then, the plated was washed three times and detection antibody (**Table 2-12**) was added for 2 hours. After this, the plate was washed three times prior adding streptavidin-HRP for 20 minutes. Then, the plated was washed and substrate solution was loaded to the plate for another 20 minutes. Lastly, the run was stopped with the stop solution, and the plate was read in a microplate reader at 450 nm.

Table 2-12: ELISA agent preparation.

Solution	*Preparation*
PBS	137 mM of NaCl, 2.7 mM of KCl, 8.1 mM of Na2HPO4 1.5 mM of KH2PO4, pH 7.2-7.4
BSA	1% BSA in PBS
Washing buffer	0.05% of Tween® 20 in PBS
Blocking buffer	1% BSA in PBS
Capture antibody	Diluted to working concentration in PBS
Detection antibody	Diluted to working concentration in 1% BSA / PBS
Standard	Diluted to working concentration in with 1% BSA / PBS

2.11. Human airway smooth muscle cells (ASM cells)

2.11.1. Recruitment of Donors for the ASM cell studies:

Primary human ASM cells were obtained from healthy subjects isolated from endobronchial biopsies performed by experienced respiratory consultants. All donors giving tissue samples gave written informed consent, and the use of these tissues for research was approved by an appropriate ethics committees (references: 4977, 04/Q2502/74 and 08/H0406/189).

2.11.2. ASM cells isolation:

Asthmatic and healthy ASM cells were isolated from bronchial tissues obtained by bronchoscopy performed by respiratory consultants at Glenfield Hospital. **Table 2-14** indicates the clinical characteristics of the different subjects used in the thesis. Using a dissecting microscope, ASM tissue was split from connective tissue and covered with media and incubated at 37 °C and 5% CO_2 until the 80-100% confluency. Then, the tissue was removed and the ASM cells were removed using trypsin, centrifuged and re-suspended in DMEM with Glutamax-1 contains 10% of FBS, 1% of non-essential amino acid, 1% of Antibiotic-Antimycotic, and 1% of Sodium Pyruvate. Next, ASM cells were plated in 75 cm^3 flasks and incubated at 37 °C and 5% CO_2 until the 80-100% confluency before ASM cells were cultured into different flasks or plates for the different experiments [95] (**Table 2-13**).

Table 2-13: Seeding number of ASM cells

Container	*DMEM volume*	*Number of cells*
75 cm^3 flask	10ml	200k
6-well plate	2ml/well	100k
12-well plate	1ml/well	40k
24-well plate	1ml/well	20k
96-well plate	0.2ml/well	2.5k – 5k

Table 2-14: clinical data of the subjects used for the different studies

	Healthy controls	*Asthmatic subjects*	*P-value*
Number	14	7	-
Ages	46.92±5.22	38±2.44	0.2301
Gender (male/female/unknown)	(3/9/2)	(3/4)	-
FEV_1 (Current)	2.784±0.18	2.56±0.29	0.2572
FEV1 % predicted	90.05±6.281	82.5±8.73	0.1218
FEV_1 / FVC %	88.33±12.76	70.36±5.72	0.1933
ICS	0	542.9±217	N/A
PC20	9.78 ± 3.0	1.988 ± 1.7	**0.0282**
atopic number (yes/ No/unknown)	(2/4/8)	(5/1/1)	-
IgE level (IU/ml)	30.36 ± 16.65	1874 ± 1181	**0.0317**
age of onset	NA	12.33 ± 3.63	NA

Data are presented as mean± SEM, and the bold values indicated significance compared to healthy controls

2.11.3.Cell counting:

To count the number of cells, traditional technique using haemocytometer and light microscopy was applied. The cells were suspended in 1 ml of media. Then, 10 µl of the cells suspension was mixed with 10 µl of trypan blue and half of the volume loaded to haemocytometer. Under the light microscopy at 20X magnification, the cells which were not stained with blue were counted at the big squares at the four corners as shown in **Figure 2.1**. Lastly, the number of living cells was calculated by using the following formula:

*Cell number (cell/ml) = (cell count / 4) * dilution factor (2) * 10000*

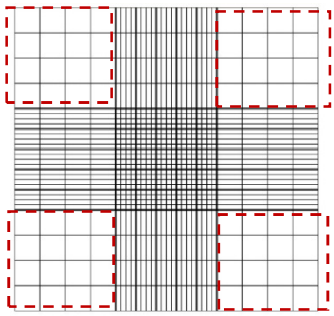


Figure 2.1: the haemocytometer grid used for cell count.

The living cells were counted in four squares shown in red.

2.12. Enzyme Linked Immunosorbent Assay (ELISA)

2.12.1.ASM cells stimulation with HLMC supernatant

Condition media experiments using ASM cells from asthmatic and healthy were in passages 4 to 6 and prepared as following (**Figure 2.2**). Firstly, ASM cells were plated in 24 well-plate, each well had 20K cells with 1 ml of feeding media (DMEM GlutaMAX-1 supplemented with 10% of FBS, 1% of NEAA, 1% of AA and 1% of SP) and incubated at 37 °C and 5% CO_2 until 90-100% confluency. Then, feeding media were discarded and the cells were washed twice and serum deprived overnight by replacing the media with the ITS media (DMEM GlutaMAX-1 with 1% of insulin-transferrin-sodium selenite (ITS), 1% of NEAA and 1% of SP) in 37 °C and 5% CO_2. ASM cells were then treated with different preprations of supernatants at ratio of 1:4 (25% v/v)) from i) activated mast cell, ii) non-activated mast cell, iii) mast cell media (control) and iv) ITS media alone (not included in the results because no difference was detected when compared to control mast cell media) for 24 hours and incubated at 37 °C and 5% CO_2, with all conditions performed in duplicate. The next day, media were discarded and the cells were washed twice with ITS media before new ITS media was added to the ASM cells containing 10 ng/ml TNFα alone, or in the presence of fluticasone propionate (FP) (100 nM) and further incubated 24 hours at 37 °C and 5% CO_2. The supernatants were then collected and stored at -20°C for later use.

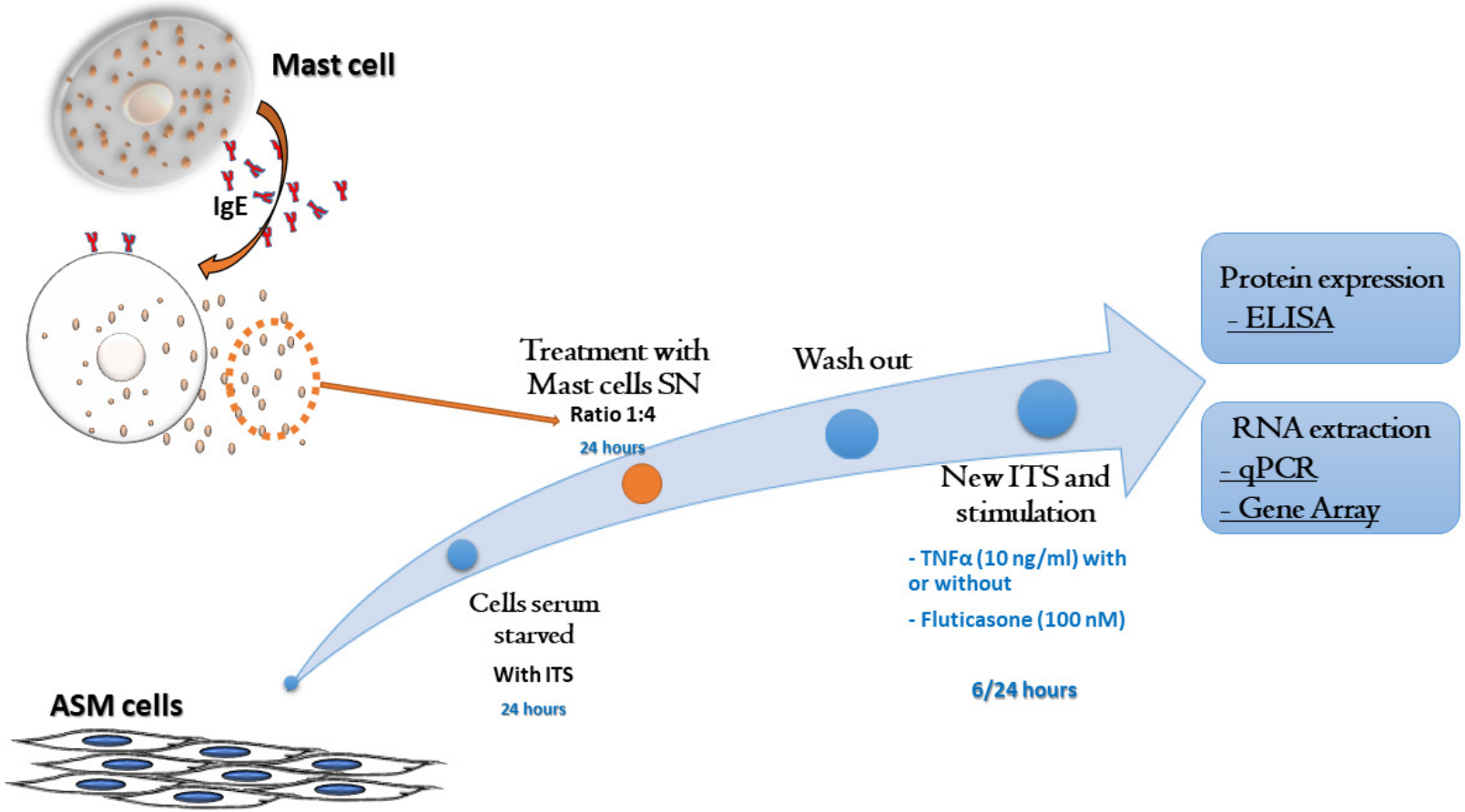


Figure 2.2: Protocol of ASM cells stimulation to assess the effect of MC supernatants on their sensitivity to fluticasone.

ASM cells at 90-100% confluence were serum-starved overnight with ITS media. The following day, ASM cells were treated with mast cell supernatant (SN) (25% v/v) for 24 hours, then washed before being treated with 10 ng/ml TNFα in the presence of absence of 100 nM fluticasone for an additional 24 or 6 hours. The cells were then prepared for the different indicated assays.

2.12.2.Chemokine measurement by ELISA:

The protein concentrations of Rantes (CCL5) Eotaxin (CCL11), CXCL8 and CXCL10 in the cell supernatants were assessed by ELISA as suggested by the manufacturer (R&D system) using the recommended antibody concentrations shown in **Table 2-15**.

Table 2-15: ELISA antibody concentration

Antibody	*CCL5*	*CCL11*	*CXCL10*	*CXCL8*
Capture	1.0 µg/mL	2.0 µg/mL	2 µg/mL	4 µg/mL
Detection	20 ng/mL	200 ng/mL	12.5 ng/mL	20 ng/mL
Standard	1000 pg/ml	1000 pg/ml	2000 pg/ml	2000 pg/ml

- To calculate the net increase [1],we followed this formula:

$$Net\ increase = TNF\alpha\ stimulation - Basal\ level$$

- To calculate the percent inhibition, we followed this formula:

$$Net\ increase = 100 * \left[\frac{(TNF\alpha\ stimulation - Basal) - (Fp\ treatment - Basal)}{(TNF\alpha\ stimulation - Basal)} \right]$$

[1] **Net increase** is the chemokine concentration following the subtraction of basal levels.

2.13. MTT assay:

This assay is colourimetric assay that has been extensively used to measure cell viability as described before [213]. MTT assay is based on the reduction of the tetrazolium dye by the mitochondrial reductase to its insoluble formazan that solubilized and is quantified by spectrophotometry. In brief, following ASM cell treatment after collection of the supernatants in each condition, 40 µl of MTT solution (**Table 2-16**) was added with 200 µl of freshly added ITS media to the ASM cells and incubated at 37 °C and 5% CO_2 for 3 hours and 30 minutes. The media was discarded and replaced with 240 µl of MTT solvent (**Table 2-16**) and incubated for 15 minutes under shaking condition. Lastly, the media was transferred to 96 well-plate, and the absorbance read by a plate reader at 590 nm.

Table 2-16 : Reagents used for the MTT assay

Solution	*Preparation*
MTT solution	5 mg of MTT in 1 ml of PBS
MTT solvent	4 mM of HCl prepared in isopropanol + 0.1% of Nonidet P-40

2.14. mRNA Quantification

qPCR assay was used to assess the expression of CXCL10, CCL5, CXCL8, mitogen-activated protein kinase phosphatase 1 (MKP-1), Glucocorticoid-Induced Leucine Zipper (GILZ), interferon regulatory factor (IRF-1), protein phosphatase 5 (PP5), β-actin and Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) in ASM cells that were pre-treated with 30-minute or 24-hour with either activated MC supernatants, non-activated MC supernatants, or control MC media. It was performed as follows:

2.14.1.Culture and stimulation of ASM cells:

ASM cells were cultured in 6 well-plates at 100k cells/well with 2 ml of feeding media containing 10% of FBS, 1% of NEAA, 1% of AA and 1% of SP, until cell reached 80-100% confluency. The cells were then serum-starved with ITS media for 24 hours before treating the cells with three different conditions, supernatants from activated (30-minute or 24-hour activation) or non-activated MCs and control (MC media). Lastly, the cells were washed twice and pre-treated with or without fluticasone propionate (100 nM) two hours prior TNFα (10ng/ml) stimulating for 6 hours at 37 ^{0}C and 5% CO_2 (**Figure 2.2**).

2.14.2.Total RNA Extraction:

The total RNA extraction was performed as recommended by the manufactory (Pure Link® RNA Mini Kit). In brief, ASM cells placed on ice were washed twice with pre-chilled PBS and lysis buffer that contains 1 % of 2-mercaptoethanol was added before the cells were scraped, transferred to RNAase free tubes and vortexed for 10 second. Then, for the homogenization step, the tubes were transferred to a Homogenizer and centrifuged at 12000g for 2 minutes. Lastly, 70% ethanol was added to the cell

homogenates, vortex, transferred to Spin Cartridge, centrifuged 12000g for 15s. The Spin Cartridge was washed, dried before RNA was eluted using 25µl of RNase-free water.

2.14.3.Reverse Transcription of total RNA:

Reverse transcription (RT) was used to synthesise cDNA from total RNA as recommended by the manufacturer (Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit). In short, we used a concentration of 10 µg/µl from each RNA sample for RT-PCR with random hexamer primer (1µl) that binds to RNA to facilitate the reverse transcriptase synthesis and topped up to 12µl and this followed by incubation at 65°C for 5 minutes. Next, 5X Reaction Buffer (4µl), RiboLock RNase Inhibitor (1µl), 10 mM dNTP Mix (2µl) and Revert-aid (1µl) were added to the RNA mix and RT-PCR was performed in a thermal cycler with a heated lid as recommended by the manufacturer, indicated in **Table 2-17**.

Table 2-17: The cycling program set up for cDNA synthesis.

Step	*Temperature, °C*	*Time*
Incubation	25	5 min
cDNA Synthesis	42	60 min
Termination	70	5 min

2.14.4.Quantitative Polymerase Chain reaction (qPCR):

cDNA was used for qPCR to assess the expression levels of different genes CCL5, CXCL10, MKP-1, GILZ, IRF-1, PP5, β-actin and GAPDH **(Table 2-18)**. We used the same primer sequences that have been used in our lab and published previously [214]. Our reference dyes were SYBR Green dye 1 and ROX™ dye which was used to

normalise the fluorescent reporter signal in real-time quantitative PCR as recommended by the manufacturer.

1.16.4.1.Efficiency test:

We assessed the amplification efficiency to determine whether the primers have 100% efficiency. CXCL10, CCL5, MKP-1, GILZ and GAPDH were checked for the efficiency test. We applied this by using different concentrations of one sample using a serial dilution of 10. Also, we tried different concentrations of the primers (0.500, 0.250 and 0.125 pmol/µl). The cycling program set up in qPCR was optimised as shown in **Table 2-19**.

Table 2-18: List of primers sequences and the optimal annealing temperatures.

Genes	*Primers Sequencing (5'-3')*		*Temperature °C*
	Forward	Reverse	
GILZ	TCTGCTTGGAGGGGATGTGG	ACTTGTGGGGATTCGGGAGC	60
MKP-1	GACGCTCCTCTCTCAGTCCAA	GGCGCTTTTCGAGGAAAAG	60
IRF-1	GCTGACCCCAGTCCGGTTGC	GCCCCTCAGCCAAAGCAGGG	60
β-actin	GCTCGTCGTCGACAACGGCTC	CAAACATGATCTGGGTCATCTTCTC	60
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG	60
CCL5	AGTCGTCTTTGTCACCCGAA	TCCCAAGCTAGGACAAGAGCA	60
CXCL8	ACTGAGAGTGATTGAGAGTGGAC	AACCCTCTGCACCCAGTTTTC	60
CXCL10	GGATGGACCACACAGAGGCTGC	GCCCCTTGGGAGGATGGCAGT	60

Table 2-19: qPCR reaction set up

Segments	*Cycles*	*Steps*	*Temperature °C*	*Time*
Segment 1	1	Initial denaturation	95	10 min
Segment 2	40	Denaturation	95	10 s
		Annealing	60	1 min
		Extension	72	30 s
Segment 3	1	Denaturation	95	30 s
Segment 4	1	Annealing	60	30 s
Segment 5	1	Denaturation	95	30 s
Segment 6	1	Annealing	60	30 s

2.14.5.Reverse Transcription of total RNA to use PCR array:

The use of PCR array from QIAGEN required a different kit to generate cDNA synthesis. We used the recommended RT² First Strand Kit (330401 QIAGEN) by QIAGEN. Briefly, 100 ng/ml of total RNA was mixed with genomic DNA elimination mix and incubated for 5 minutes at 42°C. then, the mixture was added to reverse transcription mix and incubated for 15 minutes at 42°C and followed by 5 minutes at 95°C. Lastly, the produced cDNAs were kept at -20°C until used.

2.14.6.qPCR for the RT^2 Profiler PCR Arrays:

RT^2 Profiler PCR Arrays was used to study 96 genes related to human glucocorticoid signalling pathways (PAHS-154Z) (**Table 2-21**). Real-time PCR was applied as suggested by QIAGEN. Briefly, cDNA was mixed with RT² SYBR Green/ROX PCR Master mix (330521 QIAGEN), and loaded onto the PCR array plates. then, the plated was inserted into PCR machine and the cycling program set up as shown in (**Table 2-20**).

Table 2-20: The cycling program set up for PCR array

Segments	*Cycles*	*Steps*	*Temperature °C*	*Time*
Segment 1	1	Initial denaturation	95	10 min
Segment 2	40	Denaturation	95	15 s
		Annealing	60	1 min

Table 2-21: Genes of Human Glucocorticoid Signalling in the RT^2 Profiler PCR Arrays

ADARB1	FKBP5	PDGFRB	ZFP36
AFF1	FOSL2	PDP1	ZHX3
AK2	GDPD1	PDCD7	ZNF281
AMPD3	GHRHR	PER1	ACTB
ANGPTL4	GLUL	PER2	B2M
ANXA4	GOT1	PIK3R1	GAPDH
AQP1	H6PD	PLD1	HPRT1
ARID5B	HAS2	PLEKHF1	RPLP0
ASPH	HNRNPLL	POU2F1	HGDC
ATF4	IL10	POU2F2	RTC
BCL6	IL1RN	RASA3	RTC
BMPER	IL6	RGS2	RTC
CALCR	IL6R	RHOB	PPC
CEBPA	KLF13	RHOJ	PPC
CEBPB	KLF9	SESN1	PPC
COL4A2	LOX	SGK1	
CREB1	MERTK	SLC10A6	
CREB3	MT1E	SLC19A2	
CREB3L4	MT2A	SLC22A5	
CTGF	NFKBIA	SNTA1	
CYB561	NR3C1	SPHK1	
DDIT4	TSC22D3	SPSB1	
DIRAS2	USP2	STAT5A	
DUSP1	USP54	STAT5B	
EDN1	VDR	TBL1XR1	
EHD3	VLDLR	TNF	
ERRFI1	XDH	TNFAIP3	

2.15. Protein Array

The Proteome Profiler Human Cytokine Array Kit (R&D system) was used to detect levels of 105 different human cytokines in the supernatants of activated and non-activated MCs (**Table 2-22**). Briefly, the pre-designed membranes were placed into a multi-dish with blocking buffer for 1.5 hours. Then, the blocking buffer was replaced with the different supernatant samples and incubated at 4°C overnight on rotating device. The following day, the samples were discarded, and the membranes were washed with the provided washing buffer for 10 minutes for three times. Then, detection antibody was applied for 1 hour in a shaker and followed with a washing step. Next, streptavidin-HRP was added for 30 minutes in a shaker and again a washing step was repeated. Lastly, the membranes were placed on a plastic sheet and covered with substrate solution, and chemiluminescence was used for the assessment of protein levels using Image Quant LAS 4000.

Table 2-22: List of the human cytokines assessed using Proteome Profiler Human Cytokine Array Kit

Adiponectin/Acrp30	IFN-gamma	CCL2/MCP-1
Angiogenin	IGFBP-2	CCL7/MCP-3
Angiopoietin-1	IGFBP-3	M-CSF
Angiopoietin-2	IL-1 alpha/IL-1F1	MIF
Apolipoprotein A1	IL-1 beta/IL-1F2	CXCL9/MIG
BAFF/BLyS/TNFSF13B	IL-1ra/IL-1F3	CCL3/CCL4 MIP-1 alpha/beta
BDNF	IL-2	CCL20/MIP-3 alpha
CD14	IL-3	CCL19/MIP-3 beta
CD30	IL-4	MMP-9
CD31/PECAM-1	IL-5	Myeloperoxidase
CD40 Ligand/TNFSF5	IL-6	Osteopontin (OPN)
Chitinase 3-like	IL-8	PDGF-AA
Complement Component C5/C5a	IL-10	PDGF-AB/BB
Complement Factor D	IL-11	Pentraxin 3/TSF-14
C-Reactive Protein/CRP	IL-12 p70	CXCL4/PF4
Cripto-1	IL-13	RAGE
Cystatin C	IL-15	CCL5/RANTES
Dkk-1	IL-16	RBP4
DPPIV/CD26	IL-17A	Relaxin-2
EGF	IL-18 BPa	Resistin
CXCL5/ENA-78	IL-19	CXCL12/SDF-1 alpha
Endoglin/CD105	IL-22	Serpin E1/PAI-1
EMMPRIN	IL-23	SHBG
Fas Ligand	IL-24	ST2/IL1 R4
FGF basic	IL-27	CCL17/TARC
KGF/FGF-7	IL-31	TFF3
IL-32 alpha/beta/gamma	FGF-19	TfR
Flt-3 Ligand	IL-33	TGF-alpha
G-CSF	IL-34	Thrombospondin-1
GDF-15	CXCL10/IP-10	TIM-1
GM-CSF	CXCL11/I-TAC	TNF-alpha
CXCL1/GRO alpha	Kallikrein 3/PSA	uPAR
Growth Hormone (GH)	Leptin	VCAM-1
HGF	LIF	VEGF
ICAM-1/CD54	Lipocalin-2/NGAL	Vitamin D BP

2.16. Statistical analysis

All the data obtained from the experiments were first analysed and sorted into groups in the Excel Microsoft office. The outcome was transferred to Graph Pad Prism 6 to perform statistical analysis. The data were analysed using ANOVA followed by correction for multiple compression (Tukey test) to test multiple variables. When we compared 2 variables, we have applied two tails T.test (paired or unpaired). When P-value less than 0.05, the null hypothesis was rejected to be considered as a significant change. Data were presented as Mean ± SEM. In the RT^2 profiler PCR array, student's' T.test was applied and followed by 5% False Discovery Rate (FDR) with two-stage step-up procedure of Benjamini, Krieger and Yekutieli.

3.Chapter three

Modulation of ASM sensitivity to fluticasone by mast cell conditioned media: effect of short-term activation (30-minute)

3.1. Introduction:

Some studies showed that mast cells contribute to asthma severity and AHR which are known to be less responsiveness to CS treatment [32,142]. Also, different mediators such as IL-17A [173], IL-4 and IL-2 [215], IL-13 [199], IFN-γ and IL-27[216], TGF-β1[174] and MIF [217] produced by mast cells can affect corticosteroid sensitivity in different cell types (**illustrated in chapter one pages 36-41**).

In asthma, mast cells have a potent effect on many cell types including ASM cells as a result of direct interaction (cell-cell interaction) [35] or/and indirect interaction (mast cell mediators) [35,104]. For instance, β2-agonist responsiveness in ASM cells was inhibited by mast cells (cell-cell interaction) [35] Also, preformed mast cell mediators can influence the expression of CCL11 and CXCL8 in ASM cells from healthy and asthmatic [203]. Mast cell mediators have been shown to cause epithelium permeability, mucus hypersecretion, ASM hypertrophy and ASM hyperplasia [218]. For example, tryptase (mainly produced by mast cells) can regulate the proliferation and differentiation of structural cells in the lung such as ASM cells [34,151]. Also, TGF-β1, which is also known to be produced by mast cell [174], can regulate ASM proliferation, epithelium damages and mucus secretion [31,49].

Aims:

This chapter aimed to investigate the effect of conditioned media from 30-minute activated MCs on the ability of fluticasone to suppress TNF-α-induced chemokines in healthy and asthmatic ASM cells. The studies were conducted in two phases:

Phase 1: control experiments

A. To determine the optimal method of MC activation using the β-hexosaminidase assay as a biomarker

B. To determine whether conditioned media from 30-minutes activated MCs affected viability of ASM cells.

C. To study whether conditioned media from 30-minutes activated MCs modulated TNF-α-induced chemokines.

Phase 2: Sensitivity experiments

D. To investigate whether conditioned media from 30-minutes activated MCs modulated the ability of fluticasone to repress TNF-α-induced chemokines

   a. At the protein level using ELISA

   b. At the mRNA level using qPCR

E. To investigate whether conditioned media from 30-minutes activated MCs modulated the transactivation properties of fluticasone.

F. To determinde the profile of mediators of 30-minute mast cell conditioned media before and after activation.

3.2. Results:

3.2.1. β-hexosaminidase assay and Mast cell activation:

β-hexosaminidase is commonly used as a biomarker to measure mast cell degranulation [211]. I therefore assessed β-hexosaminidase activity in the SN of activated MCs before and after cells activation using IgE and anti-IgE (1ug/ml) as described previously [109,219]. Two out of five experiments showed that MC activation was associated with a high level of β-hexosaminidase activity (experiment 3 and 5) varying from 1.86 and 3.88 fold increase over the activity seen in the SN of the non-activated mast cell, respectively, (**Figure 3.1**).

Because of the variation seen with the IgE-anti-IgE approach, I chose a different one step method using an antibody directed against FcεR1 receptor as used in [210]. In three different mast cell SN preparations, anti- FcεR1gave a consistently high level of β-hexosaminidase activity in the SNs of 30-minute activated MCs compared to that seen in non-activated MC SN with 8.62, 15.38 and 13.08 fold increase over basal values **(Figure 3.2)**. Therefore, I used this method of MC activation to determine the effect of conditioned media from the activated mast cell preparation on the sensitivity of ASM cells to fluticasone.

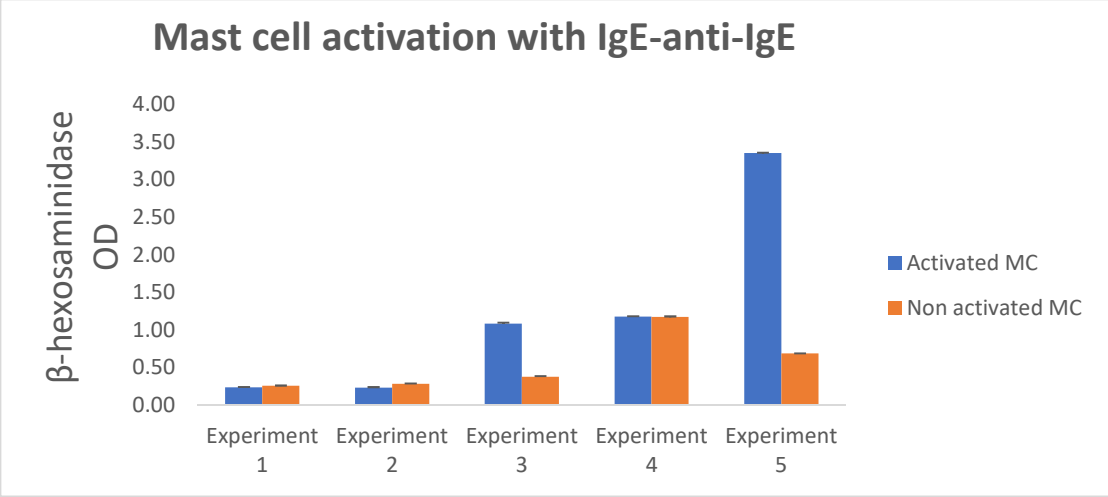


Figure 3.1: β-hexosaminidase activity in the conditioned media of 30-minute activated and non-activated mast cells.

Supernatants of 30-minute activated (Blue bars) and non-activated mast cells (red bars). IgE-anti-IgE (1ug/ml) was used for activation process and the results presented as optical density (means ±SEM).

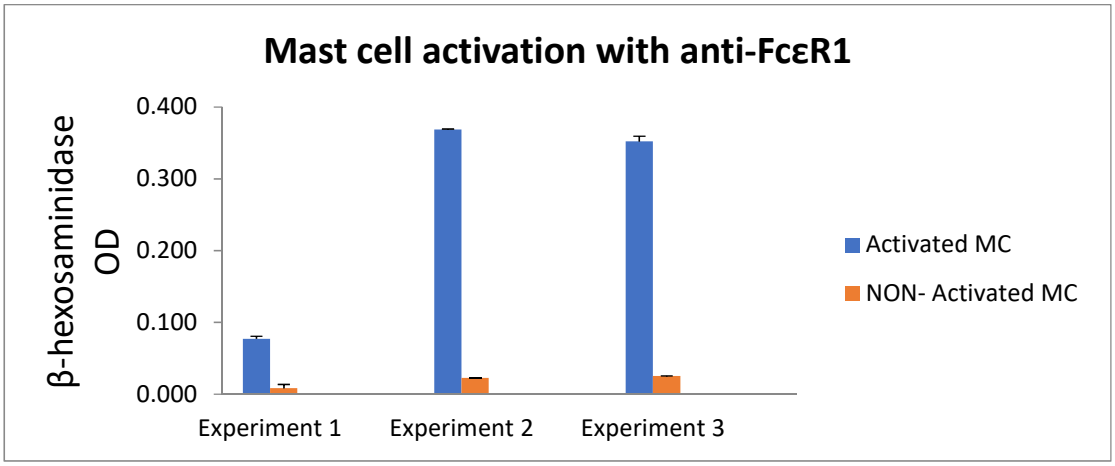


Figure 3.2: β-hexosaminidase assay applied on conditioned media from activated (using FcεR1) and non-activated (unstimulated) mast cells.

The activity of β-hexosaminidase is presented as the optical density of non-activated (red bar) and activated mast cell conditioned media (blue bar) (means ± SEM) (A).

3.2.2. Conditioned media from 30-minute activated mast cells do not affect the viability of ASM cells:

Viability of ASM cells following treatment with conditioned media from the 30-minute activated MCs was assessed using MTT assay. The absorbance values of formazan did not differ from the healthy ASM cells that were pre-treated with control mast cell media, conditioned media from 30-minute non-activated and activated MCs with values of 1.389 ± 0.31, 1.320 ± 0.27 and 1.291 ± 0.29, respectively, **(Figure 3.3A)**. Similarly, the cell viability was also not affected when asthmatic ASM cells were used with absorbance values of 0.961 ± 0.16, 0.865 ± 0.16 and 0.859 ± 0.17 in cells pretreated with control mast cell media, conditioned media from 30-minute non-activated and activated MCs, respectively (**Figure 3.3B**).

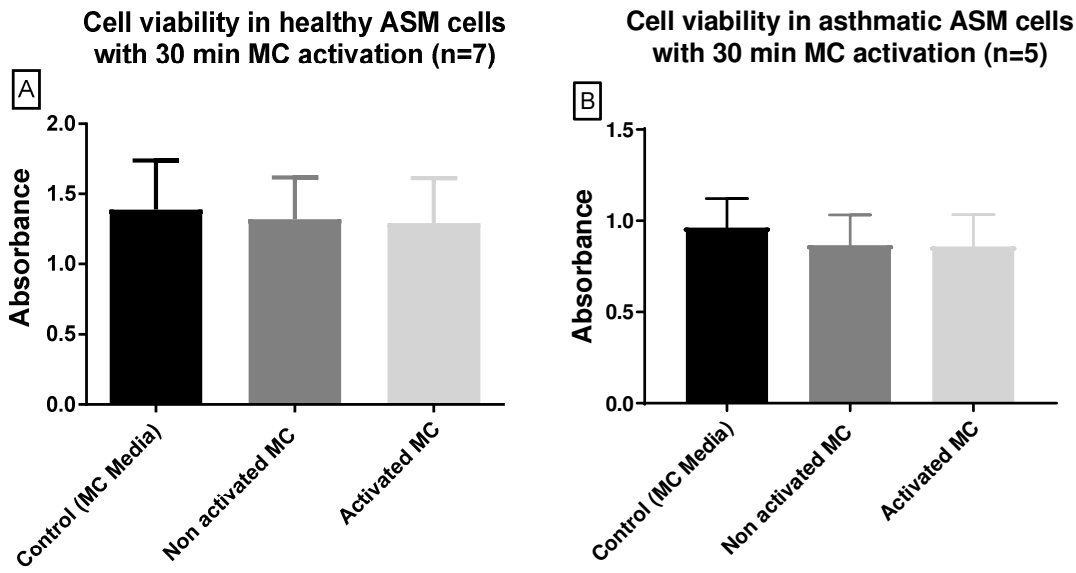


Figure 3.3: The effect of conditioned media of activated MCs on ASM cell viability.

Healthy (A) and asthmatic (B) ASM cells were pre-treated with control mast cell media (black bar), 30-minute non-activated (dark grey bar) and activated MC conditioned media (light grey bar) for 24 hours. Cell viability was assessed by MTT assay. Data are presented as optical density (means ± SEM of n=7 and 5 different cell lines).

3.2.3. Effect of conditioned media from 30-minute activated MC on TNFα-induced chemokines in ASM cells:

I first tested whether conditioned media from MCs modulate TNFα-induced expression of the pro-asthmatic chemokines CCL5, CXCL10 and CXCL8. ASM cells were first incubated 24 hours with either control MC media, conditioned media from 30-minute activated and non-activated MCs. ASM cells were then washed and stimulated with or without TNFα for 24 hours before chemokines were assessed in the supernatants by ELISA.

I first investigated whether the **net increase**[2] of chemokines production by TNFα in healthy ASM cells was affected by conditioned MC media (n=7) (**Figure 3.4**). TNFα-induced CCL5 expression in control ASM cells (4.429 ± 1.5 ng/ml) was not significantly affected in cells treated with conditioned media from 30-minute non-activated and activated MCs (7.421 ± 2.2 ng/ml and 6.224 ± 2.19 ng/ml respectively) (**Figure 3.4A**). Similarly, Production of CXCL10 by TNFα in healthy control ASM cells (50.29 ± 19.9 ng/ml) was also not significantly affected in cells treated with conditioned media from 30-minute non-activated and activated MCs (36.82 ± 12.25 ng/ml and 32.86 ± 12.4 ng/ml) (**Figure 3.4B**). Lastly, CXCL8 production by TNFα stimulation was 86.79 ± 15.79 ng/ml, 103.8 ± 18.7 ng/ml and 90.44 ± 16.49 ng/ml in healthy control ASM cells, cells treated with conditioned media from 30-minute non-activated and activated MCs respectively (**Figure 3.4C**).

I also investigated whether MC conditioned media would affect chemokine responses in ASM cells derived from asthmatic patients. I found that the net increases of CCL5, CXCL10 and CXCL8 by TNFα in asthmatic ASM cells were not differently

[2] **Net increase** is the chemokine concentration following the subtraction of basal levels.

modulated by the pre-treatment with conditioned media from 30-minute activated and non-activated MCs when compared to control media (n=5) **(Figure 3.5)**. The induction of CCL5 by TNFα in ASM cells treated with control media was 2.906 ± 1.54 ng/ml and 2.388 ± 1.2 ng/ml and 1.84 ± 0.8 ng/ml in asthmatic ASM cells pre-treated with conditioned media of 30-minute non-activated and activated MCs, respectively, with no significant difference between groups (**Figure 3.5A**). Similarly, in asthmatic ASM cells, CXCL10 induction by TNFα was 4.88 ± 2.28 ng/ml in control media and 4.27 ± 1.6 ng/ml and 4.936 ± 2.14 ng/ml in cells treated with conditioned media of 30-minute non-activated and activated MCs, respectively, with again no significant difference between groups (**Figure 3.5B**). Finally, the net increase of CXCL8 by TNFα in asthmatics ASM cells pre-treated with conditioned media from 30-minute non-activated and activated MCs was 75.58 ± 5.8 ng/ml and 59.02 ± 16.3 ng/ml, respectively while in cells treated with control media it was 54.83 ± 15.31 ng/ml, with no significant differences (**Figure 3.5C**).

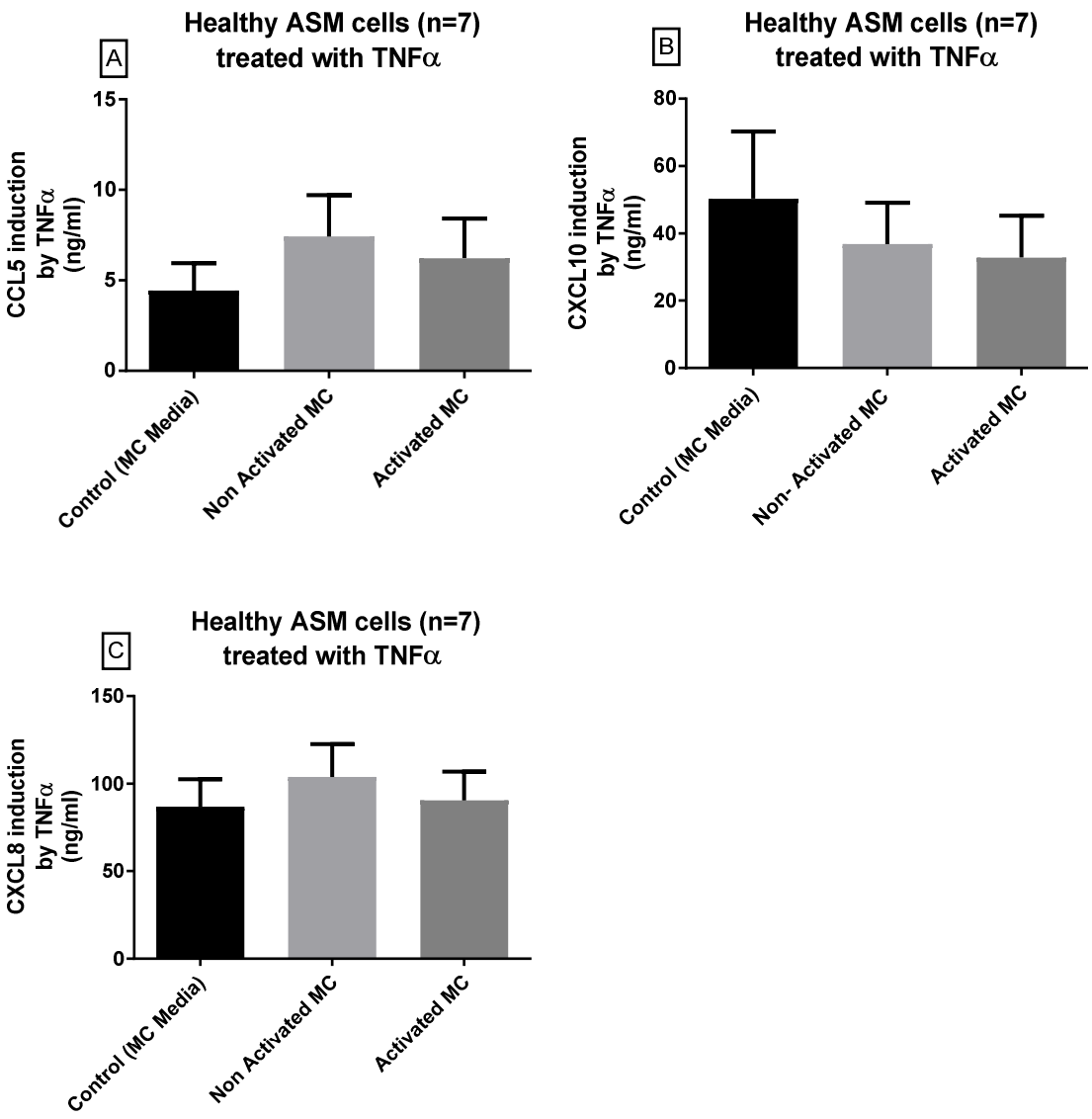


Figure 3.4: The effect of control media, conditioned media of 30-minute activated and non-activated MCs on the net increase of chemokine production by TNFα in healthy ASM cells.

Healthy ASM cells were pre-treated with control MC media (black bar), 30-minute non-activated (light grey bar) and activated MC conditioned media (dark grey bar) for 24 hours. Later, ASM cells were washed and stimulated with TNFα (10ng/ml) for 24 hours. The chemokine levels of CCL5 (A), CXCL10 (B) and CXCL8 (C) were assessed by ELISA. Data are presented as the Means ± SEM of n=7 different cell lines.

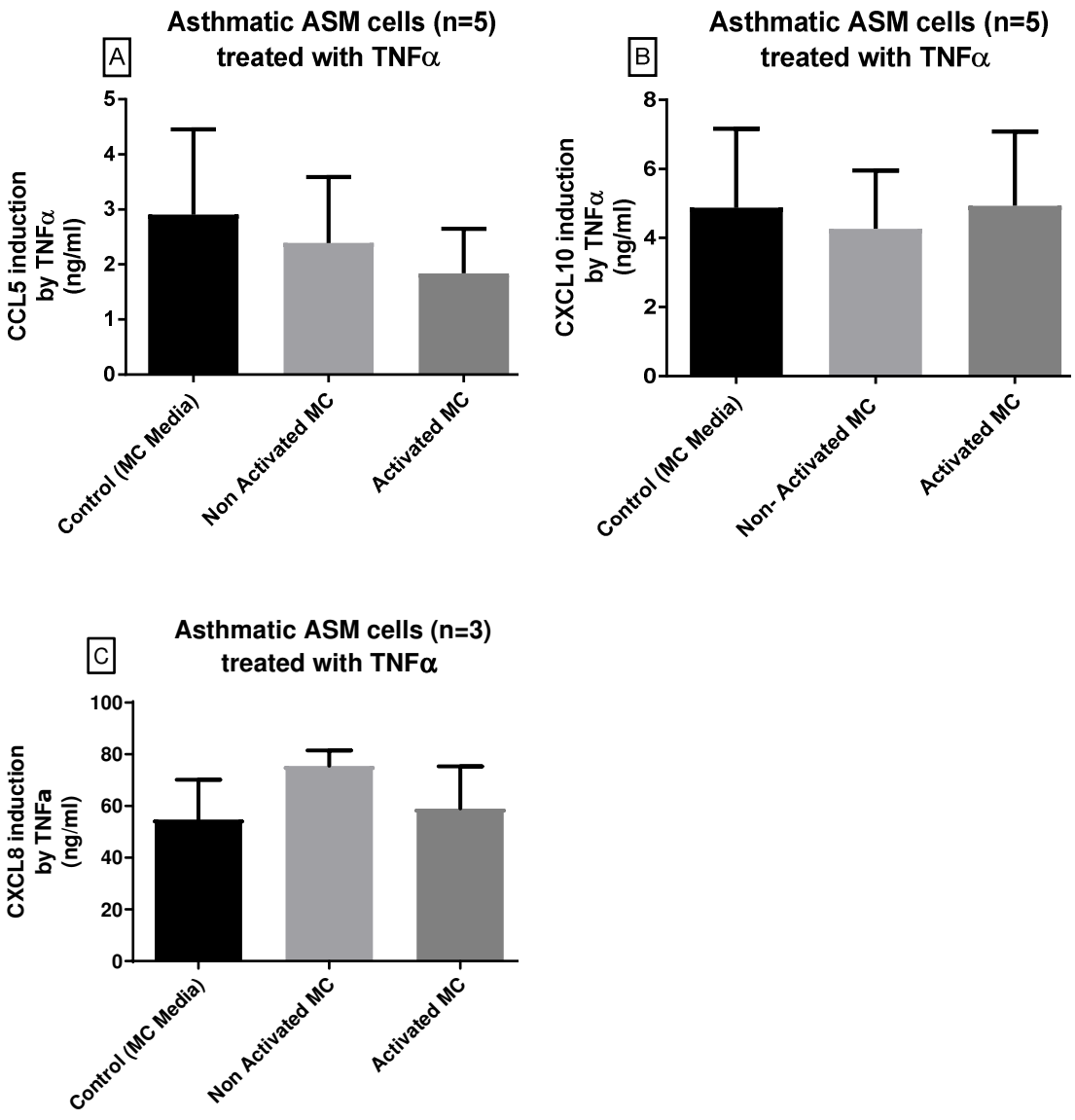


Figure 3.5: The effect of control media, conditioned media of 30-minute activated and non-activated MCs on the net increase of chemokine production by TNFα in asthmatic ASM cells.

Asthmatic ASM cells were pre-treated with control MC media (black bar), 30-minute non-activated (light grey bar) and activated MC conditioned media (dark grey bar) for 24 hours. Later, ASM cells were washed and stimulated with TNFα (10ng/ml) for 24 hours. The chemokine levels of CCL5 (A), CXCL10 (B) and CXCL8 (C) were assessed by ELISA. Data are presented as the Means ±SEM of n=5 and n=3 different cell lines.

3.2.4. Effect of MC conditioned media on the ability of fluticasone to suppress TNFα-induced-chemokine expression at the protein level:

Here, I investigated whether pre-treating ASM cells with 30-minute mast cell conditioned media can reduce the inhibitory action of fluticasone to suppress TNFα-induced chemokines. ASM cells were treated with control and conditioned media from activated and non-activated MCs before cells were washed and treated with TNFα in the presence or absence of fluticasone at a concentration (100 nM) which has been reported to almost completely suppress TNFα-induced chemokine production in healthy cells [96].

Fluticasone inhibited TNFα-induced-CCL5 by 85.33 ± 3.0 % in ASM cells pretreated with MC control media, a response that was significantly reduced to 64.21 ± 9 % ($p = 0.0204$) in ASM cells pretreated with conditioned media from 30-minute activated MCs while no change was observed (78.68 ± 6 %) in cells pretreated with conditioned media non-activated MCs, respectively, (n=7) **(Figure 3.6A)**. In asthmatic ASM cells, the percent inhibition of TNFα–induced CCL5 by fluticasone was 60.58 ± 7.8 % in control media, while in cells primed with 30-minute non-activated and activated MC conditioned media, fluticasone produced 78.92 ± 4.2 % and 77.18 ± 9 % inhibition of CCL5, with no differences between groups **(Figure 3.7A)**.

The percent inhibition of FP of TNFα-induced CXCL10 was 82.32 ± 6.4 % in healthy ASM cells primed with control media, and this was reduced to 56.06 ± 7.4 % ($p = 0.0097$) in ASM cells pretreated with conditioned media from 30-minute activated MCs while no change was observed (71.4 ± 6.4 %) in cells pre-treated with conditioned media from non-activated MC (n=7) **(Figure 3.6B)**. In asthmatic ASM cells, TNFα-induced CXCL10 was inhibited by 68.21 ± 9.7 % by fluticasone in cells pre-treated with control

media, the inhibition was further reduced to 50.95 ± 12.8 % and 41.76 ± 13.4 % in cells that were pre-treated with conditioned media from 30-minute non-activated and activated MCs, respectively **(Figure 3.7B)**. However, the apparent reduction in fluticasone inhibitory action by MC conditioned media did not reach significance.

Lastly, the inhibition TNFα-induced CXCL8 by fluticasone was not different between ASM cells pre-treated with control media and cells primed with either 30-minute non-activated or activated MC conditioned media (CXCL8 net increases were 79.23 ± 2.6 %, 71.94 ± 5.5 % and 69.9 ± 7.2 %, respectively) **(Figure 3.6C)**. Similarly, the percent inhibition of TNFα-induced CXCL8 by fluticasone in asthmatic control ASM cells was 87.23 ± 1.8 % while in asthmatic ASM cells primed with 30-minute non-activated or activated mast cell SN were 88.18 ± 4.1 % and 87.25 ± 3.4 % respectively **(Figure 3.7C)**. The percent inhibition of TNFα-induced CXCL8 by fluticasone in healthy and asthmatic ASM cell was not affected by any MC conditioned media.

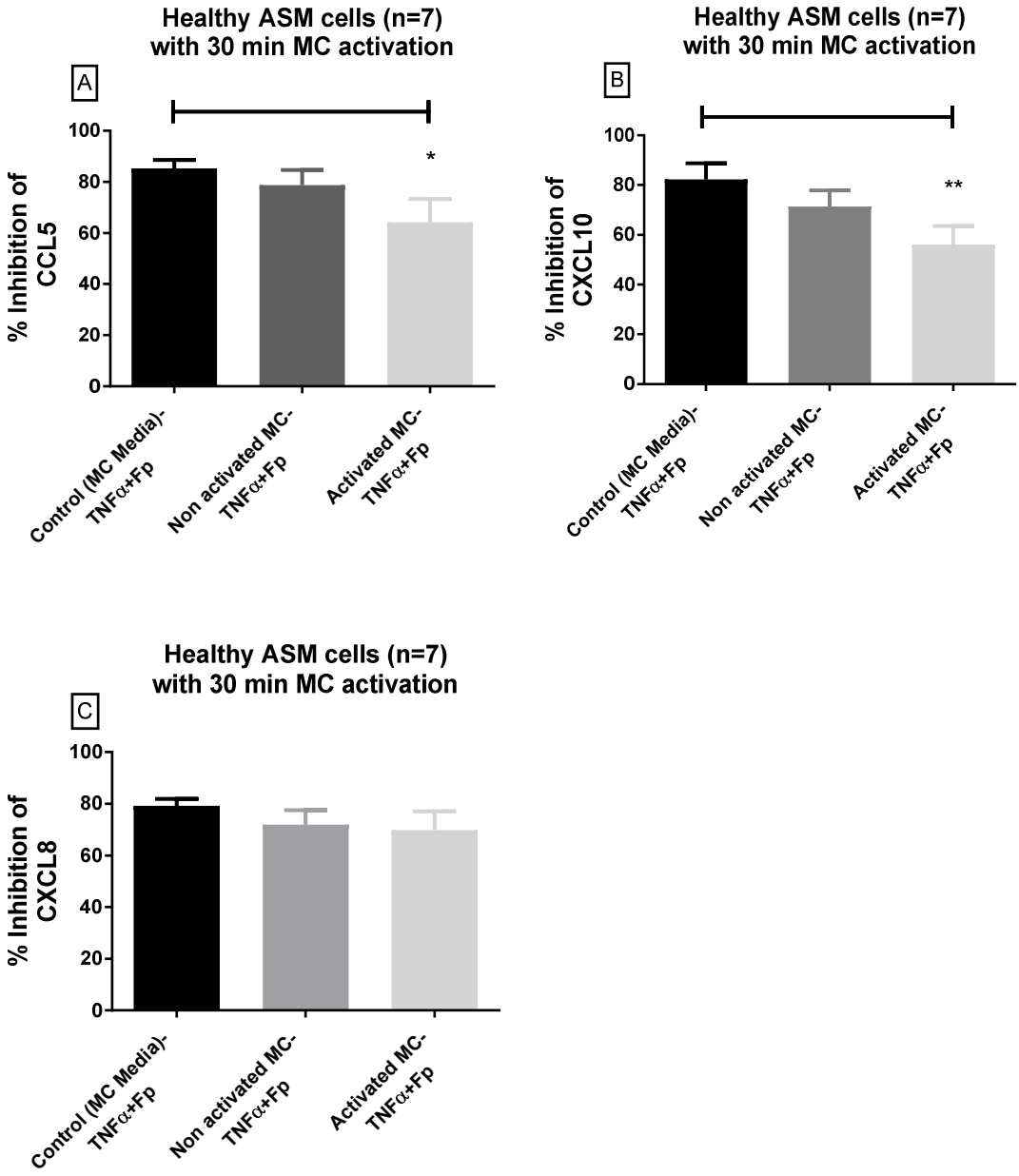


Figure 3.6: Effect of 30-minute activated MC conditioned media on the inhibition of TNFα-induced chemokine production by fluticasone in healthy ASM cells.

Healthy ASM cells were pre-treated with control MC media (black bars), conditioned media from 30-minute non-activated (dark grey bars) or activated MCs (light grey bars) for 24 hours. ASM cells were then washed and stimulated with 10ng/ml TNFα in the presence or absence of 100 nM fluticasone for an additional 24 hours. Chemokine production of CCL5 (A), CXCL10 (B) and CXCL8 (C) was assessed by ELISA and data are presented as % inhibition of the chemokine responses in cells treated with TNFα alone after normlizing to the basal (Means ± SEM of n=7 different cell lines), (= p<0.05, **= P<0.005).*

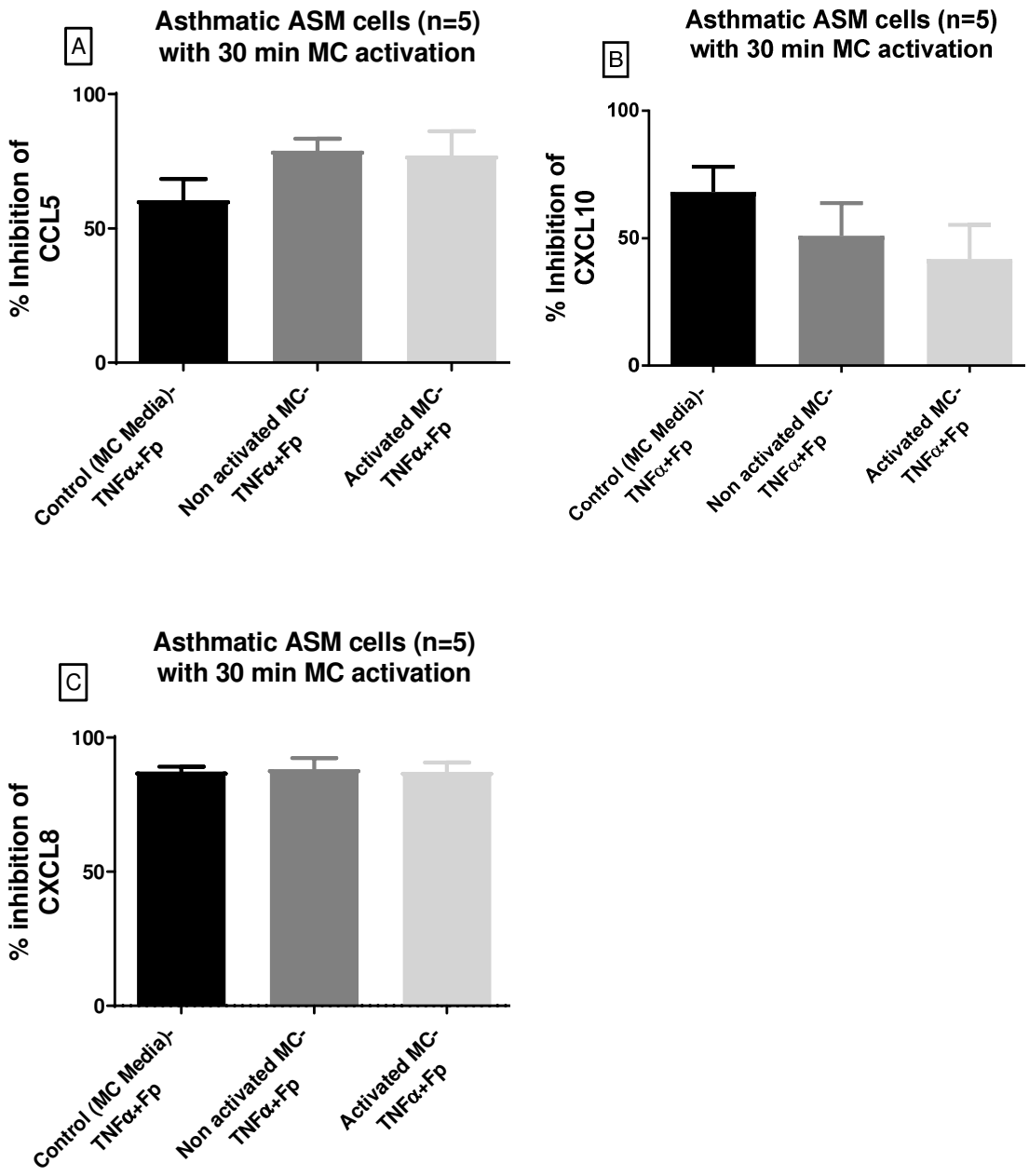


Figure 3.7: Effect of 30-minute activated MC conditioned media on the inhibition of TNFα-induced chemokine production by fluticasone in asthmatic ASM cells.

Asthmatic ASM cells were pre-treated with control MC media (black bars), conditioned media from 30-minute non-activated (dark grey bars) or activated MCs (light grey bars) for 24 hours. ASM cells were then washed and stimulated with 10ng/ml TNFα in the presence or absence of 100 nM fluticasone for an additional 24 hours. Chemokine production in CCL5 (A), CXCL10 (B) and CXCL8 (C) was assessed by ELISA and data are presented as % inhibition of the chemokine responses in cells treated with TNFα alone after normlizing to the basal (Means ± SEM of n=5 different cell lines).

3.2.5. Effect of MC conditioned media on the ability of fluticasone to suppress TNFα-induced chemokine expression at the mRNA level:

I next investigated whether the modulation of fluticasone inhibitory action by MC conditioned media on chemokine production also occurred at the mRNA level. Interestingly, I found that conditioned media from 30-minute activated MCs only reduced the inhibitory effect of fluticasone on CCL5 mRNA expression by TNFα to the same extent as the effect on the protein production (see **Figure 3.6A**). ASM cells were pre-treated with control mast cell media, conditioned media from 30-minute activated and non-activated mast cell overnight. Later, ASM cells were washed and treated with TNF-α (10 ng/ml) with or without fluticasone (100 nM) for 6 hours before total RNA isolation which is known to be the best time to detect the maximum inhibition of chemokines at the mRNA level [92].

Fluticasone suppressed TNFα-induced CCL5 mRNA by 75.84 ± 11.6 % and 74.21 ± 12.5 % in healthy ASM cells primed with control MC media and conditioned media from 30-minute non-activated MCs, while in cells primed with conditioned media from 30-minute activated MCs, this was significantly reduced to 63.14 ± 13.5 % ($p = 0.0190$) (**Figure 3.8A**). In the asthmatic cells, the repression of TNFα-induced CCL5 mRNA by fluticasone was 77.82 ± 8.12 %, 93.03 ± 2.6 % and 72.64 ± 8.6 % in cells treated respectively with control media, conditioned media from 30-minute non-activated and activated MCs (**Figure 3.9A**).

In addition, TNFα-induced CXCL10 mRNA was inhibited by fluticasone by 96.31 ± 1.5 % and 94.21 ± 2.8 % in healthy ASM cells pre-treated with control MC media and conditioned media from 30-minute non-activated MC respectively, and this response was reduced to 88.93 ± 3.89 % in healthy ASM cells which pre-treated with 30-minute

activated MCs but this effect was not significantly different compared to the effect of control media (**Figure 3.8B**). Similarly, the induction of CXCL10 mRNA by TNFα was inhibited by fluticasone by 90.07 ± 3.56 % and 90.3 ± 11.83 % in asthmatics ASM cells pre-treated with control MC media and conditioned media from 30-minute non-activated MCs, respectively, while in asthmatic ASM cells pre-treated with 30-minute activated mast cell conditioned media was reduced to 69.9 ± 11.83% (**Figure 3.9B**). The data show that MC conditioned media had no effect on fluticasone inhibitory action in asthmatic ASM cells.

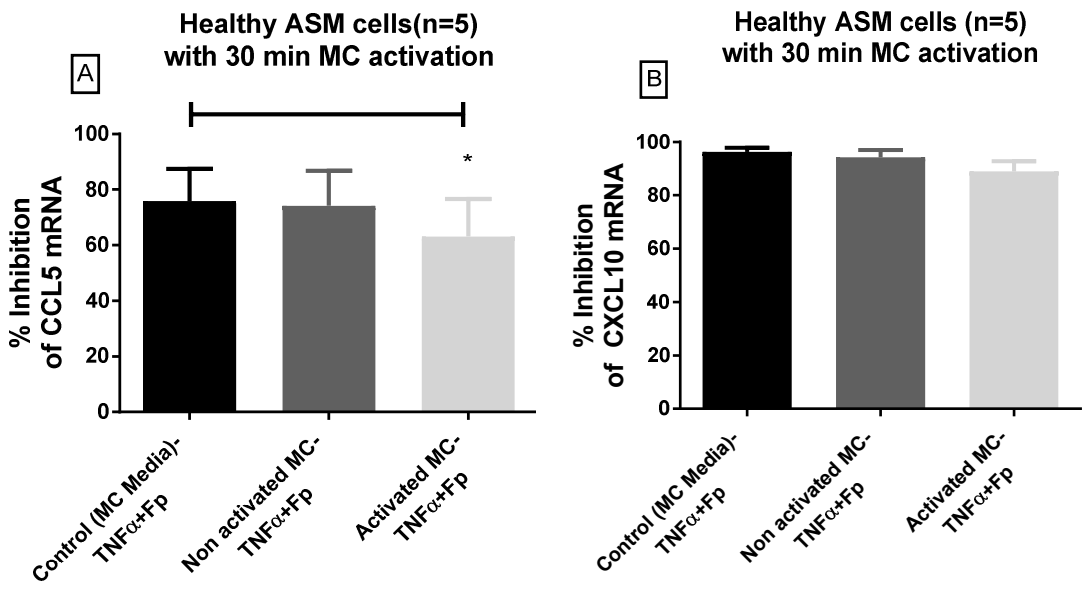


Figure 3.8: Inhibition of TNFα-induced chemokine mRNA expression by fluticasone in healthy ASM cells primed with control media or conditioned media from 30-minute activated and non-activated MCs.

Healthy ASM cells were pre-treated with control MC media (black bars), conditioned media from 30-minute non-activated (dark grey bars), 30-minute activated MCs (light grey bars) for 24 hours. ASM cells were then washed and stimulated with 10 ng/ml TNF-α and with or without 100 nM fluticasone for 6 hours. Chemokine expression of CCL5 (A) and CXCL10 (B) was assessed by real-time PCR. Data are presented as % inhibition from chemokine responses in cells treated with TNFα alone (Means ± SEM,= p<0.05, n=5).*

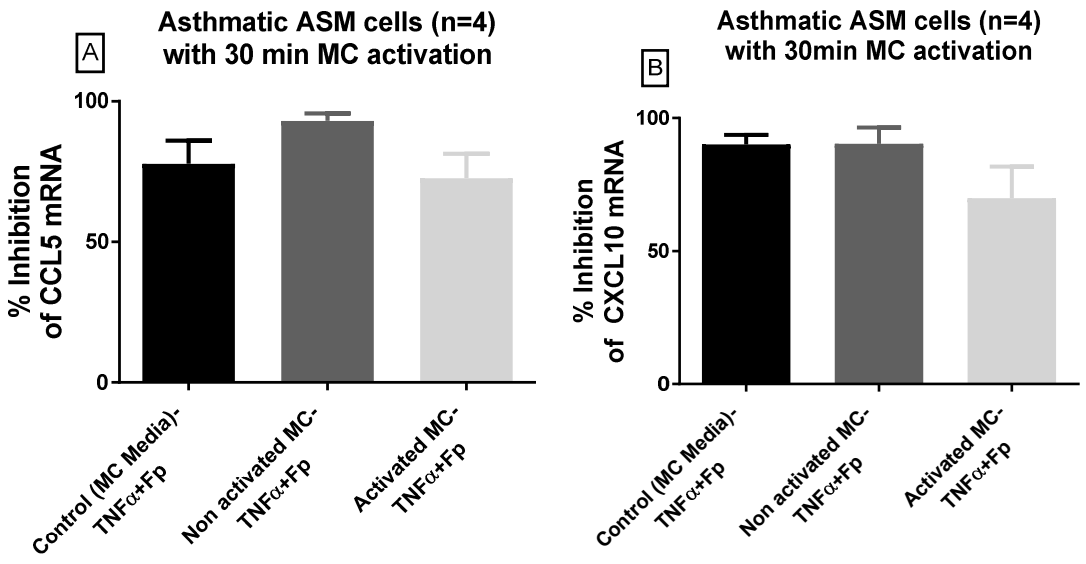


Figure 3.9: Inhibition of TNFα-induced chemokine mRNA expression by fluticasone in asthmatic ASM cells primed with control media or conditioned media from 30-minute activated and non-activated MCs.

Asthmatic ASM cells were pre-treated with control MC media (black bars), conditioned media from 30-minute non-activated (dark grey bars), 30-minute activated MCs (light grey bars) for 24 hours. ASM cells were then washed and stimulated with 10 ng/ml TNF-α and with or without 100 nM fluticasone for 6 hours. Chemokine expression of CCL5 (A) and CXCL10 (B) was assessed by real-time PCR. Data are presented as % inhibition from the chemokine responses in cells treated with TNFα alone (n=4).

3.2.6. Fluticasone transactivation property is not affected by conditioned media from 30-minute activated MC:

I next investigated whether fluticasone-induced transactivation signalling pathway was affected by MC conditioned media by looking at the expression of two well-known corticosteroid inducible genes called GILZ and MKP-1 previously known to be induced in ASM cells [92].

I found that MC conditioned media had no significant effect on GILZ and MKP-1 expression induced by fluticasone in healthy ASM cells. The fold changes of fluticasone-increased GILZ mRNA expression was 16.06±4, 17.42±5.6 and 17.88±6.8 fold in ASM cells primed with control MC media, conditioned media from 30-minute non-activated and activated MCs, respectively (**Figure 3.10A**). Also, the fold changes of fluticasone-increased MKP-1 mRNA expression was 7.08±1.2, 7.7±2.2 and 7.39±1.6 in ASM cells primed with control MC media, conditioned media from 30-minute non-activated and activated MCs, respectively (**Figure 3.10B**).

In asthmatic cells, the 13.89 ± 3.4 fold change in GILZ expression induced by fluticasone seen in cells primed with control media was not significantly reduced in cells primed with conditioned media from 30-minute non-activated or activated MCs with 11.81 ± 1.2 and 10.9 ± 2.3 fold change, respectively (**Figure 3.11A**). Also, the fold changes of MKP-1 mRNA expression by fluticasone in asthmatic ASM cells pre-treated with either control MC media, conditioned media from 30-minute non-activated or activated MCs were 3.99 ± 1.0, 2.4 ± 0.62 and 2.07 ± 0.67 over the basal conditions (**Figure 3.11B**). Although there was a trend toward a decreased of MKP-1 induction by conditioned media of activated MC, this did not reach statistical significance (p=0.084).

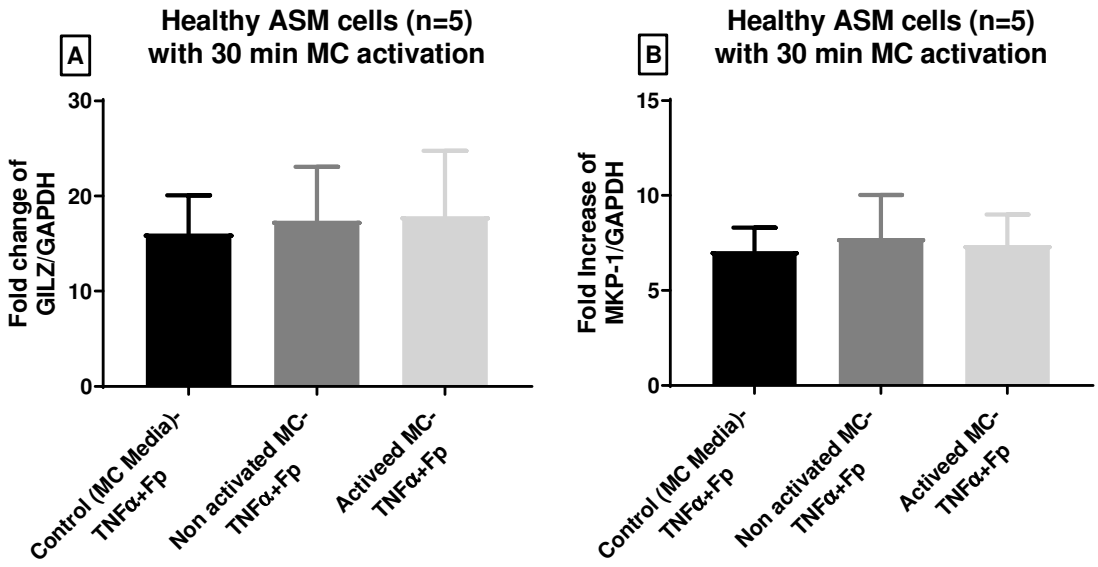


Figure 3.10: Induction of anti-inflammatory genes by fluticasone in healthy ASM cells primed with control media, and conditioned media from 30-minute non-activated or activated MCs.

Healthy ASM cells were pre-treated with control MC media (black bars), conditioned media from 30-minute non-activated (dark grey bars) or activated MCs (light grey bars) for 24 hours. ASM cells were then washed and stimulated with TNFα (10ng/ml), with or without FP (100nM) for an additional 6 hours. Expression of anti-inflammatory genes of GILZ (A) and MKP-1 (B) was assessed by real-time PCR. Data were normalised to the housekeeping gene (GAPDH) and presented as fold change over the basal condition using the $2^{\Delta\Delta ct}$ method as Means ±SEM, n=5 different cell lines).

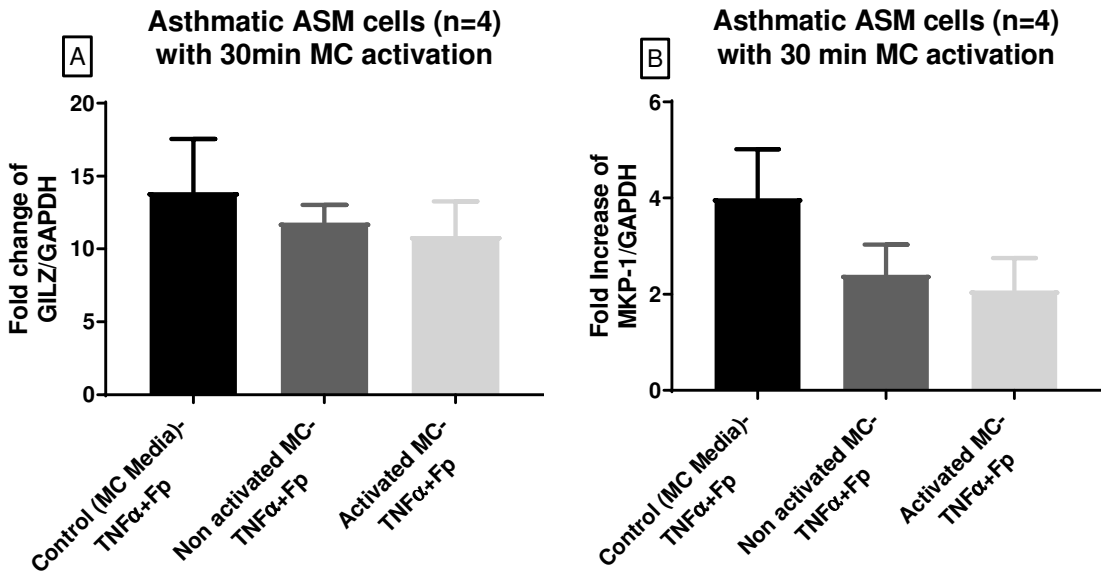


Figure 3.11: Induction of anti-inflammatory genes by fluticasone in asthmatic ASM cells primed with control media, and conditioned media from 30-minute non-activated or activated MCs.

Asthmatic ASM cells were pre-treated with control MC media (black bars), conditioned media from 30-minute non-activated (dark grey bars) or activated MCs (light grey bars) for 24 hours. ASM cells were then washed and stimulated with TNFα (10ng/ml), with or without FP (100nM) for an additional 6 hours. Expression of anti-inflammatory genes of GILZ (A) and MKP-1 (B) was assessed by real-time PCR. Data were normalised to the housekeeping gene (GAPDH) and presented as fold change over the basal condition using the $2^{\Delta\Delta ct}$ method as Means ±SEM, n=4 different cell lines).

3.2.7. Profile of the mediators produced in the conditioned media from activated MC:

In vitro corticosteroid insensitivity in various cell types can be induced by different inflammatory cytokines [96,175]. To determine the profile of inflammatory mediators released by MC following FcεR1 activation, I used the Proteome Profiler™ Antibody (membrane-based) Arrays to determine which of the following 80 different key cytokines, chemokines and acute phase proteins (the complete list is described in **Table 3-6**) were produced by MC following activation. The results showed that 9 different mediators were produced at resting conditions (**Table 3-1**). Following MC activation, mediators were released with different magnitude that could be divided into 4 different categories based on their fold increase over that seen in non-activated MC; <1 fold change (**Table 3-2**), between 1-5 fold change (**Table 3-3**), between 5-100 fold change (**Table 3-4**) and >100 fold change (**Table 3-5**).

Interestingly, some of the mediators that can affect the CS response such as IL17, IL2 with IL4 and TNFα were expressed by 30-minute activated mast cell with 0.16, 0.15, 0.61 and 0.98 fold increase over the non-activated mast cell respectively. Also, macrophage migration inhibitory factor (MIF) was produced by mast cells (non-activated and activated) but higher in non-activated mast cell.

Table 3-1: Profile of MC mediators produced by non-activated MC.

Mediators	Fold increase
MIF	0.03
VCAM-1	0.08
Relaxin-2	0.09
PDGF-AA)	0.10
IL-11	0.10
Leptin	0.11
SHBG	0.20
IL-19	0.36
Myeloperoxidase	0.43

Table 3-2: Profile of MC mediators produced by activated MC with <1 fold increase over levels produced by non-activated MC.

Mediators	Fold increase	Mediators	Fold increase
ICAM-1	0.01	CD31	0.46
Serpin E1	0.08	EMMPRIN	0.53
IL-4	0.15	IL-2	0.61
IL-17A	0.16	Endoglin	0.63
CD40 ligand	0.17	ENA-78	0.64
Resistin	0.17	IP-10	0.65
Lipocalin-2	0.18	ST2	0.65
IL-22	0.18	TIM-3	0.66
RBP-4	0.20	MIP-3beta	0.66
DPPIV	0.22	IL-3	0.66
Kallikrein 3	0.25	IL-18 BPa	0.67
Adiponectin	0.29	RANTES	0.69
SDF-1alpha	0.33	FGF-19	0.70
IL-6	0.38	EGF	0.76
GRO-alpha	0.42	TNF-alpha	0.98
uPAR	0.44		

Table 3-3: Profile of MC mediators produced by activated MC with <1-5> fold increase over levels produced by non-activated MCs.

Mediators	Fold increase	Mediators	Fold increase
IL-8	1.00	Dkk-1	2.05
CD14	1.15	LIF	2.28
Pentraxin-3	1.40	CD30	2.42
FGF basic	1.42	Osteopontin	2.53
Thrombospondin-1	1.45	VEGF	2.84
IL-1alpha	1.58	IL-1ra	3.06
MCP-1	1.72	IL-10	3.63
Angiopoietin-2	1.83	IL-1beta	3.92
GDF-15	1.87	Chitinase 3-like 1	4.00
GM-CSF	1.88	BAFF	4.04
I-TAC	1.94	MMP-9	4.86

Table 3-4: Profile of MC mediators produced by activated MC with <5-100> fold increase over levels produced by non-activated MCs.

Mediators	Fold increase
Cripto-1	18.64
HGF	20.88
IGFBP-2	27.62
IL-33	37.65
M-CSF	38.86
IL-23	41.20
IL-16	78.56
IL-15	97.86

Table 3-5: Profile of MC mediators produced by activated MC with <100 fold increase over levels produced by non-activated MCs.

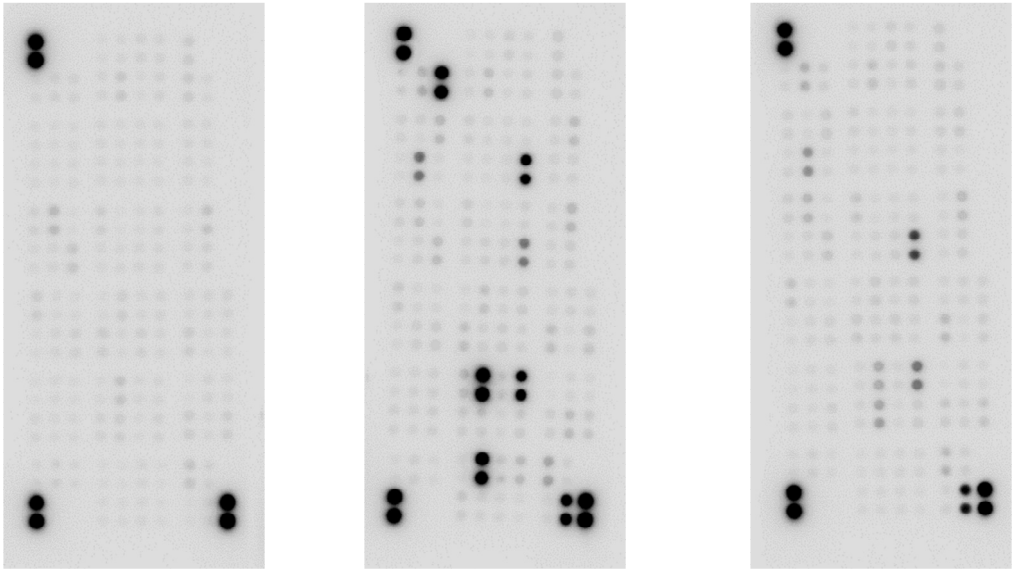
Mediators	Fold increase
Angiopoietin-1	102.14
Flt-3 Ligand	103.59
Cystatin C	149.26
C-Reactive Protein	213.87
Complement Component C5/C5a	230.93
BDNF	238.86
G-CSF	254.25
Apolipoprotein A-I	313.32
Angiogenin	430.16
Complement Factor D	1324.61

Table 3-6: List of all mediators which can be detected by the protein array assay.

Adiponectin	Chitinase 3-like 1	Fas Ligand	HGF	IL-4	IL-17A	IL-34	MIF	RAGE	TFF3	VCAM-1
Apolipoprotein A-I	Complement Factor D	FGF basic	ICAM-1	IL-5	IL-18 Bpa	IP-10	MIG	RANTES	TfR	
Angiogenin	C-Reactive Protein	FGF-7	IFN-gamma	IL-6	IL-19	I-TAC	MIP-3alpha	RBP-4	TGF-alpha	
Angiopoietin-1	Cripto-1	FGF-19	IGFBP-2	IL-8	IL-22	Kallikrein 3	MIP-3beta	Relaxin-2	Thrombospondin-1	
Angiopoietin-2	Cystatin C	Flt-3 Ligand	IGFBP-3	IL-10	IL-23	Leptin	MMP-9	Resistin	TNF-alpha	
BAFF	Dkk-1	G-CSF	IL-1alpha	IL-11	IL-24	LIF	Myeloperoxidase	SDF-1alpha	uPAR	
BDNF	DPPIV	GDF-15	IL-1beta	IL-12 p70	IL-27	Lipocalin-2	Osteopontin	Serpin E1	VEGF	
Complement Component C5/C5a	EGF	GM-CSF	IL-1ra	IL-13	IL-31	MCP-1	PDGF-AA	SHBG	Vitamin D BP	
CD14	ENA-78	GRO-alpha	IL-2	IL-15	IL-32	MCP-3	PDGF-AB/BB	ST2	CD31	
CD40 ligand	Endoglin	Growth Hormone	IL-3	IL-16	IL-33	M-CSF	Pentraxin-3	TARC	TIM-3	

Figure 3.12: Proteome Profiler™ membranes exposed to control MC media, conditioned media from 30 min activated and non-activated MCs.

Control MC media Activated MC SN Non-activated MC SN



3.3. Discussion:

Previous studies have shown that a 24 or 48 hours incubation with conditioned media from activated MC regulated a number of different functions in ASM cells including the expression of CCL11 and CXCL8 [203], CXCL10 [104], and β2-agonist responsiveness [35]. I here tested whether the sensitivity of ASM cells to corticosteroids was affected by mediators present in the supernatants of activated MCs.

Justification of the experimental model. The choice of the concentration of fluticasone (100 nM) was based on previous studies from our group and others showing that this concentration was effective in inhibiting the production of pro-inflammatory mediators induced by TNFα [95]. I also used a 24 hours incubation period with MC conditioned media as Amrani's lab has used this approach to show that corticosteroid insensitivity can be induced by pre-treating ASM cells with TNFα/IFNγ combination [96]. Finally, I found that the IgE-anti-IgE method of MC activation gave inconsistent results that could be explained by the use of antibodies coming from different lot numbers. This is in contrast with the anti-FceR1 method which showed that all MC preparations were effectively activated using this approach using the β-hexosaminidase assay [210].

Conditioned media from 30-minute activated MCs modulate ASM cells sensitivity to fluticasone. The data in this chapter provide the first evidence of a link between MC and corticosteroid sensitivity in ASM cells. The results show that the ability of fluticasone to suppress the protein expression of two major chemokines CCL5 and CXCL10 was significantly reduced by pre-treating ASM cells with conditioned media from activated MC compared to control or non-activated MC media (**Figure 3.6**). The mechanisms likely involved multiple pathways as activated MC conditioned media only suppressed mRNA induction by fluticasone of CCL5 but not that of CXCL10. It is known

that corticosteroids suppress the expression of inflammatory genes by acting at different levels [17,78]. Therefore, it is possible that corticosteroid signalling in ASM cells is affected by 30-minute activated MC conditioned media at both transcriptional and non-transcriptional levels. We also found that the "so-called" dissociated steroid compound-A, a dissociated glucocorticoid receptor alpha ligand, also regulated chemokine expression by acting at both protein and mRNA levels [220]. The effect of MC conditioned media was not due to an effect of chemokine expression as responses to TNFα were not affected (**Figure 3.4** and **Figure 3.5**). It was interesting to note that the effect of 30-minute activated MC conditioned media was not present in cells from asthmatics. We and others have shown that asthmatic ASM cells have a reduced response to corticosteroids [20,92]. I also found that the efficacy of fluticasone to suppress CCL5 and CXCL10 was reduced in asthmatic vs healthy ASM cells. This pre-existing reduced corticosteroid sensitivity seen in asthmatic cells could possibly be explained by the lack of effect of activated MC conditioned media. Although asthmatic patients had similar spirometry measurements when compared to healthy subjects, these patients had an impaired bronchial responsiveness (PC20) despite being treated with ICS. The sensitivity of asthmatic patients to CS is usually defined by the analysis of different clinical parameters which are all not equally affected by CS therapy. I also found that an alteration of the transactivation function of corticosteroids may not explain the inhibitory action of activated MC conditioned media as I found no effect on the mRNA expression of two genes, GILZ and MKP-1 (**Figure 3.8**). Additional studies are required to confirm that the protein expression of both MKP-1 and GILZ was also not modulated by MC conditioned media. The reasons for studying transactivation signalling pathway come from the studies showing that different cytokines inhibit cell response to corticosteroids by interfering with the induction of anti-inflammatory genes such as GILZ, MKP-1, IkBα [192,221]. It

is possible that corticosteroid transrepression signalling pathway may be affected by 30-minute activated MC conditioned media.

MC activation leads to the release of various types of mediators. I used a membrane based protein array to show that mast cell activation resulted in the release of various mediators including **cytokines** (*IL-1alpha, IL-1beta, IL-1ra, IL-2, IL-3, IL-4, IL-6, IL-10, IL-11, IL-15, IL-16, IL-17A, IL-18 Bpa, IL-19, IL-22, IL-23, IL-33, BAFF, BDNF, G-CSF, GM-CSF, LIF, M-CSF, MIF, ST2 and TNF-alpha*), **chemokines** (*GRO-alpha (CXCL1), ENA-78 (CXCL5), IL-8 (CXCL8) IP-10 (CXCL10), I-TAC (CXCL11), SDF-1alpha (CXCL12), MCP-1 (CCL2), RANTES (CCL5), Complement Component C5/C5a and Osteopontin*) and **growth factors** (Cripto-1, IGFBP-2, Flt-3 Ligand, PDGF-AA, EGF, FGF basic, VEGF, FGF-19, HGF, GDF-15).

Some of these mediators have been reported to regulate critical pathways in asthma including airway inflammation (*IL-4, IL-33 and TNF-alpha*), airway remodelling (*IL-4*, *CXCL1, CXCL10, CXCL11, CCL2, CCL5*), airway hyper-responsiveness (*TNF-alpha* and IL-17). The possible mediators that could regulate ASM cells response to corticosteroids include IL-2 with IL-4, IL17A, IL17A with IL23 and MIF based on previous studies showing that these mediators can induce corticosteroid insensitivity in various cell types [173,215,217,222].

In conclusion, conditioned media from 30-minute activated MCs impaired only fluticasone ability to suppress TNFα-induced CCL5 (at the protein and mRNA level) and CXCL10 (at the protein level only) in healthy ASM cells but not CXCL8. Also, conditioned media from 30-minute activated MCs had no effect on TNFα signalling pathway in ASM cells and appear not to be due to changes in corticosteroid transactivation properties.

4.Chapter four

Modulation of ASM sensitivity to fluticasone by mast cell conditioned media: effect of long-term activation (24-hour)

4.1. Introduction:

As discussed before, the number of MCs correlated with markers of asthma severity and AHR, features which are known to be less responsiveness to corticosteroid therapies [32,142]. Inhibiting MC activation using omalizumab in patients who are poorly controlled by corticosteroids leads to reduced exacerbations and symptoms [158-160]. The mechanisms by which MCs could play a role in corticosteroid-resistant features in asthma have not been elucidated, but several studies have shown that cytokines, synthesised and released by activated MCs can regulate corticosteroid responses in different cells types. Indeed, MC mediators such as IL-17A [173], IL-4 and IL-2 [215], IL-13 [199], IFN-γ and IL-27[216], TGF-β1[174] and MIF [217] have been shown to blunt corticosteroid response in different cell types via multiple mechanisms including the inhibition of the transactivation of anti-inflammatory genes (summarized in the **chapter One**).

More importantly, MCs can impact multiple functions of ASM cells via direct interaction (cell-cell interactions) [35] or/and indirect interaction (production of MC mediators) [35,104]. For instance, we showed that β2-agonist responsiveness in ASM cells was modulated by mast cells via cell-cell interaction involving CADM1 and SCF [35]. Also, co-culturing mast cells with ASM cells or conditioned media from 24-hours activated mast cell can influence the production of various key chemokines including CCL11, CXCL8 and CXCL10 in ASM cells [104,203,223]. Mast cell mediators have also been associated with ASM hypertrophy and ASM hyperplasia [218]. For example, tryptase (mainly produced by mast cells) can regulate the proliferation and differentiation of structural cells in the lung such as ASM cells [34,151]. TGFβ1, which is known to be produced by MCs [174], also regulates ASM proliferation, epithelium damage and mucus secretion [31,49].

Aims:

Because a number of synthesised MC mediators, when tested individually, can inhibit the anti-inflammatory action of corticosteroids in different cell types, I hypothesised that conditioned media from 24-hour activated MCs (which contains various *de novo* synthesised lipid mediators, cytokines, and chemokines) may modulate the inhibition of TNF-α-induced chemokines by fluticasone in healthy ASM cells. The studies were conducted in two phases:

Phase 1: control experiments

A. To confirm MC activation by assessing β-hexosaminidase assay and TNFα levels in the conditioned media.

B. To determine whether conditioned media from 24-hour activated MCs affected viability of ASM cells.

C. To investigate the effect of conditioned media from 24-hour activated MCs on the net increase of chemokines induced by TNFα.

Phase 2: Sensitivity experiments

A. To investigate whether conditioned media from 24-hour activated MCs modulated the ability of fluticasone to repress TNFα-induced chemokines

    * At the protein level using ELISA.

    * At the mRNA level using qPCR.

B. To investigate whether conditioned media from 24-hour activated MCs modulated the transactivation properties of fluticasone in the presence of TNF-α.

C. To determine the profile of mediators produced in the 24-hour mast cell SN before and after activation and identify the potential players that reduce CS sensitivity in ASM cells.

4.2. Results:

4.2.1. Confirming MC activation by assessing the TNF-α level and β-hexosaminidase activity:

For all the experiments, MCs isolated and cultured in MC media that contained SCF (1 ng/ml), an essential survival factor [109]). The media from resting MCs were used as 24-hours non-activated MC conditioned media while MCs, where media containing anti-FcεR1 (1:300) was added to the cells for 24-hours, was used as 24-hours activated MC conditioned media.

As shown in the previous chapter, β-hexosaminidase activity was measured to assess MC degranulation in the conditioned media of 24-hour activated MCs. MCs are known to have large amounts of β-hexosaminidase in granules [224]. The β-hexosaminidase assay showed that conditioned media from both 24-hour non-activated and activated MCs showed β-hexosaminidase activity, although the activity was higher following MC activation **(Figure 4.1A)**. The β-hexosaminidase activity expressed as fold increased over that seen in conditioned media from non-activated MCs was 2.80±0.40 (P= 0.0205) in n=4 different MC preparations **(Figure 4.1A)**. Also, we assessed the MC degranulation efficiency by measuring β-hexosaminidase activity in both supernatants and pellets of 24-hour activated MCs. The results were expressed as ratio of β-hexosaminidase activity in supernatant/pellet 30:49 (n=3) (Data not included). ELISA was also used to further confirm mast cell activation by measuring TNF-α level. The results indicated that the net increase of TNFα in the conditioned media of 24-hour activated MCs was 219.2 ±84.33 pg/ml of n=4 MC media (p= 0.0402) **(Figure 4.1B)**.

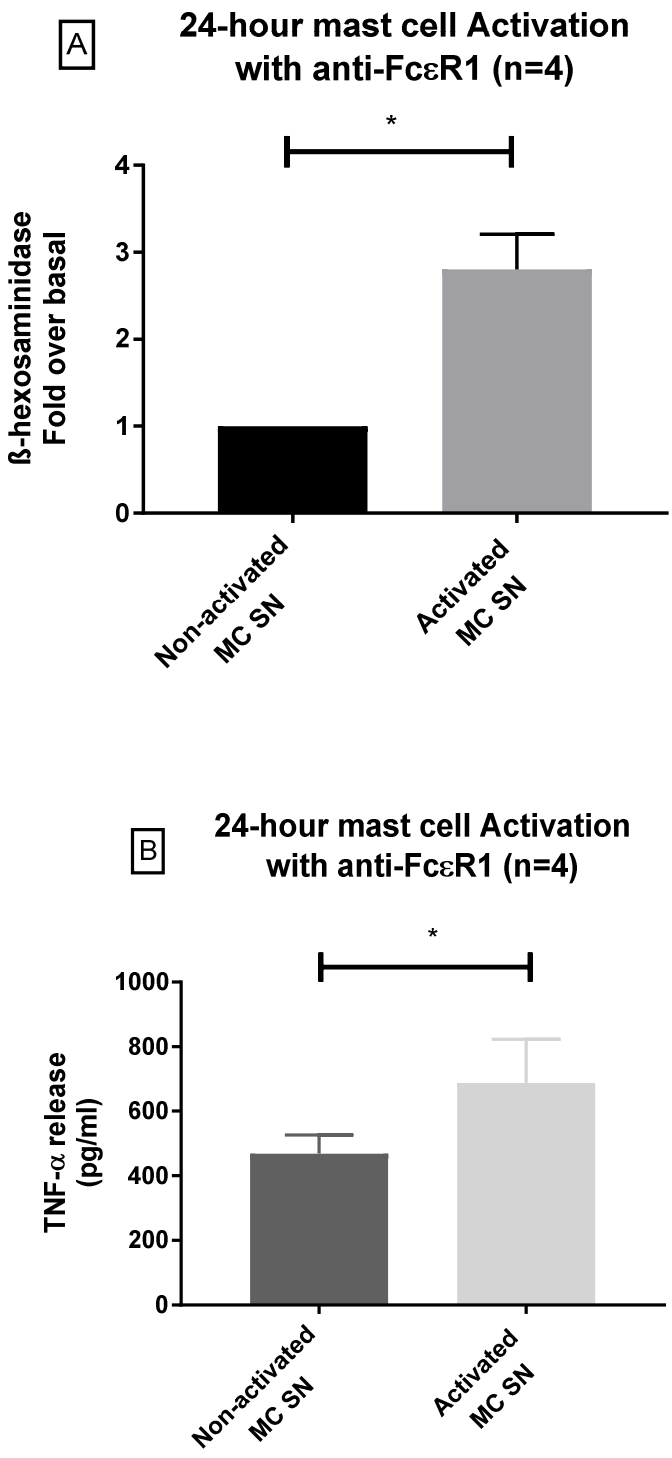


Figure 4.1: Confirmation of 24-hour MC activation by assessing β-hexosaminidase activity and TNFα levels.

*Human lung MCs were treated with anti-FcεR1 (1.7 ng/ml) for 24-hour and conditioned media from activated and non-activated MCs were used to determine β-hexosaminidase activity (A) and TNF-α level using ELISA (B). (Means ±SEM of n=4 MC media, *=P<0.05). Comparisons were made using paired T-Test.*

4.2.2. Conditioned media from 24-hour mast cell activation did not affect ASM cell viability:

The viability of ASM cells was performed to determine any changes in cytotoxic effects of exposing cells to conditioned media from the 24-hour activated and non-activated MCs. MTT assay showed that the ASM cell viability was not affected by our experimental design compared to cells pre-treated with control MC media. The absorbance values of formazan were 1.531±0.26, 1.693±0.31 and 1.628±0.34 in healthy ASM cells which were pre-treated with control mast cell media, conditioned media from 24-hour non-activated and activated MCs, respectively **(Figure 4.2)**.

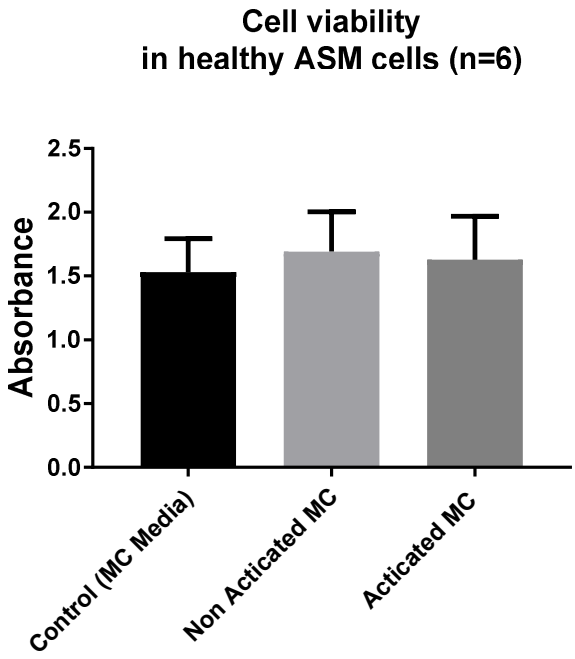


Figure 4.2: The effect of conditioned media from 24-hour mast cells (MC) on ASM cell viability by MTT.

Healthy ASM cells were pre-treated with control MC media (black bar), 24-hour non-activated (light grey bar) and activated MC conditioned media (dark grey bar). Cell viability was assessed by MTT assay. Data were presented as optical density (means ±SEM of n=6 different ASM cell lines).

4.2.3. Conditioned media from 24-hour mast cell activation do not affect TNFα-induced chemokines in healthy ASM cells:

In this set of experiment, we investigated whether the production of chemokines induced by TNFα stimulation in healthy ASM cells was affected by conditioned media from 24-hour activated and non-activated MCs compared to MC media. Previously, Alkhouri and colleagues have shown that a similar approach (24-hour conditioned MC media) modulates the production of CXCL8, CCL11 and CXCL10 by TNFα [104,203]. So, to investigate this, ASM cells were primed with control MC media, conditioned media from 24-hour non-activated and activated MCs. Later, ASM cells were washed and stimulated with or without TNFα (10ng/ml) for 24 hours and CCL5, CXCL10, CXCL8 and CCL11 in the cell supernatants were measured by ELISA. As previously described in different studies [92,104,203,220], TNFα stimulated the production of CCL5, CXCL10, CXCL8 and CCL11 significantly compared to the basal conditions in ASM cells.

The basal levels of CCL5 production in unstimulated ASM cells pre-treated with control media, conditioned media from 24-hour non-activated and activated MCs were 1.07±0.48 ng/ml, 0.84±0.35 ng/ml and 0.51±0.14 ng/ml, respectively. In cells treated with TNFα, CCL5 levels increased significantly to 12.22±6.78 ng/ml, 10.36±6.37 ng/ml and 8.65±5.97 ng/ml with a P-value less than 0.0001, 0.0001, 0.0001 respectively **(Figure 4.3A)**. Similarly, CXCL10 levels in unstimulated ASM cells pre-treated with control media, conditioned media from 24-hours non-activated and activated MCs were 4.25±2.11ng/ml, 3.04±1.79 ng/ml and 2.07±0.88 ng/ml respectively. In cells stimulated with TNF-α, the levels of CXCL10 increased significantly to 17.51±5.92 ng/ml, 18.29±6.86 ng/ml and 16.16±6.29 ng/ml with a p-value less than 0.0001, 0.0001, 0.0001

respectively **(Figure 4.3B)**. Also, the levels of CXCL8 in unstimulated ASM cells pre-treated with control media, conditioned media from 24-hours non-activated and activated MCs were 14.91±10.29 ng/ml, 8.21±2,04 ng/ml and 7.46±2.27 ng/ml respectively. In cells stimulated with TNFα, the expression of CXCL8 increased significantly to 83.03±32.55 ng/ml, 88.38±29.46 ng/ml and 89.99±29.46 ng/ml with a p-value less than 0.0001, 0.0001, 0.0001 respectively **(Figure 4.3C)**. Lastly, the basal levels of CCL11 in unstimulated ASM cells pre-treated with control, conditioned media from 24-hour non-activated and activated MCs were 127.5±91.56 pg/ml, 1216±940 pg/ml and 1227±996 pg/ml respectively. In cells stimulated with TNFα, the levels of CCL11 increased significantly to 1996±1337 ng/ml, 2365±1670 pg/ml and 2806±2127 ng/ml with a p-value = 0.004, 0.0135, 0.0016 respectively **(Figure 4.3D)**.

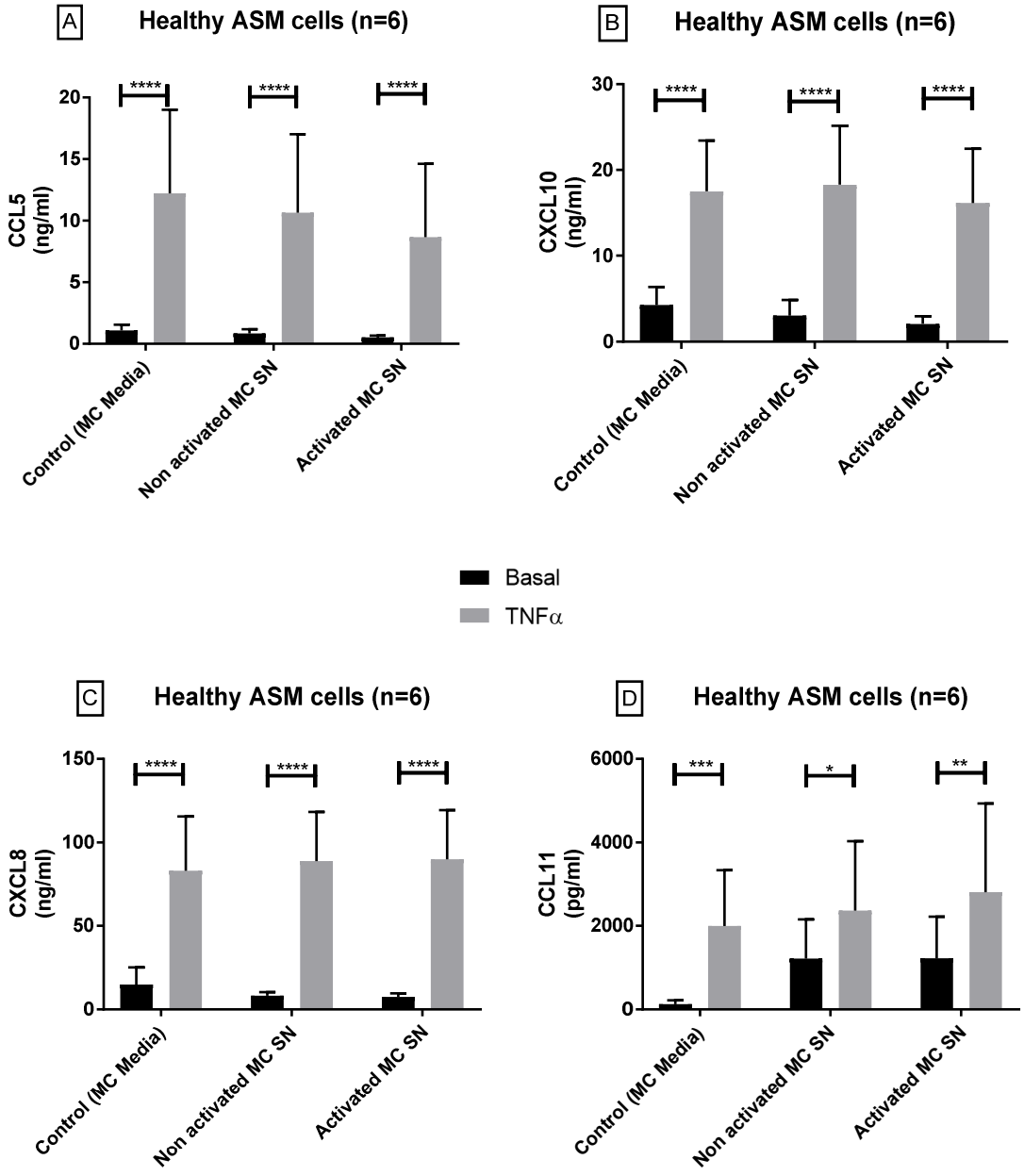


Figure 4.3: TNFα-induced chemokine production in ASM cells treated with control and 24-hour MC conditioned media.

Healthy ASM cells were pre-treated with control MC media, 24-hour non-activated and activated MC conditioned media for 24 hours. ASM cells were then washed and stimulated with TNF-α (10ng/ml) (grey bars) or without stimulation (black bars). The chemokine levels of CCL5 (A), CXCL10 (B), CXCL8 (C) and CCL11 (D) were assessed by ELISA. Data were presented as ng/ml or pg/ml of protein as Means ±SEM of n=6 different cell lines. Comparisons between groups were made using one-way ANOVA and Tukey correction.

4.2.4. Conditioned media from 24-hour mast cell activation do not modulate the net increase of TNFα-induced chemokines in healthy ASM cells:

In this set of experiment, we investigated if human lung mast cell conditioned media could influence the **net increase** of TNFα-induced chemokines in healthy ASM cells. Figure 4.3 and Figure 4.4 express the same data where figure 4.3 was provided to demonstrate that MC conditioned media did not affect both chemokine levels in both basal and TNFα stimulation. ASM cells were primed with control MC media, conditioned media from 24-hour non-activated and activated MCs for 24 hours. then, ASM cells were washed and stimulated with or without TNF-α (10ng/ml, for 24 hours). CCL5, CXCL10, CXCL8 and CCL11 were measured by ELISA. Overall, there were no significant differences in the net increase of CCL5, CXCL10, CXCL8 and CCL11 by TNFα in healthy ASM cells primed with three different conditions (control MC media, conditioned media from 24-hour non-activated and activated MCs).

The net increase of CCL5 by TNFα in control ASM cells was 10.64 ± 6.48 ng/ml, while in ASM cells pre-treated with conditioned media from 24-hour non-activated and activated MCs were 9.45 ± 6.14 ng/ml and 8.07 ± 5.92 ng/ml respectively, without any significant changes **(Figure 4.4A)**. Similarly, the net increase of CXCL10 by TNFα was 13.29 ± 4.0 ng/ml, 15.25 ± 5.54 ng/ml and 14.9 ± 5.77 ng/ml in ASM cells pre-treated with control MC media, conditioned media from 24-hour non-activated and activated MCs, respectively, without any significant changes **(Figure 4.4B)**. Again, the production of TNFα-induced-CXCL8 in control ASM cells was 68.11 ± 24.1 ng/ml, while in ASM cells which pre-treated with conditioned media from 24-hour non-activated and activated MCs were 80.61 ± 27.61 ng/ml and 82.58 ± 27.25 ng/ml, respectively, without any significant changes **(Figure 4.4C)**. Lastly, CCL11 induction by TNFα was 2.106 ± 1.84

ng/ml, 1.246 ± 0.902 ng/ml and 1.832 ± 1.35 ng/ml in ASM cells primed with control MC media, conditioned media from 24-hour non-activated and activated MC respectively, without any significant changes **(Figure 4.4D)**.

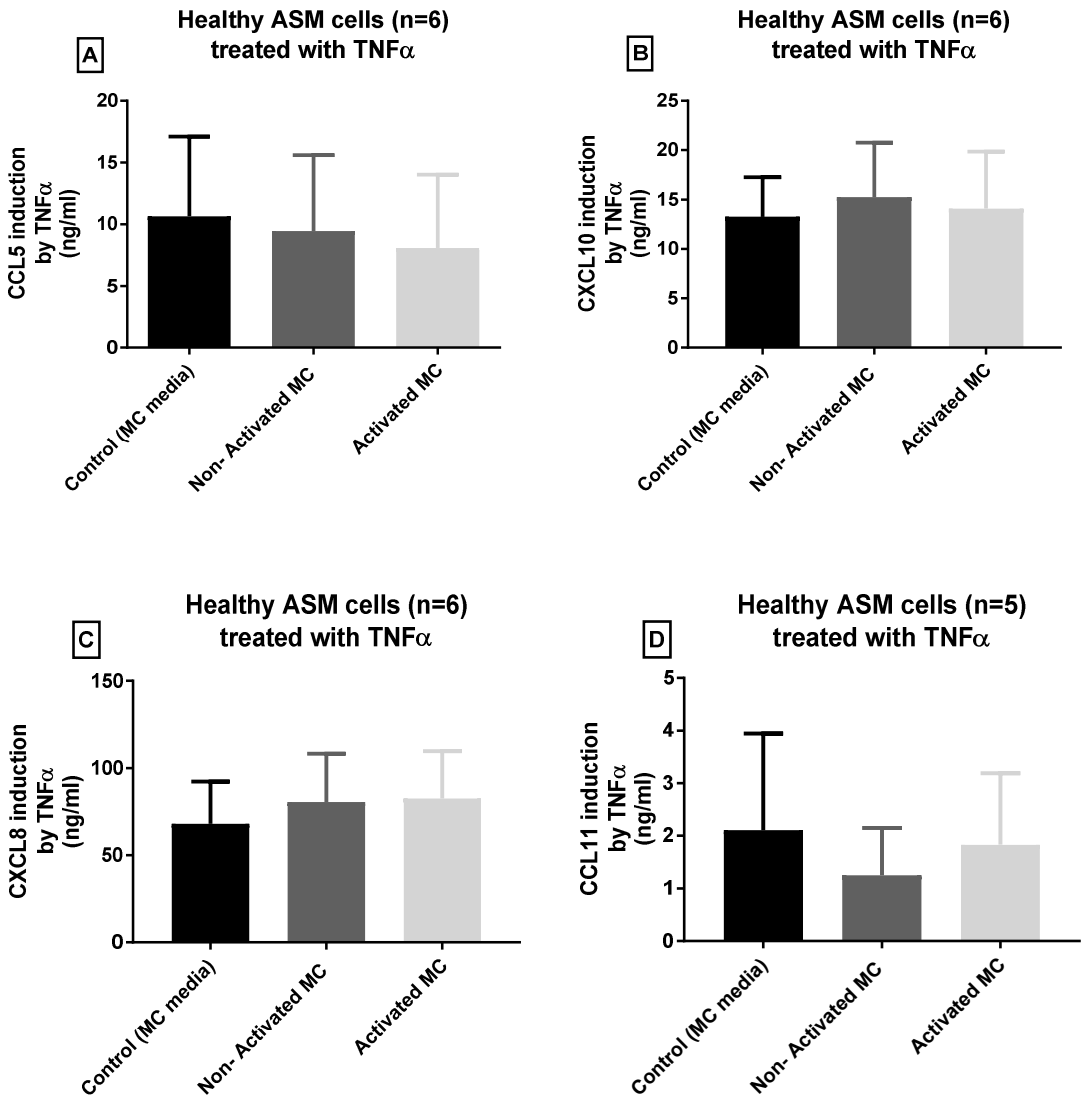


Figure 4.4: The effect of control media, conditioned media of 24-hour activated and non-activated MCs on the net increase of chemokine production by TNFα in healthy ASM cells.

Healthy ASM cells were pre-treated with control MC media (black bar), 24-hour non-activated (dark grey bar) and activated MC conditioned media (light grey bar) for 24 hours. Later, ASM cells were washed and stimulated with or without TNF-α (10ng/ml) for 24 hours. The net increase of CCL5 (A), CXCL10 (B), CXCL8 (C) and CCL11 (D) were assessed by ELISA. Data are presented as ng/ml protein and expressed as Means ±SEM of n=6 different cell lines). Comparisons between groups were made using one-way ANOVA and Tukey correction.

4.2.5. Conditioned media from 24-hour activated MCs differentially regulate the inhibitory action of fluticasone on TNF-α-induced chemokines at the protein level:

It is clear that conditioned media from 24-hour activated MCs did not modulate the signalling pathway mediating TNF-α-induced chemokines in ASM cells. I next investigated whether the ability of fluticasone to suppress TNFα-induced chemokine production was modulated. Healthy ASM cells were primed with either control MC media, conditioned media from 24-hour non-activated and activated MCs, and after overnight incubation, the cells were washed and stimulated with TNFα (10 ng/ml) and with or without fluticasone (100 nM) for an additional 24 hours. Interestingly, conditioned media from 24-hour activated MCs significantly reduced % inhibition of TNF-α-induced CCL5, CXCL10 and CCL11 by fluticasone when compared to cells treated with MC media.

The % inhibition of TNFα-induced CCL5 production by fluticasone was 71.1 ± 11.57 % in ASM cells pre-treated with control MC media which was further reduced to 61.61 ± 12.28 % and 48.84 ± 14.98 % in cells pre-treated with conditioned media from 24-hour non-activated and activated MCs, respectively. There was a significant reduction in the fluticasone action by 22.27 % in ASM cells pre-treated with conditioned media from 24-hour activated MCs compared to control condition (p= 0.0180, n=6) **(Figure 4.5A)**. Conditioned media from non-activated MCs had no effect of fluticasone % inhibition.

Also, TNFα-induced production of CXCL10 was repressed by fluticasone by 74.16 ± 9.76 % in ASM cells treated with control MC media, and 59.23 ± 8.98 % in cells pre-treated with conditioned media from 24-hour non-activated MCs. Although there

appeared to be a reduction of fluticasone % inhibition of TNF-α-induced CXCL10 of 14.94 % in ASM cells primed with conditioned media from 24-hour non-activated MCs compared to control media, this effect was not statistically significant. By contrast, ASM cells primed with conditioned media from 24-hour activated MC, the % inhibition of TNFα-induced CXCL10 production by fluticasone was significantly reduced to 53.65 ± 13.5%, compared to cells treated with control media (20.51% reduction, p=0.0208) **(Figure 4.5B)**.

Similarly, fluticasone inhibited TNFα-induced CCL11 expression by 47.51 ± 8.74 %, 43.55 ± 11.56% and 17.56 ± 10.14 % in ASM cells pre-treated with control and conditioned media from 24-hour non-activated and activated MCs, respectively. The suppressive effect of conditioned media from 24-hour activated MCs on fluticasone % inhibition of CCL11 expression was found to be significantly different compared to the effect seen in ASM cells treated with the control media and conditioned media from 24-hour non-activated MCs (29.95% and 25.99% reduction respectively) **(Figure 4.5D)**.

It is interesting to note that the inhibitory action of fluticasone on CXCL8 expression was not modulated by MC conditioned media. The % inhibition of TNFα-induced CXCL8 expression by fluticasone in ASM cells pre-treated with control MC media was 82.25 ± 5.35 %, and this did not change dramatically in ASM cells pre-treated with conditioned media from 24-hour non-activated and activated MCs (85.84 ± 4.15 % and 82.65 ± 2.28 % inhibition by fluticasone, respectively) **(Figure 4.5C)**.

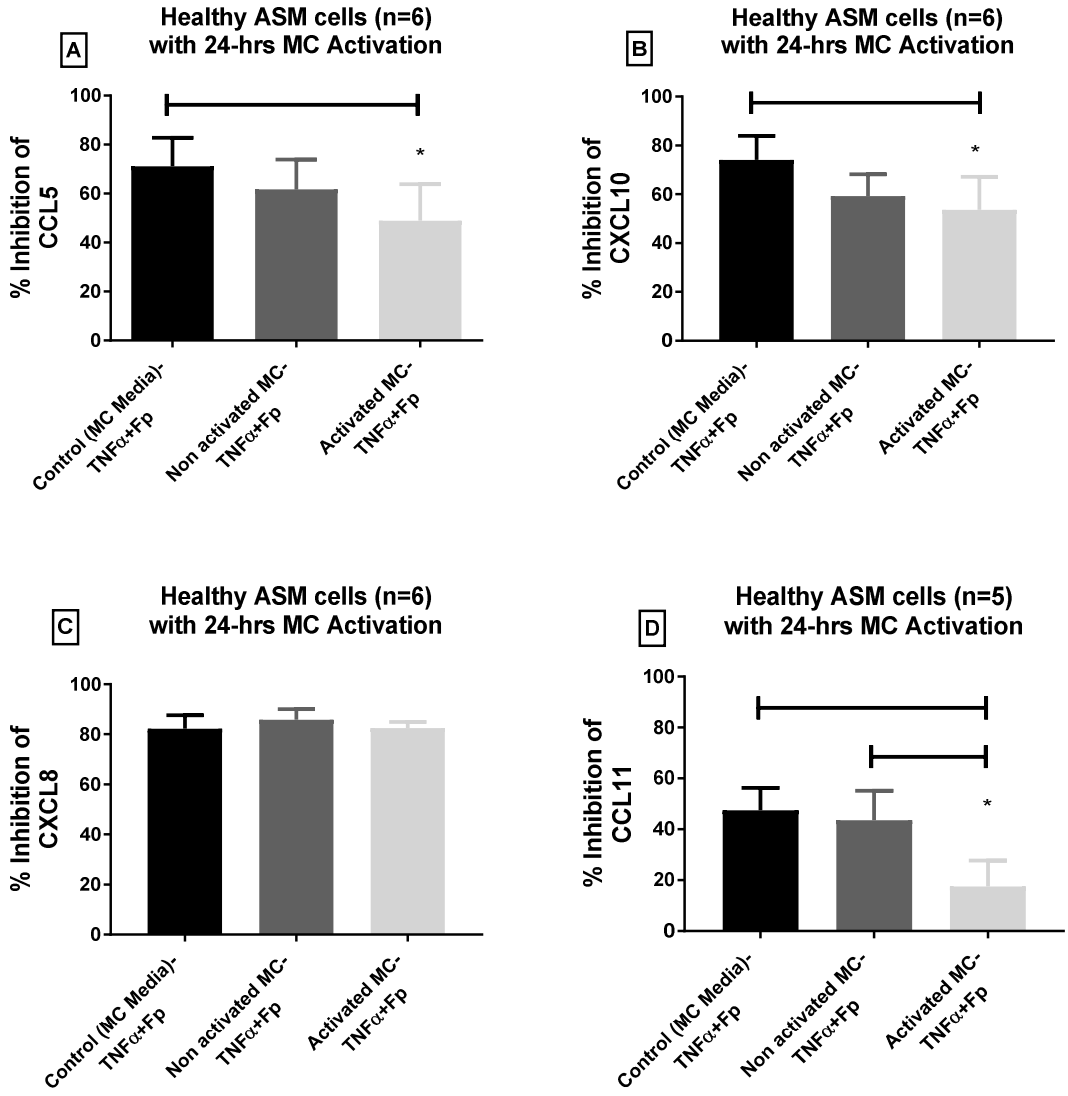


Figure 4.5: % inhibition of TNFα-induced-chemokines by fluticasone in ASM cells pre-treated with conditioned media from 24-hour non-activated and activated MCs.

Healthy ASM cells were pre-treated with control MC media (black bars), 24-hour non-activated (dark grey bars) and activated MC conditioned media (light grey bars). ASM cells were then washed and stimulated with TNFα (10ng/ml) and with or without fluticasone (100nM) for 24 hours. Expression of CCL5 (A), CXCL10 (B) and CXCL8 (C) and CCL11(D) was assessed by ELISA with data presented as % of the chemokine responses in cells treated with TNFα alone (Means ± SEM of n=6 different cell lines), (= p<0.05). Comparisons between groups were made using one-way ANOVA with Tukey correction.*

4.2.6. Conditioned media from 24-hour activated MCs impair fluticasone inhibitory action on TNF-α-induced chemokines by acting at the transcriptional level:

Healthy ASM cells pre-treated with conditioned media from 24-hour activated MCs had a reduced fluticasone action in repressing TNF-α-induced chemokines at the protein level. Additional experiments were undertaken to see whether modulation also happened at the mRNA level. Healthy ASM cells pre-treated with control MC media, conditioned media from 24-hour activated and non-activated MC for 24 hours. Following treatment, ASM cells were washed and treated with TNF-α (10 ng/ml) with or without fluticasone (100 nM) for 6 hours before total RNA isolation as described before (chapter 3). The 24-hours activated MC conditioned media had a similar inhibitory action at both mRNA and protein levels. Analysis of CCL11 expression is not included due to unforeseen technical issues.

Fluticasone repressed TNFα-induced CCL5 mRNA expression by 80.47 ± 7.45 %, 77.91 ± 8.84 and 48.46 ± 15.25 % in ASM cells pre-treated with control MC media and conditioned media from 24-hour non-activated and activated MC, respectively (Figure **4.6A)**. TNFα-induced CCL5 mRNA expression was found to be significantly inhibited in healthy ASM cells pre-treated with conditioned media from 24-hour activated MCs when compared ASM cells which pre-treated with control MC media (P=0.0483) (Figure **4.6A**).

Similarly, the % inhibition of TNFα-induced CXCL10 mRNA expression by fluticasone was 88.15 ± 1.96 %, 86.96 ± 1.86 % and 57.63 ± 11.5 % in healthy ASM cells pre-treated with control MC media, conditioned media from 24-hour non-activated and activated MCs, respectively. TNFα-induced CXCL10 mRNA expression was found to be

significantly inhibited in healthy ASM cells pre-treated with conditioned media from 24-hour activated MCs when compared ASM cells which pre-treated with control MC media (P=0.0188) (Figure **4.6B**).

Lastly, conditioned media from 24-hour non-activated and activated MCs did not affect the fluticasone inhibitory action on TNFα-induced mRNA expression of CXCL8 compared to cells pre-treated with control MC media. Fluticasone inhibited TNFα-induced- CXCL8 mRNA levels by 59.65±5.57 %, 58.36 ± 15.68 % and 65.43±14.2 % in ASM cells pre-treated with control MC media, conditioned media from 24-hour non-activated and activated MCs, respectively (Figure **4.6C**).

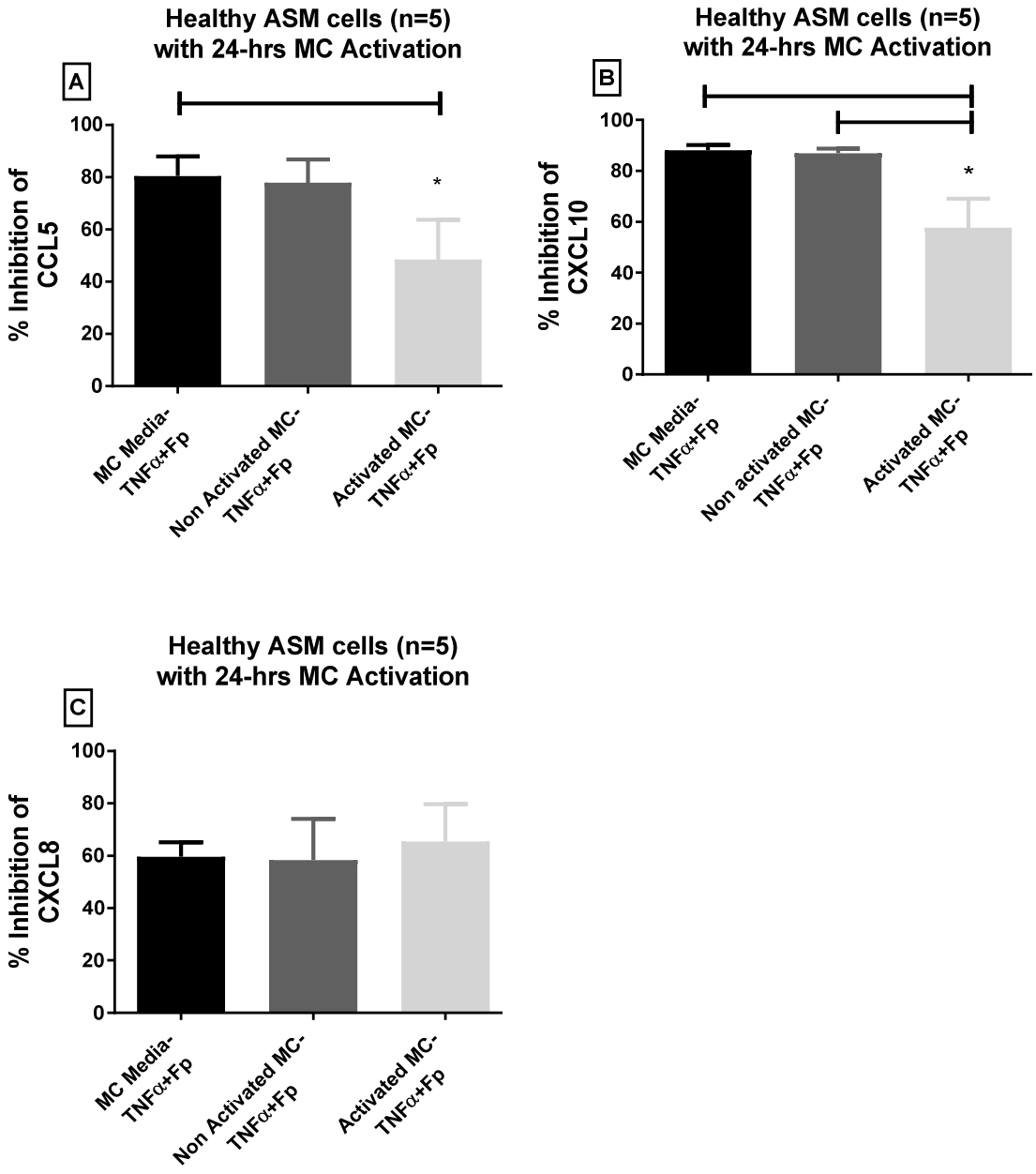


Figure 4.6: % inhibition of TNFα-induced chemokine mRNA expression by fluticasone in healthy ASM cells primed with control media or conditioned media from 24-hour activated and non-activated MCs.

*Healthy ASM cells were pre-treated with control MC media (black bars), conditioned media from 24-hour non-activated (dark grey bars), 24-hour activated MCs (light grey bars) for 24 hours. ASM cells were then washed and stimulated with 10 ng/ml TNF-α and with or without 100 nM fluticasone for an additional 6 hours. Chemokine expression of CCL5 (A), CXCL10 (B) and CXCL8 (C) was assessed by real-time PCR. Data are presented as % inhibition from chemokine responses in cells treated with TNFα alone (Means ± SEM of n=5 different cell lines, *= p<0.05). Comparisons between groups were made using one-way ANOVA and Tukey correction.*

4.2.7. Conditioned media from 24-hour activated MCs differentially modulate the transactivation of anti-inflammatory genes by fluticasone in healthy ASM cells treated with TNFα:

Here, I studied whether the transactivation of known anti-inflammatory genes by fluticasone previously reported in different cells including ASM cells (namely, GILZ and MKP-1) [92,188,225] was affected in ASM cells by the conditioned media from activated MCs activation. Human ASM cells pre-treated with control MC media, conditioned media from activated and non-activated MCs for 24 hours were washed and treated with TNFα (10 ng/ml) and with or without fluticasone (100 nM) for 6 hours before total RNA isolation.

The fold change of GILZ mRNA expression in ASM cells pre-treated with control, conditioned media from 24-hour non-activated and activated MCs was by 14.64 ± 2.4 fold, 12.74 ± 2.5 fold and 9.21 ± 2.3, respectively **(Figure 4.7A)**. Although there as a trend toward a reduced GILZ expression in cells treated with conditioned media from activated MCs, this did not reach statistical significance (p=0.355, n=7).

In addition, MKP-1 mRNA was also significantly induced in healthy ASM cells treated by fluticasone and TNF-α treatment. MKP-1 levels were induced by 17.50 ± 0.8, 5.59 ± 1.3 and 4.57 ± 0.6 fold over basal in ASM cells pre-treated with control MC media, conditioned media from 24-hour non-activated and activated MCs, respectively **(Figure 4.7B)**. Interestingly, there was a significant reduction of MKP-1 mRNA induction in ASM cells pre-treated with conditioned media from 24-hour activated MCs by ~3 fold (P=0.047) compared to levels seen in ASM cells pre-treated with control MC media.

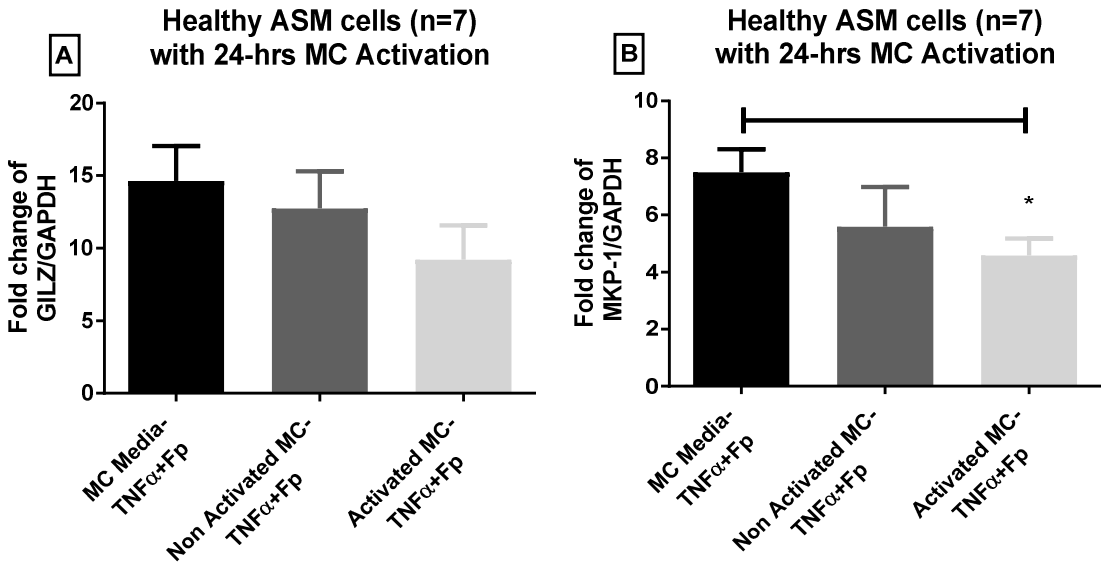


Figure 4.7: Induction of anti-inflammatory genes by fluticasone in healthy ASM cells primed with control media, and conditioned media from 24-hour non-activated or activated MCs.

Healthy ASM cells were pre-treated with control MC media (black bars), conditioned media from 24-hour non-activated (dark grey bars) or activated MCs (light grey bars) for 24 hours. ASM cells were then washed and stimulated with TNFα (10ng/ml), with or without FP (100nM) for an additional 6 hours. Expression of anti-inflammatory genes of GILZ (A) and MKP-1 (B) was assessed by real-time PCR. Data were normalised to the housekeeping gene (GAPDH) and presented as fold change over the basal condition using the $2^{\Delta\Delta ct}$ method as Means ±SEM, n=7 different cell lines (= p<0.05). Comparisons between groups were made using one-way ANOVA and Tukey correction.*

4.2.8. Profile of the mediators produced in the conditioned media from 24-hour activated MCs:

The next goal was to examine the profile of MC mediators before and after cell activation. We applied the Proteome Profiler™ Antibody (membrane-based) Arrays to conditioned media from MCs to determine the levels of various cytokines, chemokines, growth factors and acute phase proteins under basal and activated conditions. The results show that conditioned media from 24-hour activated and non-activated MCs expressed some of the mediators that can impair corticosteroid response including IL-2, IL-4, IL-17A, MIF, TNFα and IFNγ **(Table 4.1)** with the exception of IL-13 which could not be detected.

Based on their levels of expression, the 105 different MCs mediators measured by the Proteome Profiler™ were divided into two categories; those whose levels were higher in the conditioned media of non-activated MCs vs activated MCs (n=27 mediators, **Table 4.2)** and those whose levels in the conditioned media were increased following MC activation vs non-activated MCs (n=76, **Table 4.3)**. I also did a correlation test to determine the variation of my analysis and the pixel density and I found a strong correlation between the analysis and pixel density in the conditioned media from 24-hour activated MCs (r^2= 0.9974, p<0.0001) **(Figure 4.8A)** and no-activated MCs (r^2= 0.908, p<0.0001) **(Figure 4.8B)**.

Table 4.1: MC mediators are known to impair corticosteroid response found in conditioned media from 24-hour activated and non-activated MCs.

Mediators	Activated MC CM	Not Activated MC CM
IL-2	2.00	1.00
IL-4	4.69	2.16
IL-17A	11.12	9.20
MIF	30.97	34.30
IL-27	3.07	1.31
IFN-gamma	2.21	2.20
TNF-α	2.79	0.29

The results were normalised to the internal controls and represented as % expression. Red color indicated the highly expressed mediators by ≥ 2-fold over non-activated MC CM. Two fold change was deemed to be of biological significance.

Table 4.2: List of MC mediators that are highly present in conditioned media from non-activated MCs.

Mediators	Activated MC conditioned media	Non-activated MC conditioned media
PF4	0.00	0.06
IL-33	0.08	0.22
IL-15	0.02	0.24
RAGE	0.24	0.34
IL-12 p70	0.08	0.38
TFF3	0.01	0.45
MIG	0.44	0.76
IL-32	0.85	1.16
MIP-3beta	1.22	1.27
IL-1alpha	1.69	1.85
Angiopoietin-1	1.98	2.31
TfR	1.37	2.33
Lipocalin-2	2.27	2.35
TGF-alpha	2.43	2.51
Flt-3 Ligand	1.92	2.55
SHBG	2.46	2.65
Cystatin C	3.41	4.31
Angiogenin	4.79	7.40
Pentraxin-3	7.45	8.80
VCAM-1	6.23	15.40
uPAR	16.90	18.01
Endoglin	22.94	29.41
Chitinase 3-like 1	18.16	29.82
Dkk-1	7.84	32.08
MIF	30.98	34.31
IGFBP-2	17.30	39.71
IL-6	49.81	53.66

The results were normalised to the internal controls and represented as % expression. The mediators were listed by their % expression found in the conditioned media of non-activated MCs. Highlighted cell indicate the highly expressed medaitors by ≥ 2-fold over activated MC.

Table 4.3: List of MC mediators that were increased in conditioned media (CM) of MCs following activation.

Mediators	Activated MC CM	Not Activated MC CM	Mediators	Activated MC CM	Not Activated MC CM
IL-34	0.40	0	PDGF-AA	3.15	3.00
IL-31	0.45	0.01	Myeloperoxidase	3.30	2.43
IL-1beta	0.46	0.32	CD40 ligand	3.61	2.48
MIP-3alpha	0.64	0.30	EGF	3.62	1.97
IL-16	0.77	0.74	Angiopoietin-2	4.54	2.92
TARC	0.85	0.82	FGF basic	4.59	2.86
CD31	0.85	0.69	IL-4	4.70	2.16
IP-10	0.89	0.56	Resistin	4.95	4.38
MCP-3	0.98	0.34	IL-11	5.06	3.01
Cripto-1	1.04	0.05	SDF-1alpha	5.44	4.63
I-TAC	1.14	0.90	M-CSF	5.57	3.23
RANTES	1.20	0.86	IL-10	5.69	4.80
Relaxin-2	1.21	0.84	CD30	5.74	2.23
Leptin	1.25	1.04	IL-24	5.94	0.84
RBP-4	1.49	0.86	MMP-9	5.96	5.16
IL-19	1.54	1.06	FGF-19	7.30	6.52
IL-3	1.58	0.78	IL-22	8.32	2.68
FGF-7	1.64	0	CD14	8.88	6.49
BAFF	1.66	0.50	TIM-3	9.96	7.11
IGFBP-3	1.72	0.48	IL-17A	11.12	9.21
Fas Ligand	1.77	0.29	LIF	11.26	3.44
Growth Hormone	1.78	0.22	ICAM-1	14.15	8.11
IL-18 Bpa	1.97	1.40	Complement Factor D	14.91	1.18
IL-2	2.00	1.00	ST2	15.75	3.92
IL-1ra	2.04	1.38	HGF	16.05	9.83
IL-23	2.11	1.10	Thrombospondin-1	20.65	19.93
IFN-gamma	2.21	2.20	Osteopontin	25.17	11.31
C-Reactive Protein	2.24	1.10	VEGF	27.99	22.47
G-CSF	2.36	0.74	EMMPRIN	36.82	25.19
Apolipoprotein A-I	2.66	0.84	ENA-78	42.81	2.04
Kallikrein 3	2.70	2.08	MIP-1alpha/MIP-1beta	52.33	2.40
Vitamin D BP	2.78	2.66	MCP-1	56.50	32.67
TNF-alpha	2.79	0.29	GDF-15	73.68	39.23
BDNF	2.90	2.03	DPPIV	84.35	73.51
Adiponectin	2.90	2.14	IL-5	105.54	0.92
GRO-alpha	2.92	1.45	IL-8	113.92	48.83
IL-27	3.07	1.31	Serpin E1	119.68	117.56
Complement Component C5/C5a	3.08	1.11	GM-CSF	144.52	14.68

The results were normalised to the internal control and represented as % expression. The mediators were listed from low to high based on activated mast cell SN. Highlighted cell indicate the highly expressed medaitors by ≥ 2-fold over non-activated MC.

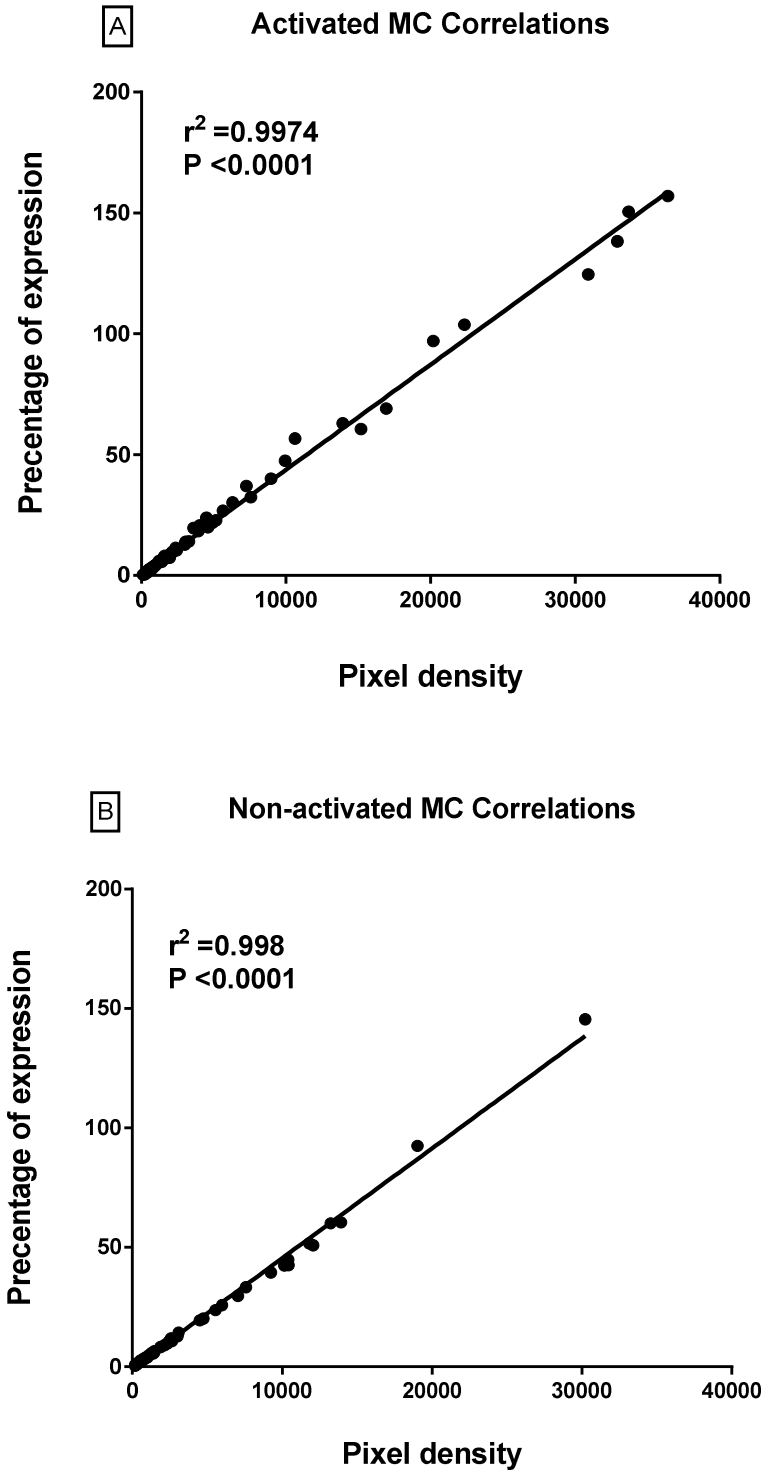


Figure 4.8: Correlation studies to determine the variability between the analysis and the pixel density.

4.3. Discussion:

MC conditioned media did not affect chemokine responses induced by TNFα: Previous studies have shown that conditioned media from 24-hour activated mast cell can regulate different response in ASM cells; inhibition of ASM proliferation [203], modulation of ASM cell contraction [34,207] and chemokine expression [104,203]. We found here that mast cell conditioned media did not suppress nor enhance the expression of different chemokines including CCL5, CCL11, CXCL8 and CXCL10. This suggests that TNFα associated signalling pathways are not affected by mast cell products in our module. Even though, a previous study showed conditioned media from 24-hour activated mast cells was able to increase IFN-γ-induced CXCL10 production from asthmatic and non-asthmatic ASM cells [104]. Also, conditioned media from 24-hour activated mast cells reduced the expression of CCL-11 in both asthmatic and non-asthmatic ASM cells while CXCL-8 expression was increased in healthy ASM cells only [203]. This suggests that the effect of conditioned media on chemokine production is highly stimuli specific.

Activated mast cell conditioned media modulate the anti-inflammatory action of fluticasone in ASM cells. My studies confirmed that fluticasone suppressed the production of chemokines by acting as the transcriptional level since their mRNAs responses were also reduced by fluticasone by more >80% for CCL5 and CXCL10 and ~60% for CXCL8. This shows that chemokine expression is not similarly suppressed by fluticasone, suggesting the existence of different mechanisms of inhibition. Similarly, Chung's lab in 2012 [20] and our lab [92] reported that the expression of CXCL8, CCL11 and CCL5 were not similarly suppressed by dexamethasone or fluticasone. The role of mast cells in the regulation of corticosteroid response is supported by multiple indirect evidence showing that different mast cell mediators have the capacity to regulate the

response to corticosteroids. For instance, treating T cells [191], macrophages [88], eosinophils [192] and PBMCs [87] with a combination of IL-2 and IL-4 for 48 hours impaired dexamethasone induced GRα nuclear translocation and reduced the expression of MKP-1 by modulating the expression and activity of PP2A. Also, TNFα and IFN-γ for 24 hours treatment in ASM cells affected the GR-α nuclear translocation and transactivation of anti-inflammatory genes such as GILZ and MKP-1 via mechanisms involving the upregulation of IRF-1 [96], GRβ [95] and protein phosphatase 5 [92].

Interestingly, activated mast cell conditioned media did not prevent nor reduce the inhibitory action of fluticasone on CXCL8 expression induced by TNFα, further supporting the concept that in ASM cells, there are different anti-inflammatory mechanisms used by corticosteroid. On note, our lab also found CXCL10 was the only chemokine induced by TNFα which was still inhibited by fluticasone in ASM cells from severe asthma in contrast to the local effect on of CCL5 and CCL11 production [92]. It is known that corticosteroids can regulate the expression of pro-inflammatory mediators by acting at different levels, transcriptional and post-transcriptional levels. For instance, activation of transcriptional factors such as MAPK, NF-κB and AP-1 can be inhibited by CS/GRα by acting at the cytoplasmic level or transcriptional level of pro-inflammatory mediators [226]. Also, corticosteroids can regulate post-transcriptional levels via the induction of zinc finger protein tristetraprolin (TTP) which interferes with the translation of TNFα inducible mRNA [227]. ASM cells from asthmatics were not studied in this chapter as increasing evidence from our lab and others show that the cells have a constitutive reduction in responsiveness to corticosteroids *in vitro.*

Activated mast cell conditioned media modulate the transactivation of anti-inflammatory genes by fluticasone in ASM cells. MKP-1 is a critical anti-inflammatory gene induced by corticosteroids which plays a crucial role in regulating the pro-

inflammatory singling pathway by inhibiting MAPK pathway resulting in the repression of TNFα-induced IL-6 in ASM cells [228]. Also, another anti-inflammatory gene induced by corticosteroids is GILZ which binds to the pro-inflammatory transcription factor NF-κB and blocks its nuclear translocation and activity [229]. My data show that the expression of corticosteroid-inducible genes MKP-1 and GILZ was decreased by activated mast cell conditioned media, although only MKP-1 was significantly suppressed. These data suggest that activated mast cell condition media regulate the transactivation property of corticosteroids in a gene-specific manner. This is important as previous studies showed that mast cell mediators such as IL-2 with IL-4, MIF, TNFα with IFN-γ and TGF-β similarly impaired the ability of dexamethasone to induce GILZ, IκB-α and MKP-1 [92,174,186,190,192].

FcεR1-dependent mast cell activation leads to the release of various types of mediators. The protein array data shows that different classes of mediators are released by activated mast cell including **cytokines** (BAFF, BDNF, Complement Component C5/C5a, G-CSF, GM-CSF, IFN-gamma, IL-10, IL-11, IL-16, IL-17A, IL-18 Bpa, IL-19, IL-1alpha, IL-1beta, IL-1ra, IL-2,IL-22, IL-23, IL-24, IL-27, IL-3, IL-31, IL-32, IL-34, IL-4, IL-5, IL-6,, LIF, M-CSF, MIF, ST2, TNF-alpha), **chemokines** (CXCL5, CXCL1, CXCL10, CXCL11, CXCL8, CXCL9, CXCL12, CCL2, CCL5, CCL7, CCL3, CCL4, CCL17, CCL19, CCL20, and Osteopontin), **growth factors** (Cripto-1, EGF, FGF basic, FGF-19, FGF-7,Flt-3 Ligand,GDF-15, Growth Hormone, HGF, IGFBP-2, IGFBP-3, PDGF-AA, TGF-alpha, VEGF) **and others** (Kallikrein 3, MMP-9, Myeloperoxidase, Serpin E1, Complement Factor D, Leptin, Relaxin-2, Resistin, Adiponectin, Angiopoietin-1, Angiopoietin-2, Apolipoprotein A-I, CD14, CD30, CD31, CD40 ligand, VCAM-1 and ICAM-1). Some of these cytokines can impair the function of CSs (summarised in **chapter one**). For example, TNFα with IFN-γ [92], IL-5 and IL2 with

IL-4 [215] can increase the expression of PP5 which can dephosphorylate GRα on ser^{211} residues leading to reduced GRα transcriptional activities [17]. Also, a combination of IL-27 with IFN-γ reduced the expression of MKP-1 protein induced by CS in order to suppress the MAP-kinase pathway [216,230]. Examples of cytokines which regulates corticosteroid sensitivity are include IL-17 and MIF via mechanism involving the phosphoinositide-3-kinase (PI3K) [179,189]. Additionally, a growth factor such as TGF-β was recently implicated in affecting the CSs sensitivity in human bronchial epithelial cells (HBECs), A549 cells, BEAS-2B cells by reducing induction of the anti-inflammatory genes IκB-α and GILZ [174,175]. Additional studies are required to determine whether the fold changes seen in the protein array data have any biological significance in functional studies using neutralizing antibodies.

In conclusion, conditioned media from 24-hour MCs impaired fluticasone ability to suppress TNFα-induced CCL5, CXCL10 (at the protein and mRNA level) and CCL11 (at the protein level only) in healthy ASM cells but had no effect on CXCL8. Fluticasone-induced GILZ and MKP-1was shown to be inhibited by mast cell conditioned media, suggesting that transactivation properties of corticosteroids were impaired by 24-hour activated mast cell mediators. Also, conditioned media from 24-hour MCs had no effect on TNFα signalling pathway in ASM cells, only modulates fluticasone induced GILZ and MKP-1 mRNA expression.

5.Chapter Five

Modulatory effect of MC conditioned media on the transactivation profile of genes induced by fluticasone

5.1. Introduction:

Previous studies have reported that MCs can, directly and indirectly, affect different responses in ASM cells including ASM hypertrophy and ASM hyperplasia [218] as well as β2-agonist responsiveness [35]. For instance, co-culturing MCs with ASM cells causes a contraction [207] and increases the proliferation of ASM cells [208], and that was related to the mast cells derived-Matrix Metalloproteinase.

In chapters 3 and 4, I presented evidence that activated MC conditioned media reduced fluticasone's ability to inhibit TNFα-induced chemokines in ASM cells by acting at both transcriptional and post-transcriptional level. The nature of the mechanisms responsible for inhibiting fluticasone action are not known but I showed that some of the corticosteroid-inducible anti-inflammatory genes (MKP-1, GILZ) by fluticasone were reduced by the conditioned media from 24-hour activated MCs **(chapter 4)**. Interestingly, a number of MC mediators (some used in combination) such as TNFα and IFNγ [17], IL-17A [173], IL-4 and IL-2 [215], IL-13 [199], IFNγ and IL-27 [216], TGF-β1 [174] and MIF [217] have been shown to reduce corticosteroid response in different cell types by involving multiple mechanisms including the inhibition of the transactivation of anti-inflammatory genes (summarized in the **Chapter One**). Impaired GRα nuclear translocation failure to induce GILZ, MKP-1 and IκB-α mRNA were the main mechanisms thought to be involved by these cytokines (summarised in **Chapter One**). In addition, severe asthma patients treated with Omalizumab (humanised monoclonal antibody that binds to circulating free IgE and prevents mast cell activation by the allergen) show a marked decreased in corticosteroid usage (both oral and inhaled), suggesting that MCs do play a key role in the patients' response to corticosteroid therapy [158,161,162]. Our group has done extensive work on the combined effect of TNF-α and IFN-γ, also known to be produced by MCs, by reporting that these cytokines reduced

corticosteroid response in ASM cells via the upregulation of different proteins (the protein phosphatase PP5 and the transcription factor IRF-1) which all inhibited the transactivation function of corticosteroid receptor GRα [17]. However, we do not know how mast cell mediators can influence fluticasone action in ASM cells.

The goal of the studies:

In this chapter, I have determined the gene transactivation profile induced by fluticasone in ASM cells and have hypothesised that pre-treatment ASM cells with conditioned media from 24-hour activated MCs would alter fluticasone-inducible genes. So the specific aims were:

A. To characterise the profile of inducible anti-inflammatory genes by fluticasone in ASM cells using the RT² Profile PCR array from QIAGEN.

B. To determine the effect of pre-treating ASM cells with conditioned media from 24-hour activated MCs on gene transactivation profile associated with fluticasone.

C. To validate some of the gene changes observed in gene array data by performing individual qPCR assays.

5.2. Results:

5.2.1. Gene transactivation profile elicited by fluticasone in healthy ASM cells:

Having shown that activated MC conditioned media might regulate the transactivation function of corticosteroid by looking at the expression of both MKP-1 and GILZ **(chapter 4)**, I used RT² Profiler PCR Array (QIAGEN) to establish a broader picture of several genes transactivated by fluticasone in healthy ASM cells. Our group has successfully used this approach to investigate basic mechanisms associated with idiopathic pulmonary fibrosis [231].

Healthy ASM cells were cultured and stimulated with or without fluticasone (100nM) for 6 hours before total RNA isolation and PCR array & analysis were performed as suggested by the manufacturer. Fluticasone was found to significantly upregulate or downregulate a number of different genes when compared to the basal condition in healthy ASM cells **(Figure 5.1 and Figure 5.2)**. The genes that were significantly up-regulated by fluticasone were FKBP5, TSC22D3 (known as GILZ), PER1, CTGF, SLC19A2, DUSP1 (MKP-1), ERRFI1, DDIT4, GLUL, PIK3R1, MERTK, MT1E, KLF13, PER2, ZFP36, NFKBIA, TNFAIP3, ADARB1, SGK1, CEBPB and STAT5B, while the genes that were significantly down-regulated by fluticasone treatment were ASPH, TBL1XR1, NR3C1, AMPD3, VDR, IL6 and POU2F2 **(Figure 5.2A and B)**. After adjusting the p-value for False Discovery Rate at 5%, only FKBP5, TSC22D3 (GILZ), PER1, CTGF, SLC19A2, DUSP1 (MKP-1), ERRFI1, DDIT4, GLUL and PIK3R1 did reach statistical significance compared to unstimulated values **(Figure 5.2A and B)**.

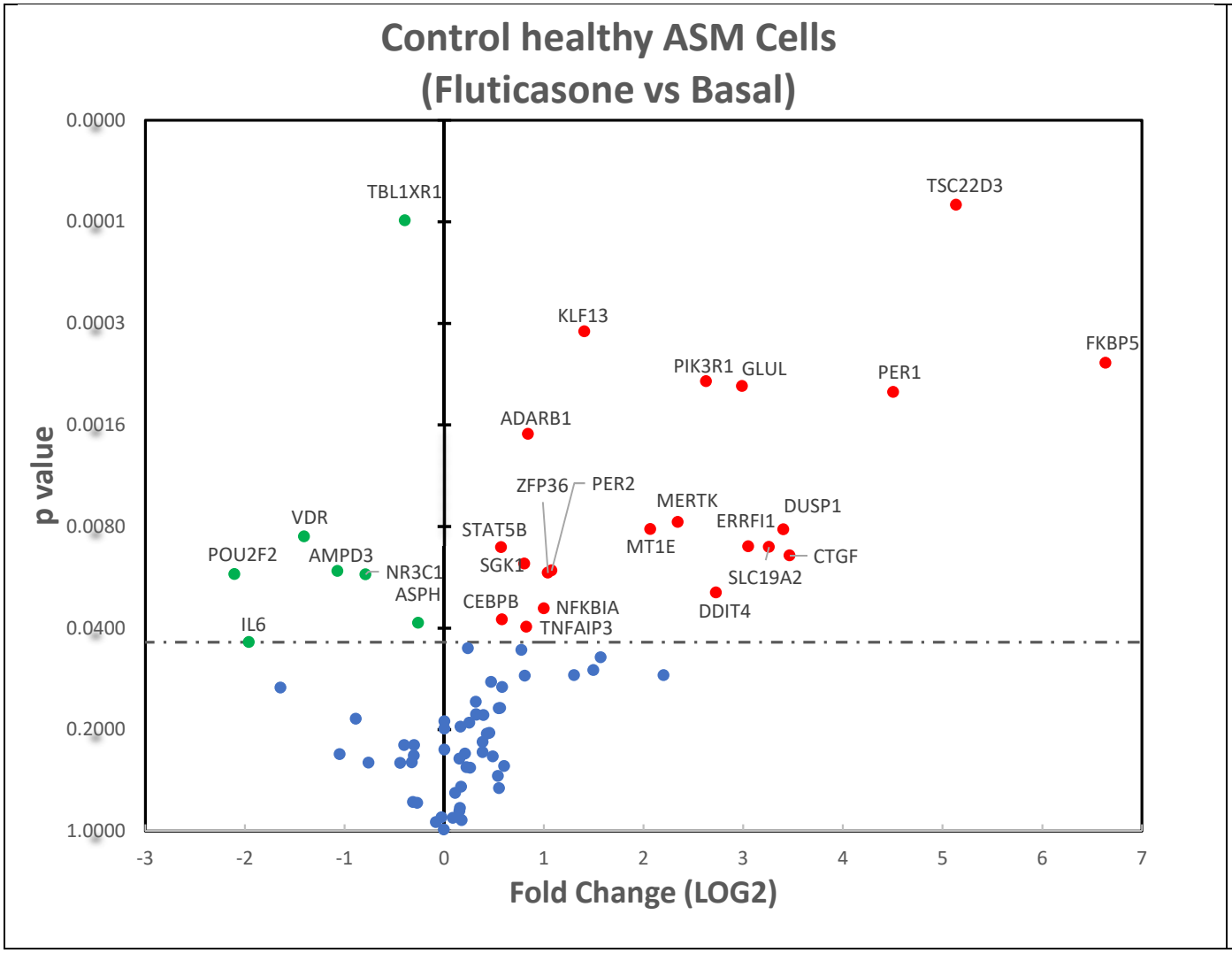
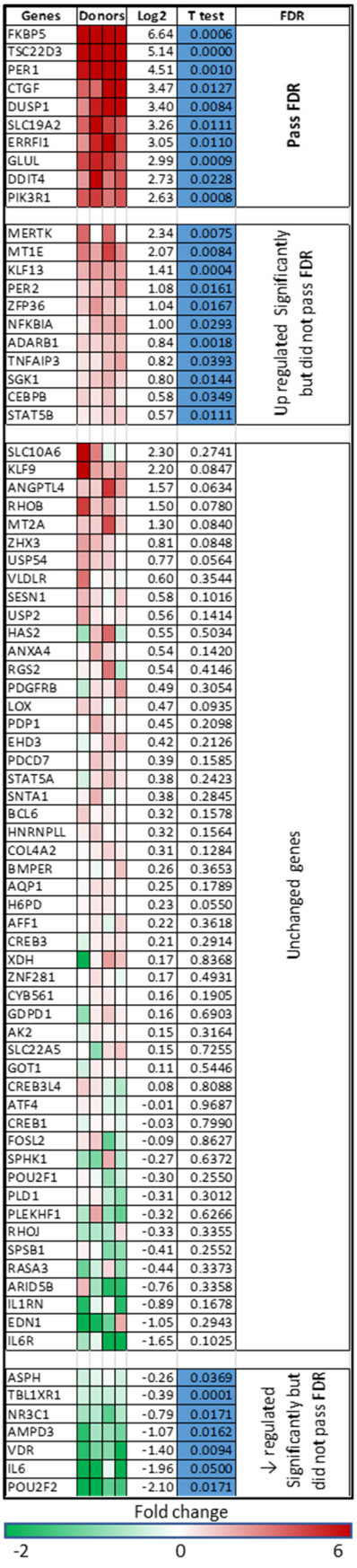


Figure 5.1: Volcano plot of fluticasone inducible genes in ASM cells detected using the PCR array.

Data are presented as statistical significance (p-value) vs fold change (LOG2) on the y-axis and x-axis respectively. All genes were normalised to housekeeping genes (GAPDH and beta-actin (Delta Ct) and presented as fold Change (2^ (-Delta Delta Ct, log 2). The red dots represent genes that are significantly up-regulated, while the green dotes are genes that are significantly down-regulated. Student's t-test was applied for statistical significance (n=4) between untreated and treated ASM cells.

(A)



(B)

GENES	Fold Change	p Value pass FDR = *
FKBP5	114.23 ↑	**0.000600** *
TSC22D3	35.77 ↑	**0.000049** *
PER1	24.67 ↑	**0.000951** *
CTGF	14.82 ↑	**0.012696** *
SLC19A2	12.69 ↑	**0.011070** *
DUSP1	12.57 ↑	**0.008401** *
ERRFI1	10.06 ↑	**0.010969** *
DDIT4	9.11 ↑	**0.022814** *
GLUL	8.21 ↑	**0.000867** *
PIK3R1	6.35 ↑	**0.000804** *
MERTK	5.08 ↑	0.007457
MT1E	4.57 ↑	0.008359
KLF13	2.66 ↑	0.000365
PER2	2.19 ↑	0.016110
ZFP36	2.13 ↑	0.016699
NFKBIA	2.09 ↑	0.029300
TNFAIP3	1.84 ↑	0.039281
ADARB1	1.80 ↑	0.001847
SGK1	1.78 ↑	0.014446
CEBPB	1.52 ↑	0.034936
STAT5B	1.50 ↑	0.011110
ASPH	0.84 ↓	0.036896
TBL1XR1	0.76 ↓	0.000063
NR3C1	0.59 ↓	0.017134
AMPD3	0.49 ↓	0.016234
VDR	0.39 ↓	0.009406
IL6	0.35 ↓	0.049958
POU2F2	0.26 ↓	0.017081

Figure 5.2: Heatmap of fluticasone-inducible genes in ASM cells established from the PCR array.

Healthy ASM cells stimulated with or without fluticasone (100nM) for 6 hours before total RNA was isolated and used for the Real-time PCR. Data were normalised to the housekeeping genes (GAPDH and B2A) and presented as fold change over the basal condition using the $2^{\Delta\Delta ct}$ method, n=4 of different ASM cell lines. ***A)*** *represent the heatmap (including log 2 fold regulation, p-value and FDR) for all the 78 genes detected in the PCR array (ranked from high to low inducible levels).* ***B)*** *List of the genes that have been significantly induced (Student's t-test for statistical significance. All the data (p-value) were then adjusted using the false discovery rate (FDR) and Q= 5%).*

5.2.2. Effect of 24-hour activated MC conditioned media on fluticasone's gene transactivation profile in ASM cells.

I next investigated whether the profile of transactivated genes by fluticasone was altered in ASM cells pre-treated of conditioned media from 24-hour activated MCs. ASM cells were cultured and pre-treated with control MC media and conditioned media from 24-hour activated MCs over-night. Later, ASM cells were washed and treated with or without fluticasone (100nM) for 6 hours prior to total RNA isolation.

The data show that in ASM cells pre-treated with conditioned media from 24-hour activated MCs, fluticasone was able to upregulate or downregulate significantly 26 out of 77 genes when compared to basal levels **(Figure 5.3)**. The genes that were up-regulated significantly by fluticasone compared to the basal condition included FKBP5, TSC22D3, PER1, CTGF, SLC19A2, DUSP1, ERRFI1, DDIT4, GLUL, PIK3R1, MT1E, KLF13, PER2, ZFP36, PDP1, TNFAIP3, CEBPB and STAT5B **(Figure 5.4A and B)**. While the down-regulated genes by fluticasone in ASM cells pre-treated with conditioned media from 24-hour activated MCs were CREB1, TBL1XR1, RHOJ, PLEKHF1, NR3C1, AMPD3, IL6 and POU2F2 **(Figure 5.4A and B)**. Interestingly, only the induction of 2 genes, namely FKBP5 and TSC22D3 (GILZ), in ASM cells primed with conditioned media from 24-hours activated MCs did reach significance when adjusting the p-value for False Discovery Rate at 5% **(Figure 5.4A and B)**.

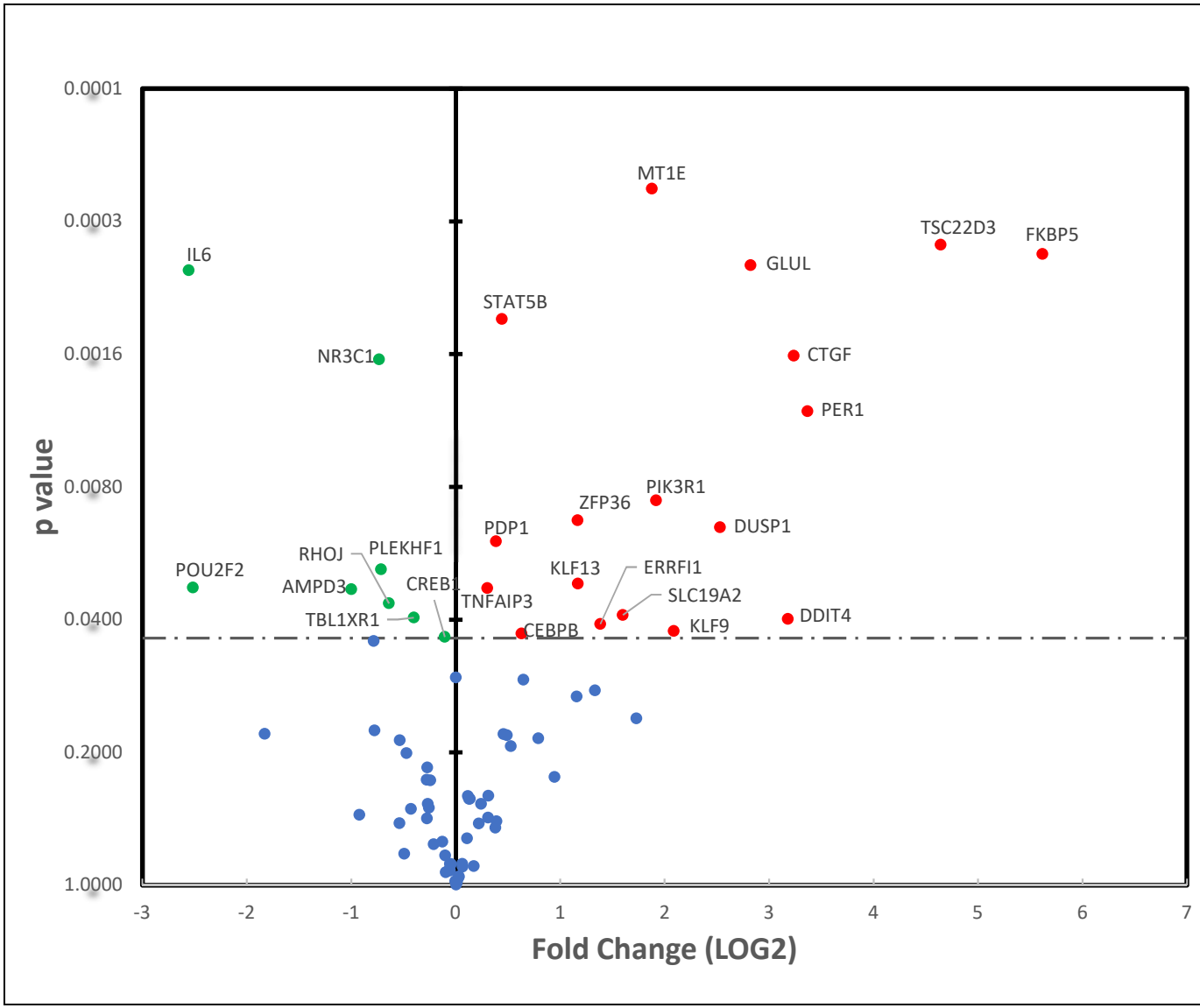
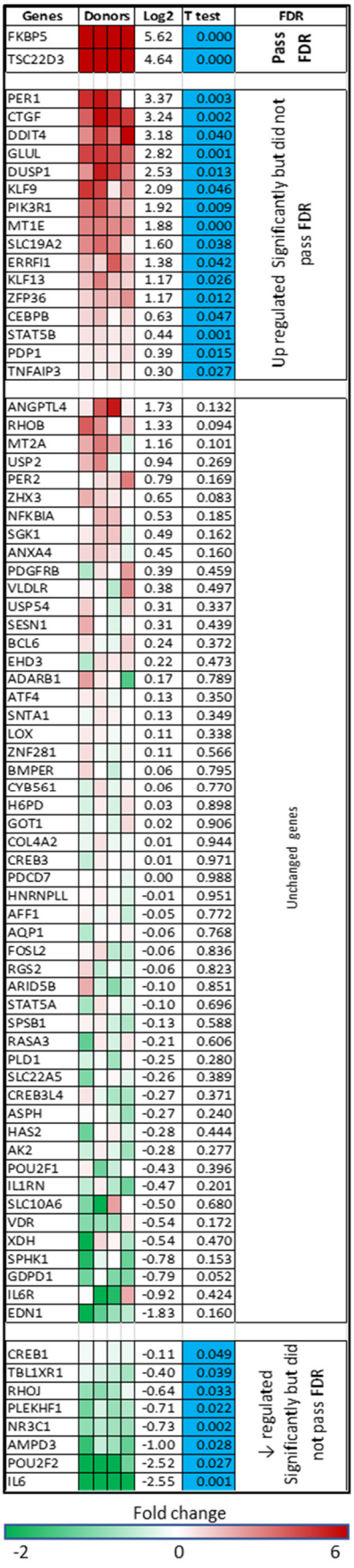


Figure 5.3: Volcano plotting of genes in fluticasone-stimulated ASM cells pre-treated conditioned media from 24-hour activated MCs and.

Healthy ASM cells primed with conditioned media from 24 activated MCs were stimulated with or without fluticasone (100nM) for 6 hours. Later, total RNA was isolated and used for real-time PCR assays. This graph presents the statistical significance (as p-value) vs fold change (as log 2) on the y-axis and x-axis respectively. All genes were normalised to housekeeping genes (GAPDH and B2A (Delta Ct) and presented as fold Change ($2^{-\Delta\Delta Ct}$, log 2). The red dots represent genes that are significantly up-regulated, and the green dotes are genes that are significantly down-regulated. Student's t-test was applied for statistical significance (n=4) between untreated and treated ASM cells.

(A)



(B)

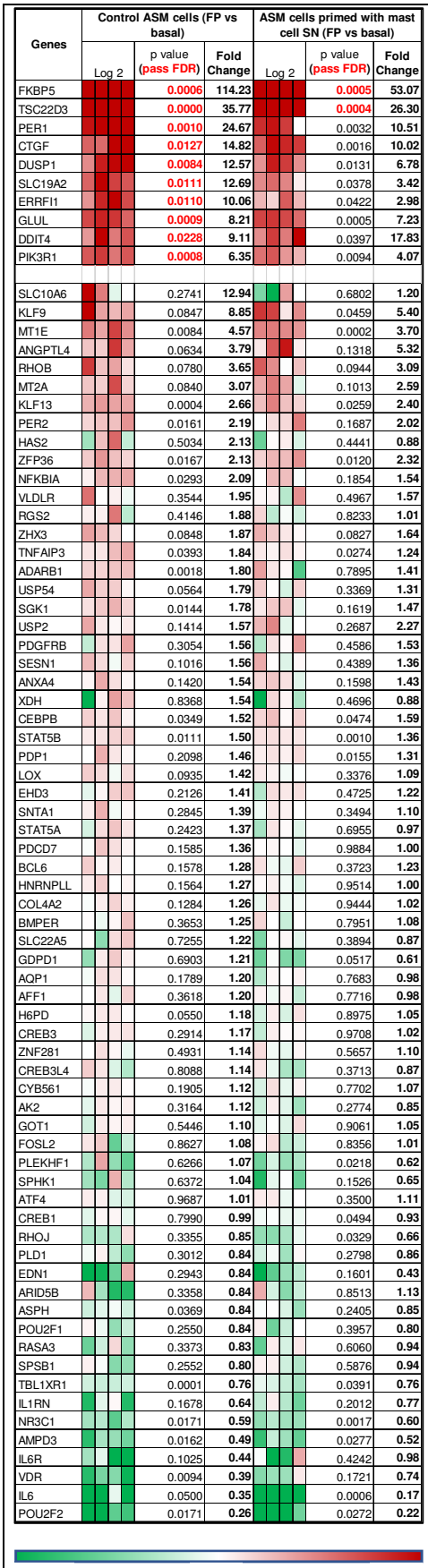
GENES	Fold Change	p Value pass FDR = *
FKBP5	53.07 ↑	**0.000475 ***
TSC22D3	26.30 ↑	**0.000426 ***
PER1	10.51 ↑	0.003204
CTGF	10.02 ↑	0.001632
DDIT4	17.83 ↑	0.039675
GLUL	7.23 ↑	0.000545
DUSP1	6.78 ↑	0.013093
KLF9	5.40 ↑	0.045889
PIK3R1	4.07 ↑	0.009422
MT1E	3.70 ↑	0.000216
SLC19A2	3.42 ↑	0.037838
ERRFI1	2.98 ↑	0.042225
KLF13	2.40 ↑	0.025892
ZFP36	2.32 ↑	0.012008
CEBPB	1.59 ↑	0.047442
STAT5B	1.36 ↑	0.001049
PDP1	1.31 ↑	0.015489
TNFAIP3	1.24 ↑	0.027354
CREB1	0.93 ↓	0.049377
TBL1XR1	0.76 ↓	0.039086
RHOJ	0.66 ↓	0.032870
PLEKHF1	0.62 ↓	0.021774
NR3C1	0.60 ↓	0.001711
AMPD3	0.52 ↓	0.027737
POU2F2	0.22 ↓	0.027168
IL6	0.17 ↓	0.000580

Figure 5.4: Heatmap of gene changes in fluticasone treated-ASM cells pre-treated with conditioned media from 24-hours activated MCs.

Healthy ASM cells were pre-treated with conditioned media from 24-hours activated MCs. 24 hours later, ASM cells were then washed and stimulated with or without fluticasone (100nM) for an additional 6 hours. Total RNA was isolated before real-time PCR assays. Data were normalised to the housekeeping genes (GAPDH and B2A) and presented as fold change over the basal condition using the $2^{\Delta\Delta ct}$ method, n=4 of different cell lines. ***A)*** *represent the heatmap (including log 2 fold regulation, p-value and FDR) for all the 78 genes which were detected in the PCR array (listed from high to low expression).* ***B)*** *Summary of the genes which have changed significantly (Student's t-test). All the data (p-value) were then adjusted using the false discovery rate approach (FDR) and Q= 5% (n=4).*

We compared the fold change in the gene expression profile induced by fluticasone in ASM cells primed with control vs conditioned media from 24-hour activated MCs. The results show that there were many genes induced by fluticasone that were not influenced the conditioned media from activated MCs when compared to the gene expression profile seen in ASM cells treated with control MC media **(Figure 5.5)**. Notably, the fold changes of various genes which were significantly induced by fluticasone in control ASM cells **(Figure 5.2)** were reduced in cells treated with conditioned media from 24-hour activated MCs **(Figure 5.4)** which are all summarised in **Figure 5.5** and **Table 5.1**. Interestingly, applying FDR approach on the gene array revealed that only 10 out 28 genes were significantly induced by fluticasone in control ASM cells including FKBP5, TSC22D3, PER1, CTGF, SLC19A2, DUSP1, ERRFI1, DDIT4, GLUL, PIK3R1) **(Figure 5.5)**. In contrast, changes in only two genes, FKBP5 and TSC22D3, were found to be significantly induced by fluticasone in ASM cells primed with conditioned media from 24-hour activated MCs (**Figure 5.5**).

Table 5.1 lists all the genes that were significantly up-regulated in both ASM cells which primed with control and conditioned media from 24-hour activated MCs compared to basal and included FKBP5, TSC22D3, PER1, CTGF, SLC19A2, DUSP1, ERRFI1, DDIT4, GLUL, PIK3R1, MT1E, KLF13, PER2, ZFP36, TNFAIP3, CEBPB and STAT5B, while the down-regulated genes were TBL1XR1, NR3C1, AMPD3, IL6 and POU2F2 **(Table 5.1A)**. Also, there were genes which were regulated significantly by fluticasone treatment in control ASM cells only including MERTK ↑, NFKBIA ↑, ADARB1 ↑, SGK1 ↑, ASPH ↓ and VDR ↓ **(Table 5.1B)**. On the other hand, there were genes which were regulated significantly by fluticasone treatment in ASM cells which primed with conditioned media from 24-hour activated MCs only including PDP1 ↑, CREB1 ↓, RHOJ ↓ and PLEKHF1 ↓ **(Table 5.1C)**.



Genes	Control ASM cells (FP vs basal)			ASM cells primed with mast cell SN (FP vs basal)		
	Log 2	p value (pass FDR)	Fold Change	Log 2	p value (pass FDR)	Fold Change
FKBP5		0.0006	114.23		0.0005	53.07
TSC22D3		0.0000	35.77		0.0004	26.30
PER1		0.0010	24.67		0.0032	10.51
CTGF		0.0127	14.82		0.0016	10.02
DUSP1		0.0084	12.57		0.0131	6.78
SLC19A2		0.0111	12.69		0.0378	3.42
ERRFI1		0.0110	10.06		0.0422	2.98
GLUL		0.0009	8.21		0.0005	7.23
DDIT4		0.0228	9.11		0.0397	17.83
PIK3R1		0.0008	6.35		0.0094	4.07
SLC10A6		0.2741	12.94		0.6802	1.20
KLF9		0.0847	8.85		0.0459	5.40
MT1E		0.0084	4.57		0.0002	3.70
ANGPTL4		0.0634	3.79		0.1318	5.32
RHOB		0.0780	3.65		0.0944	3.09
MT2A		0.0840	3.07		0.1013	2.59
KLF13		0.0004	2.66		0.0259	2.40
PER2		0.0161	2.19		0.1687	2.02
HAS2		0.5034	2.13		0.4441	0.88
ZFP36		0.0167	2.13		0.0120	2.32
NFKBIA		0.0293	2.09		0.1854	1.54
VLDLR		0.3544	1.95		0.4967	1.57
RGS2		0.4146	1.88		0.8233	1.01
ZHX3		0.0848	1.87		0.0827	1.64
TNFAIP3		0.0393	1.84		0.0274	1.24
ADARB1		0.0018	1.80		0.7895	1.41
USP54		0.0564	1.79		0.3369	1.31
SGK1		0.0144	1.78		0.1619	1.47
USP2		0.1414	1.57		0.2687	2.27
PDGFRB		0.3054	1.56		0.4586	1.53
SESN1		0.1016	1.56		0.4389	1.36
ANXA4		0.1420	1.54		0.1598	1.43
XDH		0.8368	1.54		0.4696	0.88
CEBPB		0.0349	1.52		0.0474	1.59
STAT5B		0.0111	1.50		0.0010	1.36
PDP1		0.2098	1.46		0.0155	1.31
LOX		0.0935	1.42		0.3376	1.09
EHD3		0.2126	1.41		0.4725	1.22
SNTA1		0.2845	1.39		0.3494	1.10
STAT5A		0.2423	1.37		0.6955	0.97
PDCD7		0.1585	1.36		0.9884	1.00
BCL6		0.1578	1.28		0.3723	1.23
HNRNPLL		0.1564	1.27		0.9514	1.00
COL4A2		0.1284	1.26		0.9444	1.02
BMPER		0.3653	1.25		0.7951	1.08
SLC22A5		0.7255	1.22		0.3894	0.87
GDPD1		0.6903	1.21		0.0517	0.61
AQP1		0.1789	1.20		0.7683	0.98
AFF1		0.3618	1.20		0.7716	0.98
H6PD		0.0550	1.18		0.8975	1.05
CREB3		0.2914	1.17		0.9708	1.02
ZNF281		0.4931	1.14		0.5657	1.10
CREB3L4		0.8088	1.14		0.3713	0.87
CYB561		0.1905	1.12		0.7702	1.07
AK2		0.3164	1.12		0.2774	0.85
GOT1		0.5446	1.10		0.9061	1.05
FOSL2		0.8627	1.08		0.8356	1.01
PLEKHF1		0.6266	1.07		0.0218	0.62
SPHK1		0.6372	1.04		0.1526	0.65
ATF4		0.9687	1.01		0.3500	1.11
CREB1		0.7990	0.99		0.0494	0.93
RHOJ		0.3355	0.85		0.0329	0.66
PLD1		0.3012	0.84		0.2798	0.86
EDN1		0.2943	0.84		0.1601	0.43
ARID5B		0.3358	0.84		0.8513	1.13
ASPH		0.0369	0.84		0.2405	0.85
POU2F1		0.2550	0.84		0.3957	0.80
RASA3		0.3373	0.83		0.6060	0.94
SPSB1		0.2552	0.80		0.5876	0.94
TBL1XR1		0.0001	0.76		0.0391	0.76
IL1RN		0.1678	0.64		0.2012	0.77
NR3C1		0.0171	0.59		0.0017	0.60
AMPD3		0.0162	0.49		0.0277	0.52
IL6R		0.1025	0.44		0.4242	0.98
VDR		0.0094	0.39		0.1721	0.74
IL6		0.0500	0.35		0.0006	0.17
POU2F2		0.0171	0.26		0.0272	0.22

Figure 5.5: Heatmap for glucocorticosteroid genes which were induced by fluticasone in healthy ASM cells primed with either control media or conditioned media from 24-hour activated MCs.

Healthy ASM cells were pre-treated with control MC media and conditioned media from 24-hour activated MCs for 24 hours. Later, ASM cells were then washed and stimulated with or without FP (100nM) for an additional 6 hours. Total RNA was isolated and converted to cDNA before real-time PCR. Data were normalised to the housekeeping gene (GAPDH and B2A) and presented as fold change over the basal condition using the $2^{-\Delta\Delta ct}$ method, n=4 of different cell lines. The figure represents the heatmap for all the 77 genes which were detected in the PCR array (listed from high to low expression) including log 2 fold regulation of each sample, p-value with FDR and fold change. We applied Student's t-test for statistical significance. All the data (p-value) were then adjusted using the false discovery rate approach (FDR) and Q= 5%.

Table 5.1: Summary of the expression profile of genes in response to fluticasone in healthy ASM cells treated with control MC media or activated MC conditioned media

Genes	ASM cell primed with control/ Basal vs FP condition		ASM cell primed with conditioned media from 24 MC/ Basal vs FP condition	
	Fold Change	*P Value* ***FDR=****	Fold Change	*P Value* ***FDR=****
A	**Both changed significantly**			
FKBP5	114.23 ↑	***0.000600*** *	53.07 ↑	***0.000475*** *
TSC22D3	35.77 ↑	***0.000049*** *	26.30 ↑	***0.000426*** *
PER1	24.67 ↑	***0.000951*** *	10.51 ↑	*0.003204*
CTGF	14.82 ↑	***0.012696*** *	10.02 ↑	*0.001632*
SLC19A2	12.69 ↑	***0.011070*** *	3.42 ↑	*0.037838*
DUSP1	12.57 ↑	***0.008401*** *	6.78 ↑	*0.013093*
ERRFI1	10.06 ↑	***0.010969*** *	2.98 ↑	*0.042225*
DDIT4	9.11 ↑	***0.022814*** *	17.83 ↑	*0.039675*
GLUL	8.21 ↑	***0.000867*** *	7.23 ↑	*0.000545*
PIK3R1	6.35 ↑	***0.000804*** *	4.07 ↑	*0.009422*
MT1E	4.57 ↑	*0.008359*	3.70 ↑	*0.000216*
KLF13	2.66 ↑	*0.000365*	2.40 ↑	*0.025892*
PER2	2.19 ↑	*0.016110*	2.01 ↑	*0.168667*
ZFP36	2.13 ↑	*0.016699*	2.32 ↑	*0.012008*
TNFAIP3	1.84 ↑	*0.039281*	1.24 ↑	*0.027354*
CEBPB	1.52 ↑	*0.034936*	1.59 ↑	*0.047442*
STAT5B	1.50 ↑	*0.011110*	1.36 ↑	*0.001049*
TBL1XR1	0.76 ↓	*0.000063*	0.76 ↓	*0.039086*
NR3C1	0.59 ↓	*0.017134*	0.60 ↓	*0.001711*
AMPD3	0.49 ↓	*0.016234*	0.52 ↓	*0.027737*
IL6	0.35 ↓	*0.049958*	0.17 ↓	*0.000580*
POU2F2	0.26 ↓	*0.017081*	0.22 ↓	*0.027168*
B	**Control only changed significantly**			
MERTK	5.08 ↑	*0.007457*	0.91 ↑	-
NFKBIA	2.09 ↑	*0.029300*	1.54 ↑	-
ADARB1	1.80 ↑	*0.001847*	1.41 ↑	-
SGK1	1.78 ↑	*0.014446*	1.46 ↑	-
ASPH	0.84 ↓	*0.036896*	0.84 ↓	-
VDR	0.39 ↓	*0.009406*	0.74 ↓	-
C	**ASM cells primed with MC only changed significantly**			
PDP1	1.46	-	1.31 ↑	*0.015489*
CREB1	0.99	-	0.93 ↓	*0.049377*
RHOJ	0.85	-	0.66 ↓	*0.032870*
PLEKHF1	1.07	-	0.62 ↓	*0.021774*

5.2.3. Validation of the PCR array RT^2 profiling gene array by individual qPCRs

Next, the goal was to validate the PCR Array by individual qPCRs by comparing the induction of selected anti-inflammatory genes including GILZ (TSC22D3), MKP-1 (DUSP1), FKPB5, PIK3R1 and TNFAIP3 in healthy ASM cells pre-treated with control MC media and conditioned media from 24-hour activated MCs. Healthy ASM cells pre-treated with control MC media and conditioned media from 24-hour activated MCs overnight before cells were washed and treated with or without fluticasone (100 nM) for 6 hours prior to total RNA isolation.

The results showed that fluticasone induced a 27.49 ± 0.94 and 19.65 ± 3.10 fold increase over the basal in GILZ mRNA levels in ASM pre-treated with control and conditioned media from 24-hour activated MCs, respectively. This shows that activated MC conditioned media reduced fluticasone-induced GILZ expression by ~30% compared to the response seen in control media treated ASM cells ($p = 0.0209$) **(Figure 5.6A)**.

Similarly, fluticasone induced a 11.74 ± 2.45 and 5.15 ± 1.37-fold increase over the basal in MKP-1 mRNA levels in ASM pre-treated with control and conditioned media from 24-hour activated MCs, respectively. This shows that activated MC conditioned media reduced fluticasone-induced MKP-1 expression by ~57% compared to the response seen in control media treated ASM cells ($p = 0.0339$) **(Figure 5.6B)**.

Another fluticasone-inducible gene FKBP5 was induced by 68.81 ± 6.75 and 32.61 ± 9.61-fold over basal in ASM pre-treated with control and conditioned media from 24-hour activated MCs, respectively. This shows that activated MC conditioned media reduced fluticasone-induced FKBP5 expression by ~53% compared to the response seen in control media treated ASM cells ($p = 0.0288$) **(Figure 5.6C)**.

Also, levels of PIK3R1 mRNA were induced by 5.92 ±1.5 fold and 2.25 ±0.50 by fluticasone treatment over the basal in ASM cells which pre-treated with control mat cell media and conditioned media from 24-hour activated MCs respectively. There was a significant reduction of 60% of PIK3R1 mRNA levels in ASM cells pre-treated with conditioned media from 24-hour activated MCs compared to control ASM cells ($p = 0.0087$) **(Figure 5.6D)**.

Lastly, the fold change in TNFAIP3 mRNA levels induced by fluticasone in control ASM cells was 1.56 ±0.82, while in ASM cells pre-treated with conditioned media from 24-hour activated MCs it was 0.16 ± 0.19, showing a 90% reduction by conditioned media without any significance ($p=0.078$) **(Figure 5.6E)**.

Finally, I performed Pearson correlation studies to determine the validity of the gene array data using individual qPCR data. The results shown in **Figure 5.7** demonstrate a strong correlation in the gene changes seen with gene array and qPCR data in ASM cells treated with control media ($r^2= 0.9926$, $p=0.0003$) **(Figure 5.7 A)** and activated MC conditioned media ($r^2= 0.986$, $p=0.0007$) **(Figure 5.7 B)**.

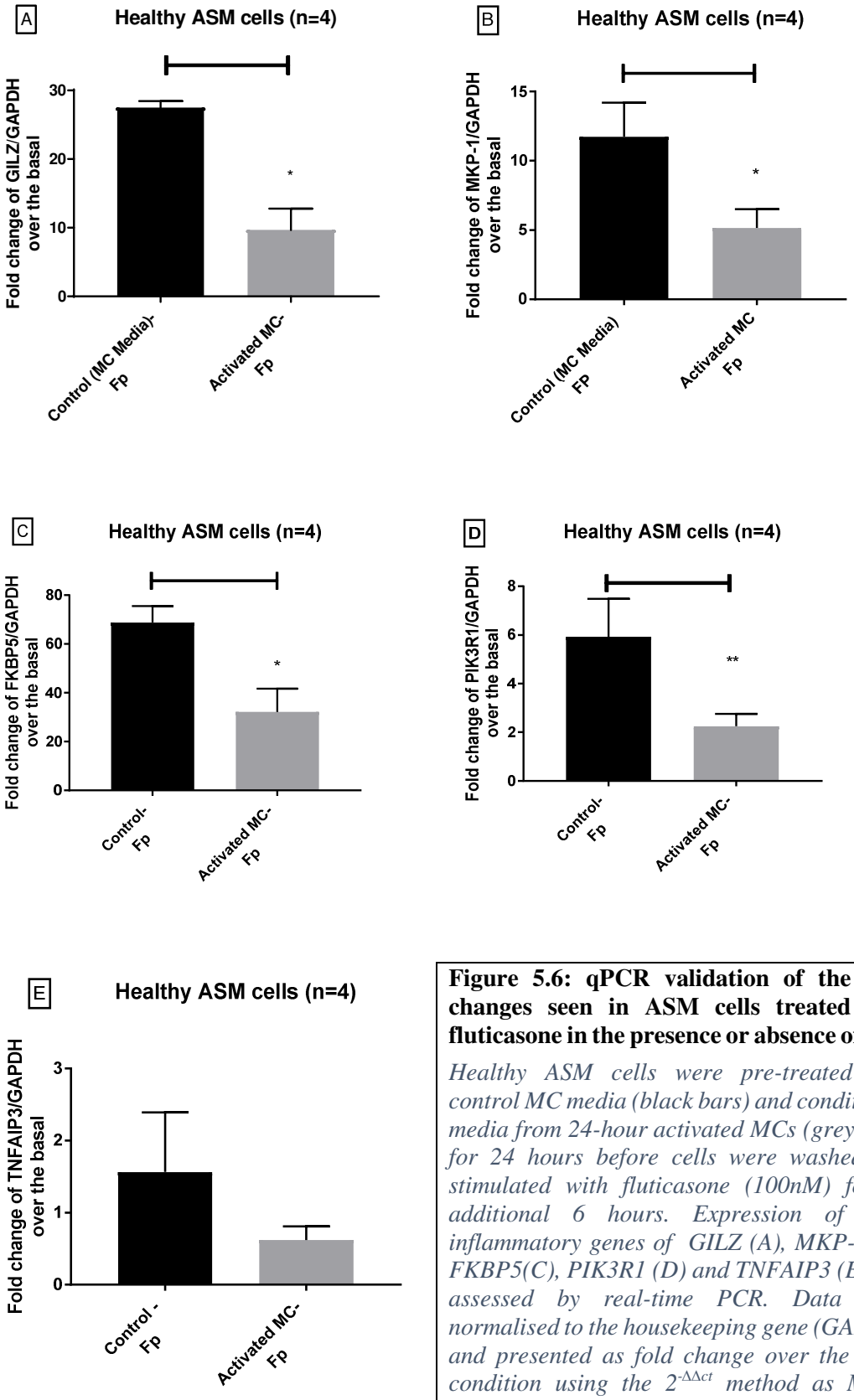


Figure 5.6: qPCR validation of the gene changes seen in ASM cells treated with fluticasone in the presence or absence of CM.

Healthy ASM cells were pre-treated with control MC media (black bars) and conditioned media from 24-hour activated MCs (grey bars) for 24 hours before cells were washed and stimulated with fluticasone (100nM) for an additional 6 hours. Expression of anti-inflammatory genes of GILZ (A), MKP-1 (B), FKBP5(C), PIK3R1 (D) and TNFAIP3 (E) was assessed by real-time PCR. Data were normalised to the housekeeping gene (GAPDH) and presented as fold change over the basal condition using the $2^{-\Delta\Delta ct}$ method as Means ±SEM, n=7 different cell lines (= p <0.05, **= p <0.005), (MC= mast cell, FP= fluticasone).*

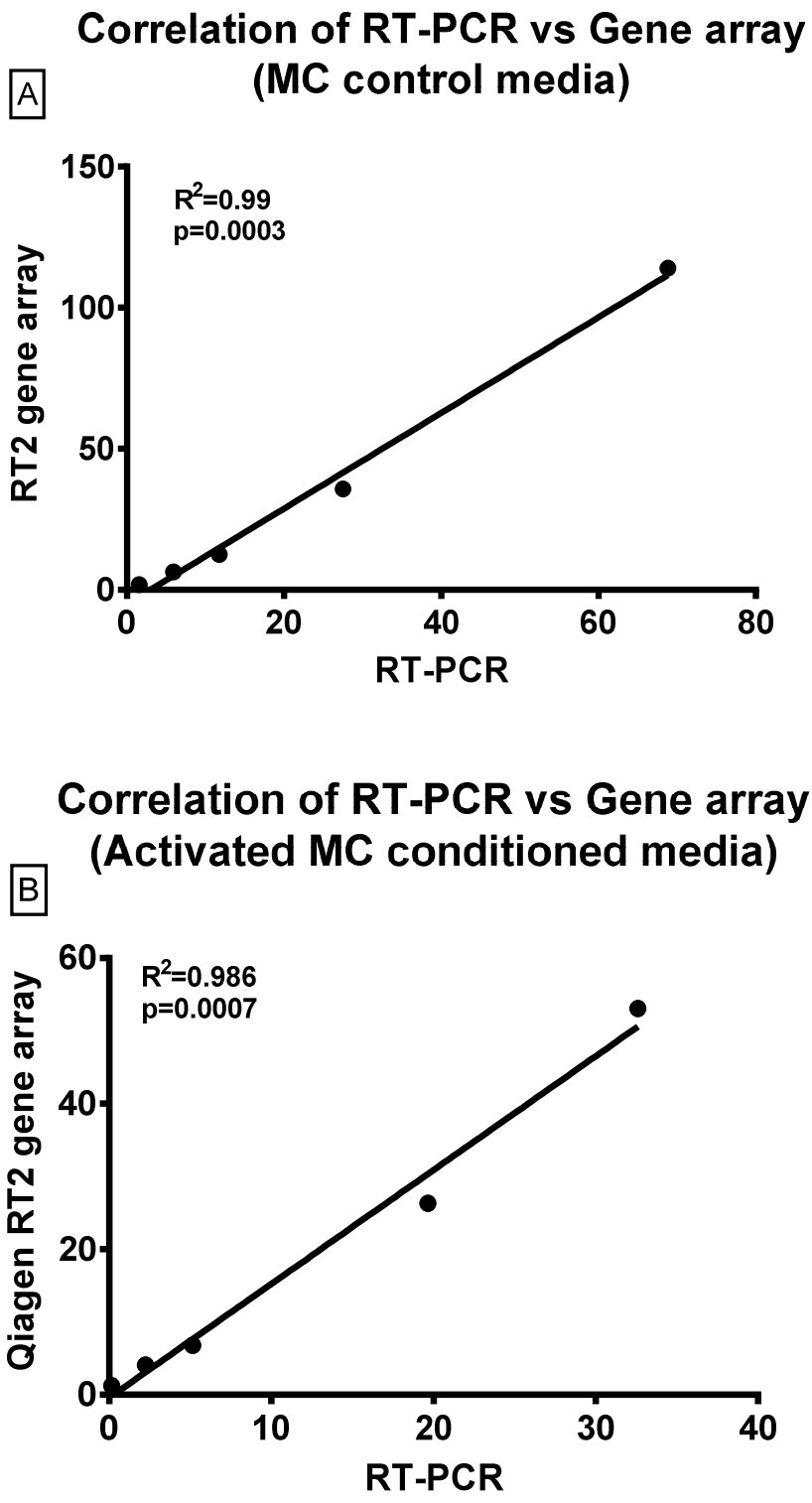


Figure 5.7: Correlation studies to determine the validity of the gene array data using individual qPCR assay.

Top graph represents the correlation in control ASM cell. Bottom graph represents the correlation in ASM cells which pre-treated with conditioned media from 24-hour activated MCs.

5.3. Discussion:

The induction of anti-inflammatory genes by corticosteroids is an essential mechanism for their anti-inflammatory action [78,79]. Previous studies in ASM cells showed that fluticasone can induce the expression of anti-inflammatory genes including GILZ, MKP-1 and IκBα among others [192,221]. These proteins interfere with the inflammatory process by acting at different levels. For example, MKP-1 has been shown to inhibit the MAPK signalling pathways while GILZ and IκBα were shown to block the activity of NF-κB pathways in various cell types [232].

Here, I used RT^2 profile gene array data to determine the impact of activated MC conditioned media on the gene transactivation profile induced by fluticasone in ASM cells. The gene array performed in ASM cells treated with control MC media showed that several genes were significantly up-regulated by fluticasone including FKBP5, TSC22D3 (GILZ), PER1, CTGF, SLC19A2, DUSP1 (MKP-1), ERRFI1, DDIT4, GLUL, PIK3R1, MERTK, MT1E, KLF13, PER2, ZFP36, NFKBIA, TNFAIP3, ADARB1, SGK1, CEBPB and STAT5B, while other genes were significantly down-regulated were ASPH, TBL1XR1, NR3C1, AMPD3, VDR, IL6 and POU2F2 **(Figure 5.2A and B)**. however, only FKBP5, TSC22D3, PER1, CTGF, SLC19A2, DUSP1, ERRFI1, DDIT4, GLUL and PIK3R1 were significant after applying FDR approach.

Our gene array showed that fluticasone induced a variety of genes, some of which have been reported to have different functions including anti-inflammatory actions such as FKBP5, GILZ, MKP-1, PIK3R1, NFKBIA and TNFAIP3. It was interesting to note that FKBP5 was the highest gene to be induced by fluticasone in agreement with previous studies in airway epithelial cells [233] as well as in ASM cells [234]. The mechanisms by which FKBP5 contribute to asthma pathogenesis have not been elucidated, but recent studies showed that FKBP5 can reduce the GRα nuclear translocation [235] and was

associated with poor response of patients to corticosteroids [233]. FKBP5 also plays an essential role in regulating the pro-inflammatory signalling pathway by inhibiting NF-kB nuclear translocation and transactivation [236]. Induction of Phosphoinositide-3-Kinase Regulatory Subunit 1 (PIK3R1) gene is also interesting since it can control the function of PI3K [237] which has been associated with multiple cellular response including abnormal ASM proliferation [238] and more importantly corticosteroid insensitivity in airway epithelium [179]. The clinical relevance of these data is supported by the increased PIK3R1 mRNA expression levels found in bronchial biopsies in patients treated with inhaled budesonide [239].

Interestingly, beside PIK3R1, fluticasone was able to induce other genes that regulate cell proliferation. Connective tissue growth factor (CTGF), which was reported to be highly expressed at the mRNA and protein level in severe asthmatic ASM cells, correlated with features of airway remodelling [240] and to increase ASM cell proliferation [241]. CTGF was also reported to be significantly upregulated by fluticasone in airway epithelial cells [242]. GLUL, an essential factor in the regulation of glutamine synthesis, promotes the proliferation of cancer cells [243,244]. GLUL was shown to be induced by dexamethasone in the hepatoma cells [245], and I present the first evidence of increased GLUL in ASM cells by fluticasone. ERRFI1 gene, which plays an important role in DNA damage response [246], can be induced by dexamethasone and was found to inhibit B-cells proliferation [247], and deletion of this gene leads to premoting human lung epithelial cells migration *in vitro* [248]. I showed that fluticasone can induce PER1 gene significantly which was previously reported in A549 cell line and in the animal model (rat) of hippocampus [249,250]. PER1 correlates negatively with cancer [251]. Induction of SLC19A2 gene by corticosieroids was also reported in Hela cells [252] and ASM cells [238]. SLC19A2 plays an essential role in cellular thiamine

transport and a mutation of this gene was linked to Thiamine-responsive megaloblastic anaemia disease [253,254].

Few studies have used gene array analysis to study the profile of genes induced by corticosteroids in ASM cells. A previous study used RNA sequencing in healthy ASM cells treated with dexamethasone for 18 hours showed a similar gene profile [255]. The study also found FKBP5, TSC22D3, PER1, DUSP1, ERRFI1 and KLF19 mRNA were among of the genes induced by dexamethasone. Another study using lung cells also investigated corticosteroid-inducible genes using RNA sequencing in A549 cell line treated with 1-hour dexamethasone [249]. These data suggest that corticosteroids can upregulate common genes in different cell types such as FKBP5 and GILZ.

The gene array preformed in ASM cells treated with conditioned media from 24-hour activated MCs showed that a number of key fluticasone-inducible genes were reduced when compared to the response seen in control media-treated ASM cells (**Table 5.1**). ***This clearly shows that conditioned media from 24-hour activated mast cells have the capacity to impair ASM cell response to corticosteroids by interfering with their transactivation property***. It is important to note that not all the fluticasone-inducible genes were similarly affected by activated MC conditioned media, suggesting that induction of these genes involved different transactivation mechanisms.

I also validated the gene array by investigating the expression of individual genes using qPCRs. In fact, qPCR assays produced comparable data as those generated in the gene array regarding the modulation of the selected genes GILZ, MKP-1, FKBP-1 and PIK3R1 by conditioned media from 24-hour activated mast cell when compared to levels seen in ASM cells treated with control MC media (**Figure 5.6**). Indeed, these studies further support the conclusion that conditioned media from 24-hours activated mast cells can blunt the transactivation property of selected genes induced by fluticasone.

6.Chapter Six

Discussion and future plans

6.1. Human lung mast cells (HLMC) and ASM responsiveness to Fluticasone:

Previous studies have reported that a 24-or 48-hour incubation with conditioned media from activated mast cells can regulate a number of different responses in cultured human ASM cells including the expression of chemokines (CCL11 [203,223], CXCL8 [203] and CXCL10 [104]), cell responses to β2-agonists [35] and migratory properties [206]. This clearly suggests that mast cell mediators can participate in asthma pathogenesis via the alteration of pro-asthmatic functions of ASM cells. Here we investigated whether mast cells may affect the sensitivity of ASM cells to the main anti-asthma therapy (i.e., corticosteroids). This hypothesis is based on previous reports showing that infiltration of mast cells into ASM bundles not only correlates with AHR but also with asthma severity which is known to be less responsive to corticosteroid treatment [32,168]. Also, our lab and others have shown that cultured ASM cells isolated from severe asthmatics have a reduced sensitivity to different corticosteroids including fluticasone and dexamethasone [20,92,95]. Although the underlying mechanisms have not been elucidated, we also observed a significant reduction of the ability of fluticasone to repress TNFα-induced expression of various chemokines in healthy ASM cells that were pre-treated with conditioned media from either 24-hour or 30-minute activated mast cells. In addition, the effect of mast cells appears to be gene-specific since only the capacity of fluticasone to inhibit TNFα-induced-CXCL10 and CCL5 expression was affected while the inhibition of TNFα-induced CXCL8 was still preserved (**Figure 3.6 and Figure 4.5**). this observation suggest that fluticasone inhibits gene expression via multiple mechanisms that are not all affected by mast cell mediators. Interestingly, levels of CCL5 and CXCL10 were found to be very high in severe asthmatics patients despite patients being on a high dosage of corticosteroid therapy [256,257]. These studies show

that the production of both chemokines is insensitive to corticosteroids and could result from the action of mast cell mediators on lung structural cells such as the ASM cells.

Notably, the modulation of fluticasone action in healthy ASM cells by conditioned media from activated mast cells (24-hour or 30-minute) was seen at both protein and mRNA levels for CCL5, while levels CXCL10 mRNA were not affected by 30-minute mast cell conditioned media. This finding suggests that fluticasone utilises different inhibitory strategies to suppress chemokine production in ASM cells and that conditioned media from activated mast cell (24-hour or 30-minute) can only affect the mechanisms used by fluticasone to repress CCL5 (transcriptional pathways) and CXCL10 (translational pathways). In contrast, pathways inhibiting CXCL8 appears to be resistant to the action of conditioned media from 30-minute and 24-hour mast cells. These findings support the fact that corticosteroids suppress the expression of inflammatory genes by acting at different levels [17,78]. We [92] and others [20] also made the similar conclusion by showing that the expression of various chemokines of CXCL8, CCL11 and CCL5 was not similarly affected by fluticasone or dexamethasone. Together, these observations strongly suggest that corticosteroids in ASM cells is differentially affected by the conditioned media from activated mast cells by regulating their action at both transcriptional and non-transcriptional levels. We also found that the "so-called" dissociated steroid Compound-A also regulated chemokine expression in ASM cells by acting at both protein and mRNA levels [220].

One possible limitation of our experimental design was the fact that we used only one single 1:4 dilution of mast cell conditioned media for our pre-treatment experiments based on studies showing the 1:4 ratio of mast cells found infiltrated within the ASM bundles [32]. Other groups showed that a high concentration of mast cell conditioned media such as 40% had a greater effect on the induction of chemokine in ASM cells

[104,203]. Therefore, it would be interesting to see whether different dilutions of mast cell conditioned media further change the anti-inflammatory action of fluticasone in ASM cells.

6.2. Effect of conditioned media from 24-hour mast cells on the expression of two well-characterised fluticasone-inducible genes, MKP-1 and GILZ in ASM cells:

We next investigated whether the transactivation property of corticosteroid was affected by activated mast cell conditioned media. Previous reports in ASM cells showed that impaired corticosteroid sensitivity seen in ASM cells from severe asthmatics was associated with a reduced expression of anti-inflammatory proteins such as GILZ [17,92,230,258]. We chose two well-characterised genes GILZ and MKP-1 since they have been shown to mediate the anti-inflammatory actions of corticosteroids by inhibiting key pro-inflammatory signalling pathways such as MAPK [228,259] and NF-κB [229]. Also, our group has demonstrated that cytokines such as TNFα and IFNγ inhibit cell response to fluticasone by preventing the upregulation of both anti-inflammatory genes such as GILZ and MKP-1 [192,221]. Together these studies suggest that transactivation property is the primary mechanism altered in corticosteroid resistant condition.

We found that induction of GILZ and MKP-1 mRNA by TNFα and FP was reduced in healthy ASM cells pre-treated with conditioned media from 24-hour activated mast cells, with MKP-1 mRNA being the only one to be significantly repressed. Previous reports also showed that in severe asthmatic patients, there was a reduction of GILZ in ASM cells [92] and MKP-1 in neutrophil and macrophages [260,261]. The mechanisms for such selective reduction in transactivation are not precise but could be related to the upregulation of p38MAPK pathways. Indeed, p38 MAPK was shown to be activated in

ASM cell when co-cultured with mast cells or with its conditioned media [223,262]. More importantly, p38MAPK could interfere with CS transrepression mechansims. It has been shown that activation of p38MAPK can directly inhibit GRα nuclear translocation and/or transactivation via the regulation of GRα phosphorylation on various serine residues (S203 and S211 residues) which leads to an impairment of both GRα transactivation and transrepression signalling pathway [90,259]. Interestingly, activation of p38MAPK was found to be increased in alveolar macrophages of severe asthmatics, which was associated with a reduction of MKP-1 levels [260,261]. Whether p38MAPKis activated by mast cell mediators remains a possibility worth investigating.

Notably, activated MC conditioned media did not prevent nor reduce the inhibitory action of fluticasone on CXCL8 expression induced by TNFα. This observation supports the concept that in ASM cells, corticosteroids use different anti-inflammatory mechanisms to suppress different inflammatory genes. Previous studies in ASM cells showed that CXCL8 expression is mediated through multiple signalling pathways including P38 MAPK and NF-κB pathways [20]. These pathways are known to be repressed by GILZ and MKP-1 in a cell specific manner [78]. It was surprising to see the CXCL8 inhibition was sustained while GILZ and MKP-1 were reduced in ASM cells which pre-treated with CM from 24-hour activated mast. The likely explanation is that CXCL8 expression may be driven by different transcription factors such as C/EBP homologous protein (CHOP) [263]. It is also known that corticosteroids can regulate the expression of the same pro-inflammatory mediator by acting at different levels, transcriptional and post-transcriptional levels, depending on the cell types.

6.3. Use of the gene array assays to determine the impact of mast cell conditioned media on the overall profile of corticosteroid inducible genes in ASM cells.

I next characterised the expression of genes induced by fluticasone using RT² Profiler PCR Array system from Qiagen which allows the study of 84 different genes known to be associated with corticosteroid signalling pathways. This study is the first to characterise the expression profile after 6 hours stimulation with fluticasone in healthy ASM cells.

6.3.1. Fluticasone-inducible genes in control ASM cells:

I found that fluticasone was able to regulate significantly 28 out of 77 genes in healthy ASM cells after 6 hours simulation including FKBP5 ↑, TSC22D3 (GILZ) ↑, PER1 ↑, CTGF ↑, SLC19A2 ↑, DUSP1 (MKP-1) ↑, ERRFI1 ↑, DDIT4 ↑, GLUL ↑, PIK3R1 ↑, MERTK ↑, MT1E ↑, KLF13 ↑, PER2 ↑, ZFP36 ↑, NFKBIA ↑, TNFAIP3 ↑, ADARB1 ↑, SGK1 ↑, CEBPB ↑, STAT5B ↑, ASPH ↓, TBL1XR1 ↓, NR3C1 ↓, AMPD3 ↓, VDR ↓, IL6 ↓ and POU2F2 ↓. As expected from previous studies, I did find that both GILZ and MKP-1 were significantly upregulated by fluticasone [74,79,92,261]. Some of the induced genes by fluticasone such as PIK3R1, PER1 and GLUL have not been investigated in ASM cells, and additional studies are needed to understand their precise role in the regulation of asthma pathogenesis. Based on their function reported in other cell types, it is possible to speculate on the anti-inflammatory role of FKBP5, TNFAIP3 and other. For instance, FKBP5 has been shown to regulate the nuclear translocation and transcriptional activity of pro-inflammatory of NF-kB in kidney cells and could participate in the fluticasone suppression of inflammatory genes induced by TNFα (**Figure 4.6 and Figure 3.8**) [236]. TNFAIP3 is also another fluticasone-inducible gene

that appears to have anti-inflammatory activity in both BEAS-2B cells and ASM cells [264-266].

Interestingly, my results seem to agree with the conclusions of other previous studies describing that FKBP5, TSC22D3 (GILZ), PER1 and DUSP (MKP-1) were the among highest genes induced by corticosteroids in various cell types (ASM cells and epithelial cells) [249,255]. In contrast, ERRFI1 and DDIT4 (both are involved DNA damage response) were the most highly upregulated gene by CSs in the brain [267]. Thus, it is clear that corticosteroids induce different expression genes profile with different magnitude (fold increase) and function (anti-inflammatory vs pro-inflammatory) in a cell-specific manner.

6.3.2. Effect of mast cell conditioned media on the gene expression profile induced by fluticasone:

I have clearly shown that the inhibition of TNF-α-induced-CXCL10 and CCL5 expression by fluticasone was reduced by conditioned media from mast cell activated for either 30-minute and 24-hour **(Figure 3.6 and Figure 4.5)**. I investigated the impact of conditioned media from activated mast cells on the gene expression profile induced by fluticasone. I here show for the first time that pre-treating healthy ASM cells with conditioned media from 24-hour activated mast cells differentially regulates the ability of fluticasone to induce expression of target genes. I found that the number of fluticasone-inducible genes was significantly lower (26 genes) following treatment of ASM cells with conditioned media with only 2 genes to be significantly upregulated (FKBP5 and GILZ) that did pass the FDR test. By comparison, in ASM cells treated with control media, 28 genes were induced significantly by fluticasone, with 10 genes which did pass the FDR test (FKBP5, GILZ, PER1, CTGF, SLC19A2, MKP-1, ERRFI1, DDIT4, GLUL, PIK3R1). Also, there was a notable reduction of fold change in some genes in ASM cells

pre-treated with conditioned media from 24-hour activated MCs compared to control ASM cells **(Table 5.1)**.

The molecular mechanisms of corticosteroid insensitivity in severe asthmatic ASM were due to the inhibitory actions of PP5 or IRF-1 [18,92] as well as p38 MAPK [260,261], known to repress various key function such as GRα translocation and transactivation [17,87,90,268]. Interestingly, p38MAPK activity increased in a coculture model of ASM cells with mast cell or following incubation with its conditioned media [223,262]. Thus, it would be essential to investigate whether p38 MAPK can be induced in ASM cells pre-treated with conditioned media from 24-hour activated mast cells and whether p38 MAPK inhibition can restore fluticasone anti-inflammatory action.

Interestingly, our PCR array showed that fluticasone was involved in another non-inflammatory signalling pathway such as the induction of CTGF and GLUL (growth gene) [241,244], ERRFI1 and DDIT4 (DNA damage response gene) [246], however, we do not know if they are involved in anti-inflammatory action and their precise role in asthma. Also, these genes were reduced in healthy ASM cells pre-treated with conditioned media from 24-hour activated MCs compared to control ASM cells.

6.4. Conditioned media from activated mast cells did not modulate TNF-α-induced chemokines in ASM cells:

The pro-inflammatory response of ASM cells varies according to the type of stimuli whether these are cytokines (TNF-α alone, TNF-α and IFN-γ) or conditioned media from 2-hour or 24-hour activated mast cells [94,96,104,203]. Although our experimental approach of ASM cell stimulation was different from these studies, it was important to determine whether cytokine production in response to TNF-α was affected by activated mast cell conditioned media. We found that the net chemokine production

of CCL11, CCL5, CXCL10 and CXCL8 in healthy and asthmatic ASM cells by TNF-α was not affected by the conditioned media from either 24-hour or 30-minute activated and non-activated MCs compared to control media. In contrast to our results, Alkhouri and colleagues found that a 24-hour exposure to 2-hour activated mast cell conditioned media reduced cytokine-induced CXCL10 expression, while 24-hour activated mast cell increased cytokine-induced CXCL10 expression [104]. Another study also found that conditioned media from 24-hour activated mast cells did reduce the production of CCL11 in both asthmatic and non-asthmatic ASM cells while CXCL-8 expression was increased in healthy ASM cells only [203]. The likely explanation for such discrepancy is the fact that in both studies cytokine stimulation was conducted in the presence of conditioned media while in our study, ASM cells were first washed before adding fresh media containing TNF-α. So, our results strongly show that in our experimental setting the modulation of corticosteroid action by mast cell conditioned media does not result from changes in TNF-α signalling pathways. This is a surprising finding as mast cells can release various preformed or synthesised mediators including TNF-α, IFN-γ, MIF, IL-4, IL-17A, IL-33, **(chapter 3 and 5)**, that have receptors on ASM cells. It remains to be seen whether repeating the experiments with both TNF-α and conditioned media present would lead to different results.

6.5. Profile of mast cell mediators produced by activated mast cells:

The Proteome Profiler™ Antibody Arrays allowed me to determine the profile of mast cell mediators released following 30-minute and 24-hour cell activation. I have used these two different time points of IgE-anti-IgE activated mast cell (30-minutes and 24-hour) to detect the different mediators, [104]. This approch was used to determine the

preformed and newly synthesised mediators produced only in the 24-hour activated and non-activated mast cell conditioned media but not in the 30-minute activated and non-activated mast cell conditioned media. We found that RAGE, IL-34, MIG, IL-31, MIP-3alpha, TARC, IL-32, MCP-3, TfR, Growth Hormone, Fas Ligand, BAFF, FGF-7, IGFBP-3, IFN-gamma, TGF-alpha, IL-27, IL-24 MIP-1alpha/MIP-1beta and IL-5 were released in conditioned media from 24-hour non-activated and activated mast cell but not from 30-minute non-activated and activated mast cell. My data confirm that the 2 time points produce different mediators released by activated mast cells that include cytokines, chemokines, growth factors, protease and others.

It was essential to confirm mast cell degranulation before exposing ASM cells to mast cells supernatants. Several studies have demonstrated that β-hexosaminidase could be used as a reliable marker of mast cell degranulation [104,203,211]. I showed consistent increased β-hexosaminidase activity in the conditioned media of mast cell activated for 30 minutes with IgE-anti-IgE when compared to non-activated cells. One important issue I faced what the variability in β-hexosaminidase activity between the different mast cell preparations. This could be explained by several factors including the issue of reproducibility of mast cell activation using the IgE-anti-IgE method and more importantly the different donors used for mast cell preparations. We did improve the mast cell stimulation procedure using one step anti-FcεR1 stimulation as an alternative option to confirm degranulation of mast cell by measuring by the β-hexosaminidase activity and TNFα concentration [211].

6.6. Potential mast cell mediators impairing the sensitivity of ASM cells to fluticasone:

The purpose of investigating the profile of mediators released by activated mast cells was to identify those which might be responsible for the changes in corticosteroid sensitivity in ASM cells. From the literature, it is clear that several mediators produced by mast cells such as IL-17A [173], IL-4 and IL-2 [215], IL-13 [199], TGF-β1 [174], MIF [217], TNF-α and IFN-γ [17] and IL17A and IL23 [222] have the potential to interfere with the inhibitory action of corticosteroids as reported in different cell types (**see chapter one**). Interestingly, impaired transactivation appears as the main mechanism of corticosteroid resistance. For example, both IL-27 and IFN-γ have been shown to affect the sensitivity to which dexamethasone in PBMC by reducing GR-α nuclear translocation and induction of the target gene MKP-1, suggesting a reduced transcriptional activity as a mechanism of corticosteroid insensitivity [230]. The protein array did show activated mast cells release all these cytokines with differences. The 24-hour activation led to the production of IL-17A, IL-23, IL-4, IL-2, IL-27, TNF-α, IFN-γ and MIF but not IL-13, while the 30-minute activated mast cell expressed IL-17A, IL-4, IL-2, TNF-α, IL-23 and MIF but not IL-13, IL-27 and IFN-γ. Interestingly, previous studies showed that ASM cells expressed receptors for IL-17A [269], IL-4 and IL-2 [270], and MIF [271], although their role in regulating corticosteroid response remains to be determined. Although we determined the mediators which were released at different time point activation (30-minutes and 24-hour), more investigations needed to know which mediators are involved in mediating CS insensitivity in ASM. Also, it remains to be determined whether specific mediators in conditioned media from 30-minutes that regulated corticosteroid response in ASM is similar to the mediators in conditioned media from 24-hour mast cell.

Interestingly, only conditioned media from activated mast cells (30-minute and 24-hour stimulation) can modulate fluticasone inhibitory action in ASM cells, while conditioned media from non- activated mast cell had no significant effect. These studies show that only factors produced by activated mast cells inhibited the response of ASM cells to fluticasone, thus excluding factors produced in high levels in basal conditions such as MIF. It would be interesting therefore to see whether IL-17A, IL-4 and IL-2 or TNF-α and IFN-γ play any role in regulating corticosteroid response in ASM cells.

6.7. Limitations and future work:

The present study has different limitations which could be improved in future studies:

- Increasing the sample size:

First, all the studies were done in cells derived from a low number of subjects which could be increased especially in PCR Array (n=4) to further support the conclusion of the data. Access to ASM cells from healthy subjects was a very difficult task due to the lack of volunteers.

- Varying dilutions of mast cell conditioned media:

The other limitation was the fact that ASM cells were pre-treated with conditioned media from mast cells with only dilution (1:4 dilution) based on previous studies that showed a 1:4 ratio of infiltrated mast cell within the asthmatic ASM cells [32]. It would be interesting to determine whether increasing this ratio or the treatment duration as previously done by others would give a different outcome on the sensitivity of cells to corticosteroids [104,203].

- Use of RNAseq to investigate expression of corticosteroid genes in our model:

Similarly, we could also repeat the gene array analysis using RNAseq to have a broader knowledge of the transcriptome profile of both known and unknown transcripts induced by corticosteroids that are affected by conditioned media from activated mast cells [272]. Western blot analysis should also be performed to validate the gene array data.

- Determining the effect of MC conditioned media on cell sensitivity to different concentrations of fluticasone (IC50):

We will be testing different time points of cell stimulation as described by others [214,249] is also an option to study the kinetics of corticosteroid-inducible genes.

- Investigate the effect of MC conditioned media on fluticasone-induced GRα nuclear translocation and phosphorylation:

I also did not investigate the precise mechanisms (mast cell mediators) behind the inhibition of fluticasone action by activated mast cell conditioned media beside the modulation of transactivation. From looking that the reduced expression of anti-inflammatory genes in response to fluticasone seen PCR array, it would be interesting to investigate whether conditioned media alter either fluticasone-induced GRα nuclear translocation and/or GRα phosphorylation by western blot or/and immune-staining as previously shown by our group [92].

- Applying RNA silencing to determine the function of different fluticasone-inducible genes:

Also, gene knockdown using RNA silencing can be applied to determine the exact role of some of the steroid-induced genes modulated by mast cell conditioned media such

as FKBP5, GILZ and MKP-1 and their contribution to the overall anti-inflammatory actions of corticosteroids.

- Determining the possible mast cell mediators involved in changing corticosteroid responses:

Neutralisation studies using specific antibodies against mediators known to alter steroid action in other cell types such as IL-17A, IL-4, IFNγ, MIF, IL-23 would help identify the possible mast cell mediators involved in changing corticosteroid responses.

- Expanding our studies to other key asthmatic mediators including CCL11:

We will study the overall impact of MC conditioned media on additional mediators including CCL11.

6.8. Conclusion:

Our study shows for the first time that ASM cell responsiveness to corticosteroids can be altered by the effects of conditioned media from activated mast cells **(Figure 6.1)**. Our study reinforces the concept that mast cells play a role in asthma pathogenesis by failing to inhibit the proinflammatory action of mediators produced by ASM cells. Multiple mechanisms appear to be responsible for the inhibitory effect of mast cell on ASM response to corticosteroids which include an inhibition of GRα transactivation.

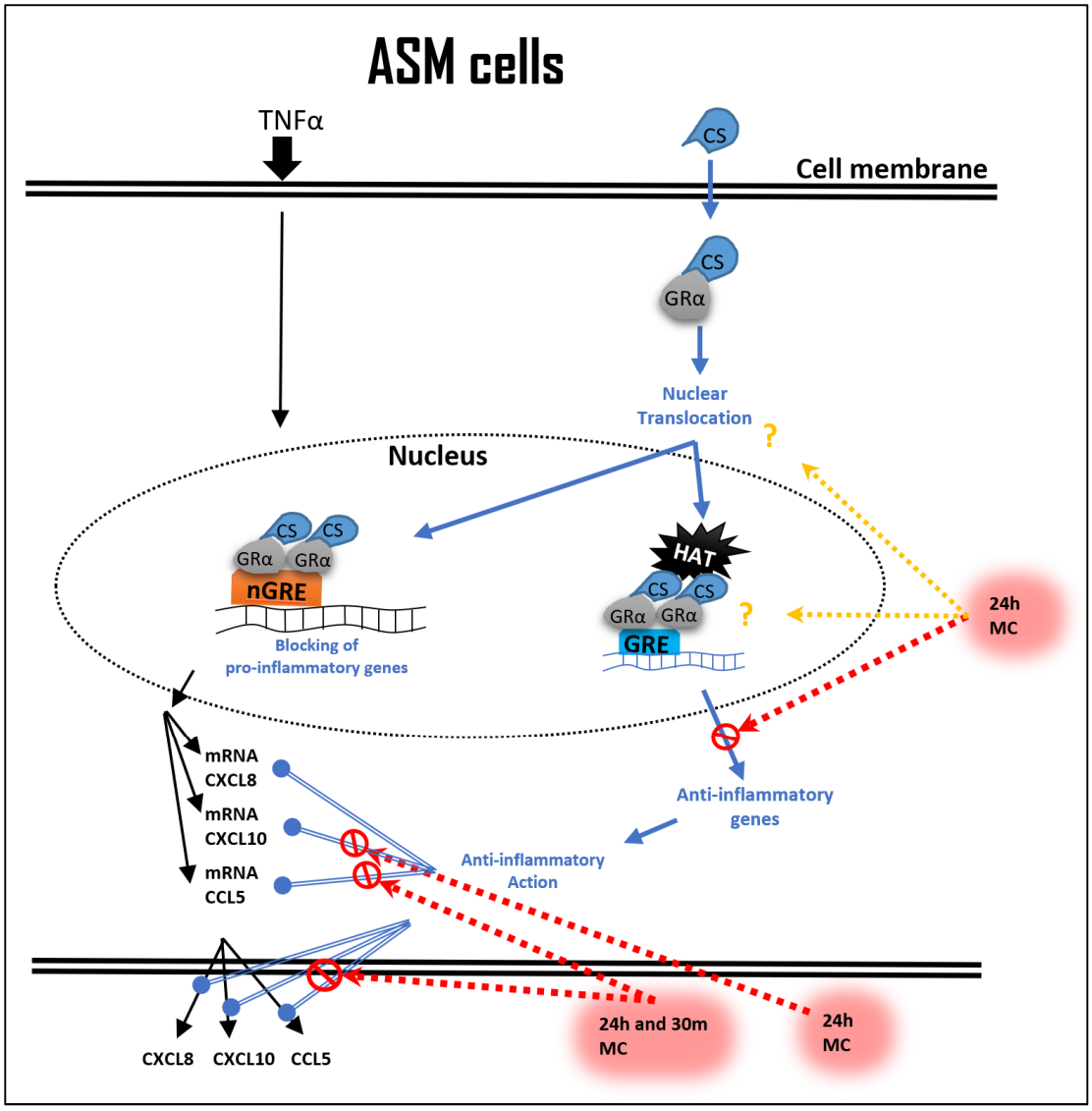


Figure 6.1: Summary of the modulatory actions of activated MC conditioned media on ASM cell responsiveness to fluticasone.

This graph summarises the main observations of this thesis uncovering multiple mechanisms involved in reducing CS responsiveness in ASM cell by mediators produced by mast cells. The red arrows show pathways suppressed by activated MC conditioned media (at both protein and mRNA levels) while orange arrrows show the underlying associated molecular mechanisms (defect in GRα nuclear translocation, phosphorylation and/or gene transcription acting on both postive and negative GRE). The nature of the MC mediators responsible for inhibiting fluticasone response is unknown (24-hour=24h, 30-minute= 30m of MC conditioned media treatment).

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