New Insight into the Molecular Mechanisms of Corticosteroid Resistance in Asthma

Thesis submitted for the degree of
Doctor of Philosophy at the University of Leicester

By
Latifa Chachi MSc (Dis)
Department of Infection, Immunity and Inflammation
University of Leicester
2014
ABSTRACT
Although corticosteroids are very efficient in managing asthma and other inflammatory diseases, a small percentage of patients affected by “severe asthma” fail to respond even to high doses of oral glucocorticoids. It is therefore important to try to understand the potential mechanisms behind this insensitivity to corticosteroid therapy in order to be able to effectively control asthma in this patient subset. We have decided to focus on one particular channel called $K_{\text{Ca}3.1}$, a calcium-activated potassium channel. First, emerging evidence in the literature to date has strongly supported a significant role for $K_{\text{Ca}3.1}$ channel in the pathophysiology of asthma. $K_{\text{Ca}3.1}$ channel is expressed by several inflammatory and structural airway cells including mast cells and human bronchial smooth muscle (HBSM). Therefore these channels might serve as new targets for the treatment of lung diseases. Here we established a cellular model of corticosteroid insensitivity consisting of primary HBSM cells exposed to two cytokines TNF-$\alpha$ and IFN-$\gamma$. Under these conditions, HBSMC exhibit a marked production of different pro-asthmatic chemokines like CCL5, CX3CL1, CCL11 and CXCL10 that are completely resistant to corticosteroid treatment. In this model, we found that although $K_{\text{Ca}3.1}$ channel expression did not change between healthy control, asthmatic and COPD subjects, $K_{\text{Ca}3.1}$channel blockers (ICA-17043 and TRAM-34) were able to inhibit the production of corticosteroid-resistant chemokines either directly via the suppression of gene expression or indirectly via the restoration of the anti-inflammatory action of fluticasone.

We also found that $K_{\text{Ca}3.1}$ channel blockers restored cell sensitivity to corticosteroid in cytokine-treated HBSMC by re-establishing the transactivation
function of fluticasone via the prevention of dephosphorylation of Glucocorticoid Receptors (GR\(\alpha\)) at Ser\(^{211}\) and induction of anti-inflammatory genes such as Glucocorticoid-induced leucine zipper (GILZ). The likely mechanism of this restoration of corticosteroid sensitivity by \(K_{Ca}3.1\) channel blockers is via the inhibition of protein phosphatase 5 (PP5) expression found to be up-regulated in steroid resistant conditions.
Writing of this thesis was not been an easy process. It has required a lot of work, time and patience. I could never been able to write this thesis without guidance and support from others. This thesis would not have been possible without contributions from several individuals and organisations. I would like to take this opportunity to thank the following

First I would like to thank all the patients and healthy volunteers who were generous to participate in the studies within this thesis.

The most significant influence on my research has come from my supervisor, Dr Yassine Amrani who has been an inspiration, encouragement and direction in this work. I am extremely grateful for his constant encouragement, support, guidance, advice and patience throughout this research and for giving me the opportunity to work with him. I could not have asked for a better mentor as I am blessed I got the best one.

I would like to thank University of Leicester, for giving me the opportunity to do Doctoral degree as part of staff development programme.

To everyone in Respiratory Medicine: to each of you I owe a huge thank you. You have all put up with me remarkably well; I would like to extend the biggest thank you to my friends Sally Stinson and Hilary Marshall, for supporting and
believing in me throughout the PhD. For that, and for being such incredible friends, thank you. To Adelina Gavrila “My flower”: Thank you for always being there to encourage and believe in me. ATS, ERS and Netherland it was certainly an experience I'll never forget.

To my husband, Ameur has been very encouraging and accommodating all along. He is the one who was inspirational that I stay focused and motivated. He stood by me and I edged my way towards the goal. He merits my sincere praise.

To my boys, Yassin and Rayaan are probably the biggest contributor of all, as they sacrificed the time that should have been given to them but was spent on the research. Thanks Yassin for saying “Don't give up. You're almost there Mama”. Thanks Rayaan for saying “I will be a Dr like you Mama!”.

And finally but most importantly to my Family in Algeria especially my Dad Ali: You made me the determined person I am and I know that finishing my PhD would have made you so proud. Your love is the reason I have come so far and been able to achieve my dreams. I wish I had told you this before it was too late, but thank you, for everything.
PUBLICATIONS AND ABSTRACTS

PEER-REVIEWED ARTICLES


ABSTRACTS


2013  L. Chachi, O. Tliba; A. Gavrila; C. Brightling; Y. Amrani. “Impaired inhibitory action of corticosteroids on chemokine expression induced by TNFα in airway smooth muscle (ASM) cells from patients with severe asthma”. ERS, Barcelona, Spain and 8th YIM 2013 Groningen, Netherlands. Poster presentation.

2013 A, Gavrila; L, Chachi, O. Tliba; C, Brightling; Y, Amrani. “Compound A (CpdA) suppressed production of corticosteroid-resistant chemokines via GR-independent mechanisms in airway smooth muscle (ASM) cells” (ERS, Barcelona, Spain, poster presentation).

2012 L. Chachi, Gavrila, A; Tliba,O; Brightling, C; Amrani; Y “The dissociated steroid receptor ligand from plant origin called compound A (CpdA) inhibits the production of steroid-resistant chemokines induced by TNFα/IFNγ in airway smooth muscle (ASM) cells in both asthma and healthy subjects” (ERS, Vienna, Austria, poster presentation).

2012 H. Pandya; A. Gassama, L. Chachi; H. Pearson, E, Gaillard; Y, Amrani “TNFα stimulates the expression different chemokines including CXCl10, CCL5 and CXCL8 in developing airway smooth muscle (ASM) cells: Modulation by fluticasone and TNFα receptors” (ERS, Vienna, Austria, poster presentation).

2012 A. Gavrila ; L. Chachi, O. Tliba; C. Brightling, Y. Amrani “Activation of both transcription factors STAT5 and IRF-1 is insensitive to corticosteroids in asthmatic bronchial smooth muscle cells exposed to TNFα/IFNγ” (ERS, Vienna, Austria, poster presentation).

2010 L. Chachi, A. Shikotra, A. Sutcliffe, C. Brightling, P. Bradding, Y Amrani “KCa3.1 ion channel blockers restore corticosteroid sensitivity in cytokine-treated airway smooth muscle (ASM) cells from both COPD and asthmatic patients” (BTS, London, oral presentation “nominee BTS young investigator Prize

LIST OF TABLES

Table 1.1:  Structural alterations contributing to airway remodelling.

Table 1.2:  Examples of pro-asthmatic genes: synergistically induced by interferons and other cytokines in BSM cells.

Table 1.3:  Important diseases associated to KCa3.1 dysfunction, localization and possible treatments.

Table 2.1:  Subjects demographics and clinical characteristics.

Table 2.2:  Number of the cells plated according to plate sizes.

Table 2.3:  The working concentrations for the different chemokines used.

Table 2.4:  Primary antibodies used for the western blot assays.

Table 2.6:  Primer sequences for quantitative real-time PCR.

Table 2.7:  Primary and Secondary antibodies used for immunofluorescence.
LIST OF FIGURES

Figure 1.1: Initiation of the inflammatory cascade after allergen (pollen) inhalation and interaction with airway cells.

Figure 1.2: Bronchial smooth muscle is an important player in the pathogenesis of asthma.

Figure 1.3: Bronchial smooth muscle a potential target for asthma therapy.

Figure 1.4: Diagram of the potential mechanisms of corticosteroid insensitivity in asthma.

Figure 1.5: Role of TNF-α in the pathogenesis of asthma.

Figure 1.6: Molecular mechanisms by which the combined action of TNF-α/IFN-γ in BSM cells could play a role of in pathogenesis of asthma.

Figure 1.7: Schematic representative of K$_{Ca}$3.1 structure.

Figure 1.8: The chemical structures of selective K$_{Ca}$3.1 channel blockers (TRAM-34 and ICA-17043).

Figure 1.9: Role of K$_{Ca}$3.1 channels in different cell types.

Figure 2.1: Light Microscopy of BSMC cells in culture.

Figure 3.1: Dose-dependent induction of CCL11 by TNF-α.

Figure 3.2: Dose-dependent induction of CCL5 by TNF-α.

Figure 3.3: Dose-dependent induction of CXCL10 by TNF-α.
Figure 3.4: Potentiating effect of IFN-γ on TNF-α-induced chemokine induction.

Figure 3.5: Effect of the fluticasone on cytokine-induced CCL11 and CCL5 production.

Figure 3.6: Effect of the steroid fluticasone on cytokine-induced CX3CL1 and CXCL10 production.

Figure 4.1: In vivo $K_{Ca}3.1$ expression in HBSM bundles from a healthy control.

Figure 4.2: In vivo $K_{Ca}3.1$ expression in HBSM bundles from an asthmatic subject.

Figure 4.3: Representative Immunohistochemistry staining at higher magnification (X1000) showing the nuclear expression of $K_{Ca}3.1$ channel in the HBSM bundles.

Figure 4.4: $K_{Ca}3.1$ expression in nuclear extracts.

Figure 4.5: Nuclear expression of $K_{Ca}3.1$ channel in cultured HBSMC.

Figure 4.6: $K_{Ca}3.1$ expression in HBSMC in healthy and asthmatic subjects.

Figure 4.7: Effect of TNF-α/IFN-γ on $K_{Ca}3.1$ expression in HBSMC.

Figure 4.8: Current-voltage curves showing the lack of detectable $K_{Ca}3.1$ currents in HBSMC.

Figure 5.1: TNF-α/IFN-γ-induced CCL5 and CXCL10 in HBSMC from healthy control and patients with asthma and COPD.

Figure 5.2: TNF-α/IFN-γ-induced CCL11 and CX3CL1 in HBSMC in healthy control and patients with asthma and COPD.
Figure 5.3: Effect of TRAM-34 or ICA-1743 on HBSMC viability.

Figure 5.4: Effect of K\textsubscript{Ca}3.1 blockers alone or in combination with fluticasone on corticosteroid-resistant CCL5 expression.

Figure 5.5: Effect of K\textsubscript{Ca}3.1 blockers alone or in combination with fluticasone on corticosteroid-resistant CCL11 expression.

Figure 5.6: Effect of K\textsubscript{Ca}3.1 blockers alone or in combination with fluticasone on corticosteroid-resistant CX3CL1 expression.

Figure 5.7: Effect of K\textsubscript{Ca}3.1 blockers alone or in combination with fluticasone on corticosteroid-resistant CXCL10 expression.

Figure 5.8: Transduction efficiency of GFP-shRNA adenovirus in HBSMC.

Figure 5.9: Effect of K\textsubscript{Ca}3.1 shRNA adenoviruses on HBSMC apoptosis.

Figure 5.10: Quantitative analysis of percentage of live, apoptotic, or dead HBSMC.

Figure 5.11: Silencing of K\textsubscript{Ca}3.1 channel expression using K\textsubscript{Ca}3.1 shRNA adenoviruses in HBSMC.

Figure 5.12: K\textsubscript{Ca}3.1 shRNA adenoviruses modulate TNF-\textalpha/IFN-\gamma–induced chemokine expression.

Figure 5.13: TNF-\textalpha/IFN-\gamma induced mRNA expression of inflammatory chemokines in HBSMC.

Figure 5.14: K\textsubscript{Ca}3.1 blockers modulate TNF-\textalpha/IFN-\gamma induced CCL5 expression at the mRNA level.

Figure 5.16: K\textsubscript{Ca}3.1 blockers modulate TNF-\textalpha/IFN-\gamma induced CCL11 expression at the mRNA level.
Figure 6.1: Fluticasone Propionate increases GRα phosphorylation at Ser211 in a time-dependent manner.

Figure 6.2: Impaired fluticasone-induced GRα phosphorylation expression by cytokines is prevented by K_Ca3.1 inhibitors.

Figure 6.3: Effect of K_Ca3.1 blockers on fluticasone-induced GILZ expression in steroid resistant state.

Figure 6.4: Effect of K_Ca3.1 blockers on fluticasone-induced MKP-1 expression in steroid resistant state.

Figure 6.5: Effect of TNF-α/IFN-γ on PP5 expression.

Figure 6.6: PP5 upregulation by TNF-α/IFN-γ is inhibited by K_Ca3.1 inhibitors.

Figure 6.7: Expression of PP5 in bronchial smooth muscle bundles.

Figure 7.1: Role of K_Ca3.1 in mediating corticosteroid insensitivity in HBSMC.
LIST OF ABREVIATIONS

α-actin  Alpha smooth muscle actin
ADAM  A Disintegrin and metalloproteinase
ANOVA  Analyses of variance
AHR  Airway hyper-responsiveness
APC  Antigen presenting cells
ATS  American Thoracic Society
BTS  British Thoracic Society
BALF  Bronchoalveolar Lavage fluid
B cells  B lymphocytes
BCR  B cell receptor
BSA  Bovine serum albumin
bFGF  Basic fibroblast growth factor
Ca$^{2+}$  Calcium
CCL  Chemokine ligand
CXC  Chemokine (C-X-C motif) ligand
cAMP  Cyclic adenosine monophosphate
COPD  Chronic obstructive pulmonary disease
DMEM  Dulbecco’s modified eagles medium
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FcεRI</td>
<td>The high-affinity IgE receptor</td>
</tr>
<tr>
<td>FEV1</td>
<td>Forced expiratory volume in 1 second</td>
</tr>
<tr>
<td>FP</td>
<td>Fluticasone propionate</td>
</tr>
<tr>
<td>FVC</td>
<td>Forced vital capacity</td>
</tr>
<tr>
<td>GC</td>
<td>Glucocorticoids</td>
</tr>
<tr>
<td>GINA</td>
<td>The Global Initiative for Asthma</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte/macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>GRE</td>
<td>Glucocorticoid-response element</td>
</tr>
<tr>
<td>H₂SO₄</td>
<td>Sulphuric acid</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidise</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular cell adhesion molecule 1</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>ITS</td>
<td>Insulin-Transferrin-Selenium</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage - colony stimulating factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HBSMC</td>
<td>Human bronchial smooth muscle cells</td>
</tr>
<tr>
<td>IPF</td>
<td>Idiopathic pulmonary fibrosis</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IgM</td>
<td>Immunoglobulin M</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus tyrosine kinases</td>
</tr>
<tr>
<td>K⁺</td>
<td>Potassium</td>
</tr>
<tr>
<td>LABA</td>
<td>Long-acting β2-agonist</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>Mab</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MBP</td>
<td>Major basic protein</td>
</tr>
<tr>
<td>MC</td>
<td>Mast cells</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein 1</td>
</tr>
<tr>
<td>MLC</td>
<td>Myosin light chain</td>
</tr>
<tr>
<td>MLCK</td>
<td>Myosin light chain kinase</td>
</tr>
<tr>
<td>MLCP</td>
<td>Myosin light chain phosphatase</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloprotease</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide</td>
</tr>
<tr>
<td>Na3VO4</td>
<td>Sodium orthovanadate</td>
</tr>
<tr>
<td>NAF</td>
<td>Sodium fluoride</td>
</tr>
<tr>
<td>Na⁺</td>
<td>Sodium</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor-κB</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>NP-40</td>
<td>nonidet-40</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PGD2</td>
<td>Prostaglandin D2</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethanesulfonyl fluoride</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation Assay</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SABA</td>
<td>Short-acting β2-agonist</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem cell factor</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducers and activators of transcription</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered Saline containing 0.001% Tween®20</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>T cells</td>
<td>T lymphocytes</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-beta</td>
</tr>
<tr>
<td>Th</td>
<td>Helper T cells</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-alpha</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cells</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
VCAM-I  Vascular cell adhesion molecule-I
Table of Contents

Chapter 1 INTRODUCTION.................................................................1

I. ASTHMA.........................................................................................2
   1. Definition and Diagnosis............................................................2
   2. Asthma is a major health issue...................................................3
   3. Causes of Asthma....................................................................4

II. PATHOGENESIS OF ASTHMA...................................................5
   1. Airway inflammation – the classical view......................................5
   2. Airway hyper-responsiveness....................................................10
   3. Airway remodelling.................................................................10

III. BRONCHIAL SMOOTH MUSCLE IS AN IMPORTANT PLAYER IN THE PATHOGENESIS OF ASTHMA ..............................................16
   1. Potential mechanisms by which BSM may contribute to asthma pathogenesis.........................................................17
      1.2 Airway hyper-responsiveness via an abnormal BSM contractile function ............................................................19
      1.3 Airway remodelling via increased BSM migration ....................20
      1.4 Airway inflammation via the increased production of inflammatory mediators by BSM ..............................................20
   2. Treating asthma means treating Bronchial Smooth Muscle ..........22

IV. ASTHMA THERAPIES ...............................................................24
   1. β2-receptor agonists therapy.....................................................24
   2. Corticosteroid therapy...............................................................25
      2.1 Why are corticosteroids so important in asthma? ..................25
      2.2 Anti-inflammatory mechanisms of glucocorticoids..................26
   3. Other drugs for asthma: ............................................................29

V. CORTICOSTEROID INSENSITIVITY IN ASTHMA.......................30
   1. Potential mechanisms of reduced corticosteroid sensitivity in asthma ...31
      a) Defects in ligand binding/GR number ....................................31
      c) Cross-talk with the transcription factor AP-1 ..........................33
2. Other factors contributing to glucocorticoid resistance .......................... 34

VI. CROSS TALK BETWEEN TNF-α AND IFNS IN THE PATHOGENESIS OF ASTHMA .......................................................... 37
   1. TNF-α is key player in asthma................................................. 37
   2. IFN-γ as a mediator in asthma............................................. 41
   3. TNF-α/IFN-γ combination leads to pro-asthmatic responses in BSM cells 42

VII. KCa3.1 CHANNELS AND ASTHMA .............................................. 46
   1. Ion channels and asthma ................................................. 46
   2. Potassium (K⁺) Channels ................................................ 47
   3. Ca²⁺-activated K⁺ channels ............................................ 47
   4. The intermediate conductance Ca²⁺-activated K⁺ channel (KCa3.1 channel) ................................................................... 49
      4.1 KCa3.1 channel structure ............................................. 49
      4.2 Pharmacology of KCa3.1 channels ................................. 51
      4.3. KCa3.1 channel Activators ........................................ 52
      4.5 Roles of KCa3.1 channels in health and disease ............... 54
      4.5.1 KCa3.1 channel and cell proliferation ....................... 54
      4.6 Pathophysiological roles of KCa3.1 channels ................. 56

VIII. EMERGING ROLE OF KCa3.1 CHANNELS IN ALLERGIC ASTHMA ... 58

Chapter 2 MATERIALS AND METHODS ...................................................... 63

I. BRONCHIAL SMOOTH MUSCLE (BSM): ......................................... 64
   1. Human subjects............................................................... 64
   2. BSM Isolation and Culture .............................................. 65

II. Enzyme linked immune-sorbent assay [ELISA]: ............................. 68
   1. Sample preparation ....................................................... 68
   2. Chemokine Immunoassays ............................................ 69

III. WESTERN BLOTTING ................................................................. 70
   1. Protein preparation ....................................................... 70
   2. Protein Concentration .................................................. 71
   3. Protein electrophoresis .................................................. 71
   4. Stripping western membranes ........................................ 73
Chapter 3  ESTABLISHING A CELLULAR MODEL OF CORTICOSTEROID RESISTANCE USING HUMAN BRONCHIAL SMOOTH MUSCLE CELLS (HBSMC) .......................................................... 88

I.  RATIONALE ......................................................................................... 89

II.  AIM ...................................................................................................... 91

III. RESULTS ............................................................................................ 91

1.  TNF-α dose-dependently induced chemokine expression by HBSM Cells: 91

2.  IFN-γ augments TNF-α-induced chemokine expression by HBSM Cells: 97

3.  Production of CCL11, CCL5, CXCL10 and CX3CL1 by TNF-α and IFN-γ is resistant to fluticasone treatment ....................................................... 100

IV.  DISCUSSION ....................................................................................... 104
Chapter 4  K\text{Ca3.1} CHANNELS IN HBSMC IN HEALTH AND ASTHMA

I. RATIONALE: ................................................................. 109
II. AIM .............................................................................. 110
III. RESULTS ....................................................................... 111
  1. Expression of K\text{Ca3.1} in BSMC bundles: ....................... 111
  2. Nuclear expression of K\text{Ca3.1} channel in HBSM tissues and cells...... 114
  3. K\text{Ca3.1} expression in HBSMC is not changed between healthy control and asthmatic patients: ................................................................. 117
  4. K\text{Ca3.1} expression and activity are not altered in corticosteroid insensitive conditions.............................................................................. 119
    4.1 K\text{Ca3.1} expression .................................................................. 119
    4.2 K\text{Ca3.1} channel activity .......................................................... 122
IV. DISCUSSION: ................................................................... 123

Chapter 5  K\text{Ca3.1} CHANNELS AND CORTICOSTEROID INSENSITIVITY IN HBSMC

I. RATIONALE .................................................................... 127
II. AIM: ............................................................................... 129
III. RESULTS ......................................................................... 130
  1. TNF-\alpha/IFN-\gamma combination differentially regulates the production CXCL10, CCL5, CCL11 and CX3CL1 in HBSMC from healthy controls and patients with asthma and COPD.............................................................................. 130
  2. K\text{Ca3.1} channel blockade differentially suppresses the production of steroid-resistant chemokines induced by TNF-\alpha/IFN-\gamma .............................................. 134
    2.1 Effect of K\text{Ca3.1} inhibitors on HBSMC viability ..................... 134
    2.2 K\text{Ca3.1} channel blockers (TRAM-34 and ICA-17043) modulate TNF\alpha/IFN-\gamma induced chemokines production........................................... 136
  3. K\text{Ca3.1} downregulation attenuates TNF-\alpha/IFN-\gamma induced expression of steroid-resistant chemokines ...................................................... 142
    3.1 Optimization of adenoviral transduction efficiency for silencing studies ...................................................... 142
    3.2 Silencing of K\text{Ca3.1} Channels using shRNA adenoviruses........... 144
Apoptosis assay ................................................................................................................. 144
3.3 shRNA adenovirus-mediated silencing of K_{Ca}3.1 channels .................. 147
3.4 Downregulation of K_{Ca}3.1 channel in HBSMC attenuates ................. 149
TNF-\(\alpha\)/IFN-\(\gamma\)–induced expression of steroid-resistant chemokines ........... 149
4. Inhibition of K_{Ca}3.1 channel suppresses TNF-\(\alpha\)/IFN-\(\gamma\) induced steroid-resistant chemokine at the mRNA expression ..................................................... 151

IV. DISCUSSION .............................................................................................................. 157

Chapter 6 CORTICOSTEROID INSENSITIVITY IN HBSMC INVOLVED A
K_{Ca}3.1 CHANNEL-DEPENDENT PP5 MODULATION OF GR\(\alpha\)
FUNCTION .................................................................................................................. 161

I. RATIONALE .............................................................................................................. 162
II. AIM ......................................................................................................................... 163
III. RESULTS ............................................................................................................... 164

1. Fluticasone-induced GR\(\alpha\) phosphorylation at Ser\^{211} is impaired in steroid-resistant states but restored in the presence of K_{Ca}3.1 channel inhibitors . . 164
1.1 Fluticasone induced a dose-dependent GR phosphorylation at ser\^{211}: 164
1.2 K_{Ca}3.1 blockers prevent cytokine-induced impairment of GR\(\alpha\)-phosphorylation induced by fluticasone ......................................................... 166

2. K_{Ca}3.1 blockers prevent cytokine-induced impairment of GR\(\alpha\)-dependent expression of GILZ induced by fluticasone ........................................... 168

3. PP5 expression is induced by TNF-\(\alpha\)/IFN-\(\gamma\) via K_{Ca}3.1 channel dependent pathways ........................................................................................................ 172
3.1 Time course of PP5 expression induced by TNF-\(\alpha\)/IFN-\(\gamma\) ............... 172
3.2 K_{Ca}3.1 channel inhibition suppresses cytokine-induced PP5 expression ...................................................................................................................... 174
3.3 PP5 expression within BSM ............................................................................... 176

VI. DISCUSSION ........................................................................................................... 178

Chapter 7 CONCLUSIONS AND FUTURE DIRECTIONS ................... 180

I. CONCLUSION ............................................................................................................ 181

1. Growing evidence showing the existence of corticosteroid resistance in bronchial smooth muscle in asthma ........................................................................ 181
2. Use of cultured bronchial smooth muscle cells to establish an in vitro model of corticosteroid insensitivity seen in the muscle bundles of asthmatic patients .............................................................. 182

3. KCa3.1 as a potential new target for the treatment of corticosteroid resistance in HBSMC ................................................................. 183

II. FUTURE DIRECTIONS ........................................................................ 191

References
CHAPTER 1

INTRODUCTION
I. ASTHMA

1. Definition and Diagnosis

Asthma is a chronic airway disease and a major worldwide health problem. The term asthma origins form the greek word “asthmaino” (ἀσθημαινω), demonstrating gasping, and the term was first used by Hippocrates (460-377 BC) in the Corpus Hippocraticum [1].

The Global Initiative for Asthma (GINA) defines asthma as a “Chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation is associated with airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread but variable, airflow obstruction within the lung that is often reversible either spontaneously or by treatment” [2].

There is no single test that can ascertain a diagnosis of asthma; symptoms are the primary indicators when the patient is suffering from the respiratory disorder, episodes of wheezing (tachypnea), breathlessness (dyspnea), chest tightness, and coughing. These are the clinical hallmarks of an attack of asthma which occurs particularly at night or in the early morning. The episodes of asthma are usually associated with widespread but variable airflow obstruction which is often reversible spontaneously or with treatment [3]. These episodes take place in 2 phases. The first phase is characterised by an immediate asthmatic reaction that begins 15-30 minutes after antigen exposure. This phase is often reversible spontaneously or with treatment. The second
phase, also called late phase, can begin hours after the exposure to an allergen and can last for several days. Asthma can be accompanied by increased pressure in the chest region which may cause chest pain [4].

Lung function tests such as Peak Expiratory Flow (PEF, 'peak flow') or Forced Expiratory Volume in the first second (FEV1) can support the diagnosis, both of these measurements count on the fact that airflow limitation is directly related to the luminal size of the airways (airway calibre) and the elastic properties of the surrounding lung tissue. As symptoms worsen, the airways get narrower thus making exhalations more difficult, which in turn generate lower scores on the peak flow meter or spirometer. A PEF score or FEV1 reading, then, provides an objective assessment of lung function [5].

2. Asthma is a major health issue

Asthma is a major public health concern worldwide, with an estimated 235 million individuals suffering from this disease, and it is estimated that a further 100 million people will be affected by 2025 and its frequency varies from 1% to 18% of the population in different countries. Worldwide 250,000 people die from asthma each year with a higher mortality in underdeveloped and/or low income countries. In western countries asthma is also a main cause of absence from work and school. Therefore, asthma represents a substantial burden in terms of medical costs (hospital and medication), economical costs and social impact (reduced quality of life, premature deaths, absence from school) [6, 7].

Within the UK, 5.4 million are affected by asthma of whom 1.1 million are children making it the most common long-term childhood condition in this country [8]. In 2010 there were 1,143 deaths from asthma in the UK (16 were
children aged 14 years or under). Approximately 3 people per day or 1 person every 8 hours dies from asthma. Even if some patients have mild forms of asthma that have little influence on their lives, more than half of patients experience serious symptoms [9]. In an international survey, the UK had the highest prevalence of asthma in children aged 13-14 years out of all 56 countries contributing data [10]. It is now widely accepted that the prevalence of asthma has greatly increased in the past 20-30 years, which has been particularly pronounced in children and adolescents [11-13]. There are also considerable financial costs associated with chronic asthma which is costing the United Kingdom £1 billion every year. National Health Service (NHS) estimates the costs due to asthma around £889 million reflecting the use of combination medicine, supply, general practitioner consultations and hospital admissions. Secondary costs comprise the remaining financial burden, including social security benefits and loss in work productivity due to absence. In 2008/09 Asthma UK announced that up to 1.1 million working days were lost due to lung problems or breathing [14, 15].

3. Causes of Asthma

A large body of literature has investigated the factors playing a role in the pathogenesis of asthma. This literature suggests that asthma is affected by both host-dependent and environmental dependent factors.

Host factors relates to genetic background and inheritable traits with the identification of genes associated with (i) increased production of IgE (atopy), (ii) airway hyperresponsiveness and (iii) release of inflammatory mediators such as TNF-α, TGF-β and IL-13.
A number of chromosomal regions have been associated with asthma susceptibility and a co-inheritance of the tendency to produce elevated IgE serum level with airway hyperresponsiveness has been observed [16, 17]. Genomic studies identified 79 genes differentially induced in asthma and healthy controls [18]. However, the expression of these genes is likely to be influenced in a complex interaction with multiple environmental factors. Host risk factors also include obesity and male sex, with male children who present a two-fold higher prevalence for asthma prior to the age of 14 [19]. Environmental factors that are the key players influencing the development of asthma are indoor and outdoor allergens such as house dust mite, dog dander, cat and cockroach allergens or Aspergillus species mold. Exposure to these allergens during childhood (up to 3 years) appears to be critical for developing asthma-like symptoms [20, 21]. Other risk factors are viral infections of the airways during childhood, tobacco smoke [22, 23], occupational sensitizers [24, 25] and the diet [26].

II. PATHOGENESIS OF ASTHMA

1. Airway inflammation – the classical view

In recent years, there has been an increasing amount of literature describing the central role of airway inflammation in asthma pathogenesis. This inflammatory process is a multicellular process involving mainly eosinophils, neutrophils, Th2 lymphocytes, activated mast cells, macrophages, and basophils. In atopic asthma, the airway responds to airborne inhaled allergens by a Th2 response with the release of the typical array of Th2 cytokines (Th2
paradigm) [27]. The dendritic cells in the airway epithelium and the submucosa capture inhaled allergens internalize and process them, and then present them to T lymphocytes. The sensitized T cells then produce cytokines, in particular the interleukins IL-4, IL-5, IL-6, IL-9 and IL-13, which can be found elevated in the bronchoalveolar lavage (BAL) fluid and in the serum of asthma patients indicating a predominantly Th2 mediated inflammatory response [28, 29]. The cytokine “shower” leads to a recruitment of secondary effectors cells such as macrophages, basophils and eosinophils into the airways while some cytokines such as IL-4 promote the immunoglobulin isotype switching of B cells towards IgE synthesis (Figure 1.1).

Figure 1.1: Initiation of the inflammatory cascade after allergen (pollen) inhalation and interaction with airway cells (taken from [30].)
The early inflammatory response to allergen exposure is mast-cell dependent with mast-cells infiltrating the mucosa and the deeper airways [31]. Remarkably mast cells and bronchial smooth muscle are both increased in small and large airway in chronic asthma [32]. Mast-cells are activated after binding of IgE to the high affinity IgE receptor (FcεRI) leading to the release of TNF-α, IL-4 and IL-5. Mast cells activation then acts on HBSM by the release of the contractile mediators leukotriene (LT)D4, prostaglandin (PG)D2 and histamine, which act as powerful bronchospasm agents [31, 33]. HBSMC can also release stem cell factor, other chemokines, cytokines, and growth factors such as SCF, CXCL8, and CXCL10 that all may play a role in the recruitment, differentiation, and retention of mast cells within the airways [34, 35]. Other mast cell mediators such as typtase, may also contribute to airway remodelling through its action on protease-activated receptor type 2 (PAR2). PAR2 is present on many cell types, including epithelial cells, fibroblasts, BSM, and inflammatory cells [36] and its activation induces HBSMC proliferation [37] or bronchoconstriction by stimulating muscle contraction [38].

Eosinophils have been identified as the major contributing cell population implicated in allergic asthma and it is believed to be fundamental to airway dysfunction in asthma [39]. Airway eosinophilia is a constant finding in patients with allergic asthma [40], with the exception of a subclass of patients with severe asthma who showed a mixed eosinophilic and neutrophilic inflammation, or neutrophilic inflammation alone [41]. Evidence implicating eosinophils in asthma include the increased numbers that associate with disease severity [40], and the dramatic decrease in sputum and tissue
eosinophils on treatment of asthma with inhaled or oral glucocorticoids which is associated with clinical improvement [42-44].

It is commonly believed that the Th2 cells release IL-5, contributing to the maturation of the eosinophils from CD34+ precursors [45]. Eosinophils are involved in driving airway inflammation and remodelling through the expression of many chemokines, cytokines and other mediators. Normal T cell expressed and secreted (CCL5) and CXCL-8 for example promote recruitment of more eosinophils and other inflammatory cells. A host of cytokines may be released by eosinophils [46], which not only have proinflammatory activities on other structural and inflammatory cells within the airways, but may also increase eosinophil survival [47]. Furthermore the eosinophils play a role in airway remodelling by inducing a variety of cytotoxic mediators, including major basic protein (MBP), eosinophil peroxidise (EPO), eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN), that are stored in granules and released upon activation. Eosinophils and their mediators have been shown to cause direct damage to the epithelial layer [48, 49], causing epithelial detachment and cell lysis and thereby contributing to airway remodelling. Furthermore, MBP causes increased HBSM hyperreactivity in vitro [50], and its levels in BALF associate with airway hyperresponsiveness [51]. Eosinophils attach to epithelial cells through CD18 dependent mechanism regulated by the local cytokine environment [52, 53]. This adhesion causes degranulation and together with T cells induces epithelial apoptosis which may be a contributing factor to the epithelial damage [54].
In recent years, there has been an increasing amount of literature on the role played by other inflammatory cells; the exact role of neutrophils, monocytes and basophils in the pathogenesis of asthma is remaining unclear. It has been found that neutrophils are found in increased numbers in airways and it has been argued that neutrophilic inflammation in severe asthmatics is secondary to treatment, as the majority of these patients take long term oral glucocorticoids, which increase neutrophil survival. A counterargument to this is that the controversial steroid insensitivity of neutrophils may explain the poor response to corticosteroids seen in refractory disease. Interestingly, Ordoñez et al showed significantly elevated levels of neutrophils, and the neutrophil chemokine CXCL8, in the tracheal aspirate of patients intubated for acute severe asthma, compared to patients intubated electively for non-pulmonary surgery; only five of the ten patients studied were taking inhaled corticosteroids or maintenance oral steroids [55], supporting the theory that neutrophilic inflammation is a primary characteristic of severe asthma rather than a consequence of therapy. Additionally, neutrophil numbers in severe asthma has been suggested as clinical marker of airway damage and reduced glucocorticoid therapy responsiveness [56-58]. It is believed that this neutrophilic phenotype is related with more severe disease resulting in greater tissue damage and airway remodelling. Mucus hypersecretion have been identified as a major contributing factor which has been associated with airway narrowing and accelerated decline in FEV1 in chronic asthma. Neutrophils may also contribute to this through secretion of neutrophil elastase which is a potent secretagogue for submucosal gland and goblet cells [59].
2. **Airway hyper-responsiveness**

Airway hyper-responsiveness is defined as an abnormality of lung function resulting in excessive airway narrowing in response to different stimuli (exogenous and endogenous) that provoke little effect in healthy individuals [60]. In consequence, asthma patients suffer from *airflow limitation*. Airway hyper-responsiveness is linked to both airway inflammation and airway remodelling and is partially reversible by bronchodilators that act by relaxing the bronchial smooth muscle. Airway hyper-responsiveness can be explained by excessive contraction of the HBSM which is further enhanced by the thickening of the airway wall whereby small changes in the airway wall thickness that have a little effect on baseline airway resistance can markedly increase the airway responsiveness to inhaled particles [61]. Clinically, the presence of bronchial hyper-responsiveness can be demonstrated with a bronchial challenge test using methacholine or histamine [62]. This test has been used for the diagnosis of asthma.

3. **Airway remodeling**

In addition to inflammation and bronchial hyper-responsiveness, remodelling of the airways is also a main histopathological feature of asthma. Remodelling is believed to be the consequence of an abnormality of the dynamic process of the wound repair process that leads to the reconstruction of the airway wall. In asthma airway remodelling is defined as increased thickening of the airway wall due to numerous structural modifications [63]. These modifications which could have deep physiological consequences in asthma are summarized in Table 1.1.
Table 1.1: Structural alterations contributing to airway remodeling

Thickening of the layer below the basement membrane in subepithelial lamina reticularis or reticular basement membrane (RBM) is a morphological hallmark of asthma. It is due to the increased deposition of collagen I and III, tenascin, and fibronectin [66] but not of laminin. These proteins are believed to be produced by activated myofibroblasts [72]. The degree of subepithelial fibrosis correlates with the severity [73] but not the duration of disease [74]. The thickening of the airway wall is increased in atopic asthma than nonatopic subgroup of asthma. Airway thickening has been positively associated with airway hyper-responsiveness, the frequency of asthma attacks, and the numbers of fibroblasts and "myofibroblasts" adjacent to the RBM [72, 75]. The relationship between airway remodelling and epithelial cells has been widely investigated. Epithelial abnormalities which include epithelial desquamation and goblet cell hyperplasia represent important histological characteristics of asthma. In addition, studies have demonstrated an increased number of
epithelial cells in the bronchoalveolar lavage fluids, and loss of the surface epithelium in biopsy specimens [64, 76], leading to the possibility that disturbance of the defensive and protective epithelial layer results in alterations in host defences and increased susceptibility to infections and stimulation [65, 70]. It has been suggested that an intrinsic weakness and fragility of the epithelial layer in patients with asthma contributes to epithelial desquamation. This hypothesis is supported by the observation that the columnar epithelium lining the bronchi of asthmatic patients has less contact area with the basal lamina compared to healthy controls [77]. Furthermore, studies using electron microscopy have also shown a decrease in epithelial cell desmosome length in asthmatic patients compared with healthy controls [78].

Previous studies have reported that epithelial damage occurs in vivo. Lackie et al found that expression of the adhesion molecule CD44 localized by light- and electron-microscopic immunocytochemistry cells was increased in the epithelium of asthmatic patients compared with healthy controls. Other studies have showed increased levels of staining for epidermal growth factor receptor (EGFR), involved in epithelial repair processes (a marker of epithelial activation/injury), in the bronchial epithelium of asthmatic patients compared to healthy control subjects [79, 80]. In vitro studies showed that epithelial damage caused increased fibroblast activation through release of growth factors including fibroblast growth factor (bFGF), platelet derived growth factor (PDGF), ET-1 and TGF-β [81, 82]. Therefore, the airway epithelium enters into a chronic “wound situation” that leads to the development of an abnormal epithelium-mesenchymal interaction ending in the release of pro-inflammatory cytokines.
and growth factors, such as epidermal growth factor (EGF) [79] as well as the epithelial stress, independently, to some degree, of inflammation. All these autocrine factors play a key role in the development of airway remodelling in asthma. Asthmatics patients also show a high numbers of goblet cells that secrete viscous mucus and a profound decrease of the ciliated cells. A number of studies have found that the goblet cell hyperplasia to be much more marked in patients who have died from acute severe asthma [83]. However, another study found the presence of similar variations in the airway epithelium of patients with mild to moderate asthma [84]. The mucus composition is changed with an increase of the 5AC mucin that is responsible for the high viscosity and problematic sputum expectorations in asthma [85].

Mucus hypersecretion is associated with accelerated decline in FEV1 in both non-smokers and smokers with asthma [86]. This augmented mucus secretion has been relatively undervalued in asthma compared with airway inflammation. However, mucus plugging contributes to several key features in asthma including airflow limitation, airway hyperresponsiveness and overall morbidity and mortality in asthma.

Increased vascularity has been described as another key feature of airway wall remodelling in asthma by several studies showing an increased number and size of bronchial vessels in patients with asthma compared with normal controls [69, 87]. A number of angiogenic factors are believed to be involved in these vascular modifications seen in asthmatic airways, with vascular endothelial growth factor (VEGF) and the angiopoietins, Ang1 and Ang2 as being key players. Ang1, acting at its receptor Tie2, is crucial for vessel
maturation and development [88], and is widely expressed in normal tissues [89]. Ang2 is in some perspectives a natural antagonist of Ang1. Ang2 is induced essentially at sites of vascular remodelling [89]. VEGF levels were found to be elevated in the BALF of asthmatic subjects [90]. Furthermore levels of VEGF and Ang1 were shown to be markedly higher in bronchial biopsies from patients with asthma compared to healthy controls. In contrast no changes in the VEGF receptors, VEGFR1 and 2 were observed [90]. Additionally, inhaled corticosteroids (ICS) reduced VEGF but not Ang1 staining in the airways of asthmatics treated with ICS compared with placebo [91]. Further, the increased vascularity may facilitate influx of inflammatory cells and therefore expression of inflammatory mediators [92]. The increase in vasculature may also contribute to AHR by supporting the airway smooth muscle layer, which is thickened in asthmatic airways [93].

In addition to increased vascularity of the airways, alterations in the extracellular matrix (ECM) participate to the thickening of the airway wall. Several studies showed that the ECM is altered in asthmatic patients compared to that of healthy subjects and is a consequence of increased deposition of collagen I, III,V, fibronectin, tenasin, hyaluronan, versican and laminin [94, 95], whereas collagen IV and elastin deposition is decreased [96]. Increased ECM deposition is believed to result from the imbalance between the activity of Matrix metalloproteases MMPs and tissue inhibitors of metalloproteases TIMPs which may be altered in asthma [87]; mucosal biopsies from asthmatic patients have shown enhanced MMP-9 mRNA expression in eosinophils [97], and enhanced MMP-2 and MMP-9 levels have been detected in sputum from
asthmatics [98, 99]. In addition to granting support to the airway tissue, ECM proteins have been shown to modulate HBSMC cells, epithelial cells and fibroblast development, migration, and proliferation [69].
III. BRONCHIAL SMOOTH MUSCLE IS AN IMPORTANT PLAYER IN THE PATHOGENESIS OF ASTHMA

Figure 1.2: Airway of a healthy control (left panel), a patient with mild-to moderate asthma (middle panel), and a patient with asthma who died of status asthmatics (right panel). The asthmatic airway presents a thickening of the basement membrane (1), and an increased mass of smooth muscle cells (2). [100]

There has been major progress in the field of bronchial smooth muscle (BSM) biology over the last few years. Numerous studies have suggested that BSM could play a significant role in the pathogenesis of asthma, not only BSM is responsible for the acute episodes of airway obstruction, but also because BSM is now seen as a source of various pro-asthmatic mediators such as cytokines, chemokines and growth factors, which act either in a paracrine or autocrine mode [101-103].

In addition to regulating the local airway inflammation, these inflammatory mediators can also enhance the contractile properties of BSM
leading to an increase sensitivity of the airways known as airway-hyper-responsiveness [104]. A recently developed method known as bronchial thermoplasty has confirmed the causal relationship between BSM and impaired lung function in severe asthmatics [105]. This technology is based on the release of thermal energy via a bronchoscope to remove specifically the layer of BSM [106]. The effect of thermoplasty has been assessed for a 2 years period on asthmatic patients suffering from mild to severe asthma. The results showed that a reduction in BSM mass led to airway dilatation and a significant reduction of airway hyper-responsiveness. However, the long-term side effects of this treatment are still to be ascertained [107].

1. **Potential mechanisms by which BSM may contribute to asthma pathogenesis**

1.1 **Airflow obstruction via an increase in BSM mass**

Many studies have shown that there is an increase in BSM mass in the airways of patients with asthma compared to healthy subjects. The first study exploring the amount of BSM in asthmatic airways dates back to 1922 [108]. This study compared the airways of patients with fatal asthma to those of patients who had died suddenly of non-respiratory conditions. This study showed an increase in smooth muscle layer thickness in the asthmatic group. Later studies have confirmed the presence of this finding in patients with fatal asthma [71, 109]. This increase in BSM thickness forms part of the remodelling process observed in asthmatic airways, which seems to correlate with disease severity. Many potential mechanisms could participate to the excessive BSM
mass within the remodelled airways of asthmatic patients. Increased BSM cell size (hypertrophy) has been reported in specimens from fatal [110, 111], severe [112, 113] and allergic asthma [114].

Similarly, the literature is disagreeing regarding whether the increased BSM seen in asthmatic airways is due to hypertrophy or hyperplasia. Woodruff et al showed an increase in cell number but not size in the airways of patients with mild to moderate asthma compared to normal controls [115]. In contrast to Woodruff study, Benayoun argues that BSM cells were increased in size across all asthma groups, those with intermittent, persistent mild-to-moderate and persistent severe asthma [112]. An additional study defined two subgroups of asthmatic patients, one in whom only hyperplasia of the larger bronchi was detected, with no evidence of cellular hypertrophy, and a second group in whom hypertrophy dominated and was observed at all levels of the bronchial tree, with only mild hyperplasia seen [111]. The quantity of BSM in the airway is likely to be determined by the balance between cell proliferation and cell death. A number of growth factors such as FGF(b) and TGF-β have been shown to promote BSM proliferation in vitro, several of which are found in elevated amounts in the asthmatic airway [116].

Another mechanism which is less well studied area is BSM cell apoptosis. Freyer et al showed that various components of the extracellular matrix (ECM) such as fibronectin and collagen I, which are known to be deposited in increased amounts in asthmatic airways [87], inhibit BSM cell apoptosis and increase cell survival which may in turn be responsible for the increase in BSM mass [117].
Another potential contributing factor towards the increased smooth muscle mass observed in the airways of asthmatics is that smooth muscle progenitors may migrate from the blood to the smooth muscle bundles. Mast cells infiltration within the BSM bundles could promote smooth muscle migration. BSM migratory mast cell derived stimuli have been described by different studies and include interleukin-1β (IL-1β), TGF-β [118], PDGF [118, 119], basic fibroblast growth factor (bFGF) [120], chemokine ligand 11 (CCL11) [118], and CCL19 [31].

1.2 Airway hyper-responsiveness via an abnormal BSM contractile function

Airway hyper-responsiveness can be explained by either excessive contraction of the BSM or thickening of the airway wall or both phenomenons. Most evidence supporting an alteration of BSM contractile function comes from in vitro studies. Using isolated airway preparations from tracheal and bronchial rings different species studies have demonstrated that the incubation of airway tissues with inflammatory stimuli (including cytokines, or asthmatic serum) can dramatically affect the sensitivity of BSM to contractile agonists and may augment BSM contraction by affecting intracellular calcium signalling, or other pathways including increased RhoA/Rho kinase pathway activation, persistent β2-adrenoceptor activation or increased phosphodiesterase 4 expression [121-123]. These studies demonstrate that the increased airway narrowing seen in asthmatic patients could result from inflammation-dependent changes in BSM contractility, possibly as a consequence of altered calcium signalling in the BSM
itself. It remains unclear whether asthmatic BSM has an intrinsic difference in contractile properties.

1.3 Airway remodelling via increased BSM migration

Cell migration could play a role in BSM hyperplasia and remodelling in asthma. BSM cells migrate in response to different mediators including cytokines [124] as well as the composition of ECM proteins such as collagen III, V and fibronectin [125]. PDGF also stimulates the migration of BSM cells by modifying the balance of MMPs and TIMPs [126]. Some chemokines such as CCR3 ligand eotaxin, CCL5 or CXCL8 (produced by the epithelium) can also promote migration of BSM cells in vitro [124, 127]. Studies of BSM migration are still in their beginning and it remains unclear BSM migratory properties play any role in the increased BSM mass seen in asthmatic airways. However, if cell migration does indeed make a significant contribution to airway remodelling, this would provide an exciting new target for therapeutic intervention in asthma.

1.4 Airway inflammation via the increased production of inflammatory mediators by BSM

Over the last decade or so, it has become clear that BSM cells could represent key contributors to the inflammatory and remodelling processes seen in the asthmatic airways [128, 129]. Inflammatory mediators may be produced by many resident cells within the airways as well as by cells that have infiltrated into the airway from the circulation which include eosinophils, mast cells, macrophages, neutrophils and lymphocyte [130, 131]. A number of in vitro
studies have indeed shown that BSM secrete a variety of different inflammatory mediators such as chemokines.

BSM cells are capable of secreting transforming growth factor-β (TGF-β) and stem cell factor (SCF), which are chemotactically attractive for mast cells [132], interleukin-8 (IL-8), which recruits neutrophils [133] and Regulated Upon Activation Normal T cell Expressed and Secreted (RANTES, or CCL5) and CCL11, which recruits eosinophils [134]. These studies suggest that BSM also contribute to the increased numbers of inflammatory cells infiltrated within the asthmatic airways when compared with non-asthmatics [32, 114].

Furthermore, BSM cells from asthmatics have been shown to produce different ECM proteins compared to BSM cells from non-asthmatics; they produce more perlecan (PLC) and collagen I and less laminin-alpha1 and collagen IV [135]. Augmented ECM proteins expression by asthmatic BSM cells was shown to be the result of increased production of connective tissue growth factor (CTGF) [136]. This altered ECM proteins produced by the asthmatic BSM cells enhanced the proliferation rate of BSM cells (asthmatic or non-asthmatic), indicating that BSM cells, by producing ECM proteins, may themselves accentuate the remodelling of BSM in asthma. Indeed, ECM proteins control the proliferation, synthetic capacity and phenotype of BSM cells.

BSM cells express various adhesion molecules, both constitutively and in response to several pro-inflammatory mediators which facilitates airway infiltration by inflammatory cells [137]. For example, BSM stimulated with TNFα upregulates VCAM-I, ICAM-I and CD44 which enhances neutrophil adherence to BSM [138, 139]. Finally, several chemokines and cytokines produced by
BSM also have angiogenic properties. For example, BSM produces GM-CSF [140], CXCL8 and CCL11, all of which have angiogenic potential [141-143]. Furthermore matrix metalloproteinases have an essential role in remodelling the ECM to allow endothelial cell invasion and association to form nascent blood vessels [144].

2. Treating asthma means treating Bronchial Smooth Muscle

As discussed previously in asthma, one of the key end-effector of airway obstruction is BSM [102, 121, 123]. BSM also participate in asthma pathogenesis via different mechanisms including via the regulation of Inflammation, airway hyper-responsiveness and remodelling of the airways. Targeting pathways in BSM such as those involved in its “secretory function” could offer a novel alternative approach to treat asthmatic patients.

One approach to treat BSM contraction is through targeting Myosin light chain Kinase (MLCK), a key enzyme in BSM contraction via the phosphorylation of the MLC20 [145]. Several studies showed that MLCK expression is upregulated in models of asthma and in human asthmatic BSM [112, 146]. Targeting Rho kinase pathways has been also described as another therapeutic approach to relax BSM contraction by the reduction of MLC20 phosphorylation [147].

Overcoming airway smooth muscle remodeling through targeting growth factors [148], and/or cell cycle regulator (anti c/EBPα) [100]. Overcoming airway smooth muscle inflammation through targeting the synthesis function of BSM [149]. Together these studies provide important insights into the use of BSM
features to treat patients may lead to the development of better therapeutic strategies which can be summarized in the Figure 1.4.

**Figure 1.3: Potential therapeutic targets in BSM.** Different signaling pathways reported in the literature by various groups regulate key pro-asthmatic responses in BSM that include airway remodeling, contraction and inflammation. These signaling molecules could represent novel therapeutic targets for the treatment of asthma.
IV. ASTHMA THERAPIES

Asthma does not have a specific curative therapy because of its complex etiology. However, asthmatic episodes are controlled by treatments targeting various aspects of this disease. Asthma therapy is planned according to the disease severity and based on different guidelines (BTS and GINA) [150]. A mixture therapy which has been in use for several years which consists of the anti-inflammatory drugs corticosteroids and the bronchodilators short or long-acting β2-agonists [151].

1. β2-receptor agonists therapy

Worldwide, β2-adrenergic agonists represent the treatment of choice to treat bronchoconstriction that occurs in asthma and COPD patients. β2-agonists can be administered by both ways inhalers or orally. These drugs activate specifically β2 receptors on the BSM surrounding the airways to evoke a rapid bronchodilatation [152], which helps to relieve the asthma symptoms like dyspnea (shortness of breath). The action of β2-agonists starts within minutes after inhalation and lasts for about ~4 hours for short acting β2-agonists (SABAs) in contrast to the long-acting β2-agonists (LABA) which confer bronchoprotection for ~12 hours. At the cellular level, β2-agonists activate the receptor-coupled adenylate cyclase which leads to an increase in cyclic adenosine 3’5’-monophosphate (cAMP) levels by the signal-transducing Gs, finally activating the protein kinase A (PKA) cascade. This leads to BSM relaxation and bronchodilation through the inhibition of myosin light-chain kinase activity and opening of Ca²⁺ channels [153].
Because of their rapid onset of action, SABAs such as albuterol and terbutaline are used as reliever for patients who experience an acute shortness of breath but, because of their short duration of action, several doses of β2-agonists are often necessary each day. Depending on the severity and the control of asthma, patients are also treated with LABAs which are used as controller medicine. LABAs such as salmeterol or formoterol exhibit a slower onset of action but a longer duration of action (12 hours) and need only be taken twice a day. LABAs are mostly prescribed for disease control in combination with steroids. The common side effect of β2-agonist treatment include anxiety, tremor, palpitation, and low blood potassium [151].

2. Corticosteroid therapy

2.1 Why are corticosteroids so important in asthma?

Corticosteroids are a group of steroid hormones that mediate their actions through binding at the glucocorticoid receptor. In health, glucocorticoids have a relevant and extensive role in physiological homeostasis. This comprises effective anti-inflammatory activity that is a critical component of the negative feedback response to stress [154]. In diseases, steroids have been used for the treatment of various inflammatory conditions including asthma since early 1949 [155]. Since that time corticosteroids have become the cornerstone of asthma management due to their consistent ability to reduce airway inflammation and hyper-responsiveness, control asthma symptoms and improve in lung function [156]. Like β2-agonists, corticosteroids can be prescribed as inhaler and oral medicine. The inhaler corticosteroid fluticasone propionate (FP) has been developed with better pharmacological profile (lesser
systemic side effects due to low oral bioavailability and high potency) and represents the first line therapy in the United States and UK. FP treatment has been shown to improve pulmonary function and asthma symptoms [157].

Inhaled steroids have few side effects, especially at lower doses. Oral candidiasis, a fungal infection of the mouth and throat, can be seen in patients taking high doses of inhaled corticosteroids. The risk of developing oral thrush is reduced if the patient rinses their mouth out after taking their inhaler [158]. Overall, the BTS and SIGN 2003 guidelines, showing high quality research, determine that "Inhaled steroids are the most effective preventer drug for adults and children for achieving overall treatment goals" [12].

2.2 Anti-inflammatory mechanisms of glucocorticoids

The mechanisms of corticosteroid action have not been fully understood. Corticosteroids enter the cell via passive diffusion through the plasma membrane where it binds to its main glucocorticoid receptors (GRs). Unbound glucocorticoid receptor (GRα) is found within the cytoplasm in an inactive oligomeric complex, coupled to molecular chaperones. Ligand interaction with the receptor leads to a conformational change of GRα allowing its dissociation from the chaperone proteins (HSP-90). Activated ligand-GRα complex translocates to the nucleus where it binds as a dimer to DNA on Glucocorticoid Response Elements (GRE) to positively or negatively regulate gene transcription. Within the nucleus, the complex exerts a number of different effects on gene transcription activity that are not fully investigated. In broad terms, glucocorticoid mechanism of action may be divided into two types [159]:

26
i. **Direct regulation of gene transcription or trans-activation.**

Glucocorticoid receptor homodimers interact with specific DNA sequences, called glucocorticoid responsive elements (GREs) and regulates GRE specific gene transcription [156, 160, 161]. This transactivation requires the interaction of GR-\(\alpha\) with different transcriptional co-activator molecules such as GRIP-1, pCAF and CREB binding protein (CBP). These proteins have intrinsic histone acetyl-transferase (HAT) activity and thus can induce histone acetylation, which unwinds DNA and increases gene transcription. Inflammatory genes activated by corticosteroids include the anti-inflammatory proteins such as IL-10 or MAP kinase phosphatase-1 (MKP-1) which inhibits mitogen-activated protein kinase (MAPK) pathways, glucocorticoid inducible leucine zipper (GILZ) which interferes with the function of AP-1 and NF-kB [162], the inhibitor of NF-κB (IκB), all of which have been involved in repressing the expression of pro-inflammatory genes, such as IL-8, TGF-\(\beta\) [163]. It is believed that most of the side effects such as cataracts, osteoporosis, growth retardation, metabolic effects and fragility seen with at high doses of corticosteroid therapy have been linked to transactivation function of corticosteroids [164].

ii. **Indirect regulation of gene transcription, also known as trans-repression.** Transrepression describes the suppression the transcriptional activity of different pro-inflammatory genes by the glucocorticoid-GR\(\alpha\) complex as monomers. Typically, transrepression usually occurs at much lower doses of glucocorticoid than those required for mediating transactivation and produces a broader anti-inflammatory effect. It is also
considered to be the *main anti-inflammatory mechanism* of glucocorticoids [163]. The inhibition of pro-inflammatory gene expression occurs via different mechanisms that are activated in a cell-, stimuli and tissue-dependent manner:

*Direct inhibition.* The complex glucocorticoid-GRα physically interacts with different inflammatory transcription factors such as NF-κB and AP-1, both of which are usually activated in the airways of asthmatic patients and COPD patients [165].

*Indirect inhibition.* The inhibition results from the induction of chaperone proteins that prevent activation of inflammatory transcription factors such as the NF-κB chaperone IκB [163, 165]. More recently, Amrani’s group showed that corticosteroid also inhibits the expression of inflammatory genes via the suppression of an additional transcription factor called Interferon-Regulatory Factor-1 (IRF-1) [166].

*Co-factor competition.* Competition for common transcriptional co-activators such as the glucocorticoid receptor interacting protein 1 (GRIP1) required by both the GRα and inflammatory transcription factors can also reduce inflammatory gene expression [167].

*Epigenetic mechanisms.* The GR-α can change the behaviour of inflammatory transcription elements through actively recruiting multiprotein complexes capable of adding or removing covalent compounds such as acetyl and phosphate groups. This activity alters their affinity for their response elements and hence their behaviour, trans-repressor activity is mediated primarily by interference with chromatin remodelling. Chromatin is an active
structural matrix that modulates DNA conformation and has a relevant physical role in transcriptional regulation. Glucocorticoids alter the balance of enzyme activity in favour of histone deacetylation via the recruitment of Histone deacetylase (HDAC)2 and inhibition of Histone acetyltransferase (HATs) [160, 163, 168].

iii. Non-genomic mechanisms: non-genomic refers to several non-specific mechanisms that are indirectly related with gene transcription and do not require nuclear GRα-mediated transcription or translation. These actions are believed to be mediated by the activation of signal transduction pathways such as MAPK pathway [169]. It has been reported that high concentrations of glucocorticoids can promote instability of some pro-inflammatory mRNA resulting in increased degradation of inflammatory gene complex expression such as VEGF [170]. However, the clinical relevance of these mechanisms in vivo is unclear.

3. Other drugs for asthma:

In recent years, there has been an increasing interest in developing a new therapeutics that successfully target many other features of asthma pathogenesis. Anti-IgE antibodies (e.g. Omalizumab) targeting the allergen-specific IgE which is the key mediator of the inflammatory allergic reactions are successfully used in the control of severe allergic asthma [171]. Omalizumab binds to the region of IgE that binds to the high-affinity IgE receptors (FceRI) on inflammatory cells such as mast cells and basophils, thus preventing the IgE-mediated degranulation of these cells. Clinically, omalizumab therapy has been associated with a reduction of the risk of exacerbations and hospitalization by
improving lung function in patients with severe persistent asthma [172, 173]. Leukotriene receptor antagonists (LTRA) (e.g. montelukast) represent another powerful treatment recommended for a better asthma control. LTRAs act by blocking the action of leukotriene C4, D4 and E4 at the receptor which are thought to play a role in bronchoconstriction and mucus secretion [174, 175]. Other novel therapeutics are still being evaluated in clinical trials include Pitrakinra a recombinant proteins that binds IL-4 receptor [176], suplatast tosilate a Th2 cytokine inhibitor that prevent the synthesis of IL-4 and IL-5, [177]. Other examples include Etanercept (a fusion protein) or infliximab (an monoclonal antibody) which blocks TNFα action have shown mixed results in asthmatic subjects [178]. Recently Pavord’s Group suggested that Mepolizumab (anti IL-5) therapy could reduce exacerbations and improve AQLQ scores in patients with refractory eosinophilic asthma by reducing the circulating and sputum eosinophils (>80%) [179, 180].

V. CORTICOSTEROID INSENSITIVITY IN ASTHMA

Whilst the majority of patients with asthma can be controlled with inhaled therapies described above, a small percentage of patients (about 10%) fail to show the expected improvements in response to corticosteroids therapy; these patients have the worst prognosis with a number of exacerbations and/or hospitalizations [9]. These corticosteroid-insensitive patients consume more than 50% of the total health care costs related to asthma and are at high risk of dying from the disease and/or from side effects when high doses corticosteroids are used [163]. As well as their anti-inflammatory effects, steroids have a role in
many metabolic processes. Thus long term steroid treatment in patients with poor steroid sensitivity can cause serious systemic side effects that might be life threatening.

Clinically, glucocorticoid insensitivity in asthma can be defined as the failure to demonstrate an increase in baseline FEV1 by greater than 15% after a course of oral prednisolone 20 mg daily for the 1st week followed by 40 mg daily for 2nd week [181].

1. Potential mechanisms of reduced corticosteroid sensitivity in asthma

   A number of studies have tried to understand the molecular mechanisms underlying corticosteroid insensitivity in asthma. Different groups, including the renowned Adcock’s group, made significant findings about the potential causes of corticosteroid insensitivity. Most studies in asthmatics have focused mostly on immune cells (PBMC, alveolar macrophages, T cells). Below is a brief description of some of the proposed mechanisms:

   a) Defects in ligand binding/GRα number

   This phenomenon might be due particularly to IL-2, IL-4 and IL-13 which are overexpressed in patients with steroid-resistant asthma. Sher et al were able to show two patterns of ligand-binding abnormalities in patients with corticosteroid resistance; the first defect, termed type 1, was the most commonly found specifically in T cells (15/17 patients) where there was a marked decrease in GRα binding affinity which was reversible after 48 hr in culture media. The type 2 defect was rare (2/17 patients) and an irreversible phenomenon present not only in T cells but in all mononuclear cell population
was characterized by a low number of GRα [182]. The likely mechanisms explaining the defect in ligand binding to GRα include an increased expression of the dominant negative isoform of GRα called GRβ which has been shown to block GRα in various cell types including peripheral blood cells [183]. Other mechanisms include increased GRα phosphorylation on various inhibitory serine residues (serine 226) by p38MAPK, raising the possibility that p38 MAPK inhibitors may have a role in restoring steroid sensitivity in corticosteroid resistant asthma [184].

b) **Defects in GRα nuclear translocation and GRα-DNA binding**

Investigations also showed that asthmatic patients with corticosteroid resistance had a low level of GRα-DNA binding interactions compared to that seen in patients with corticosteroid sensitive and non-asthmatic individuals after stimulation of PBMCs with dexamethasone [168]. Alteration in GRα-GRE binding has also been connected with increased levels and/or activation of the pro-inflammatory transcription factor AP-1 and subunit JNK and c-Fos in response to inflammatory stimuli, such as TNF-α [185]. Some studies found no change in the binding affinity of GRα but the number of GRα available for DNA binding was reduced in asthmatic patients with corticosteroid resistance [186]. In some patients, the nuclear localization of GRα was normal, however their corticosteroid insensitivity can be explained by a defect in histone H4 acetylation that normally enables GRα to stimulate anti-inflammatory proteins [186]. Other studies found corticosteroid resistant patients have increased levels of MAPK proteins (p38 and JNK) which inhibit GRα function via direct
phosphorylation on serine 226 residues [187]. The expression of p38MAPK-γ isoform was also found to be increased in PBMCs from individuals with severe asthma, and knockdown of this γ-isoform prevented corticosteroid insensitivity induced by IL-2 and IL-4 combination. c-Jun N-terminal kinase (JNK) was also shown to impair corticosteroid sensitivity via the phosphorylation GRα on Ser226 in HeLa and COS-7 cells [188, 189]. JNK1 was involved in GR phosphorylation in PBMCs from individuals with severe asthma [190, 191].

c) Cross-talk with the transcription factor AP-1

PBMCs isolated from corticosteroid resistant patients show increased levels of AP-1 DNA binding in the nuclei as well as a failure of GRα to repress AP-1 level [192]. Increased activation of AP-1 could be due to the exaggerated activity of the upstream pathways c-fos and JNK in patients with CSR [193].

d) Cellular mechanisms

Neutrophils are involved in the development of corticosteroid resistance in asthma. Their survival is augmented in severe asthma patients and apoptosis inhibited by corticosteroid treatment [185, 194]. This is related with high constitutive levels of GR-β in circulating neutrophils and decreased production of GR-α upon entering the airway [195]. This particular modification may make airway neutrophils especially insensitive to corticosteroid treatment. Continued exposure to neutrophil promoting factors may facilitate neutrophil tissue damage and further oxidative stress induced modifications to corticosteroid responsiveness [181].
Th17 cells and their cytokines have also been implicated in corticosteroid insensitive asthma. IL-17, a hallmark cytokine of Th17 responses is increased with neutrophilia in asthma [196]. Th17 cells are insensitive to corticosteroid both in vitro and in vivo. Additionally, Th17 cell transfer decreased responsiveness to corticosteroids in animals previously sensitive to their effects [197]. Whether IL-17 represents a distinct pathway of corticosteroid insensitivity or can occur alongside the other potential mechanisms is unknown. IL-17 increases the production of GR-β in airway epithelial cells to a greater extent than in normal cells and is not suppressed by corticosteroids in vitro [198, 199]

2. Other factors contributing to glucocorticoid resistance

Cigarette smoking is known to reduce dramatically the therapeutic response to inhaled and oral corticosteroids [200, 201]. Cigarette smoke causes oxidative stress and, remarkably, markers of oxidative stress are also enhanced in severe corticosteroid resistant asthma [202]. These findings suggest that oxidative stress may contribute to steroid resistance. These findings have been supported by further studies showing that the level in exhaled breath condensate of one marker of oxidative stress, 8-isoprostanone, was unchanged by treatment with inhaled corticosteroids in children with asthma [202]. Cigarette smoking is associated with increased levels of corticosteroid insensitive sputum cytokines such as IL-6, IL-7 and IL-12 [203]. Modifications in the levels and activity of HDACs can probably reduce steroid responsiveness in association with cigarette smoking. In smokers with COPD, HDAC2 expression and activity is reduced in BAL fluid macrophages [204] and overexpression of HDAC2 in BAL fluid macrophages obtained from GC-insensitive COPD Patients restored
GC function [205]. Some studies have suggested that rhinoviral infection and bacterial infection can decrease GRα nuclear translocation and reduced corticosteroid function in severe asthma patients [206, 207]. Thus, whilst these in vitro findings enhance our understanding of the possible causes driving steroid insensitivity in asthma (summarized in Figure 1.4), there is no clear consensus among experts in the field about the mechanisms and factors responsible for causing steroid resistance in patients.
Figure 1.4: Diagram summarizing the potential mechanisms of corticosteroid insensitivity in asthma: Several environmental stimuli such as cigarette smoke, viral and bacterial infections have been reported to impair corticosteroid therapy in asthma. Although the mechanisms have not been clearly established, studies using immune cells from asthmatic patients showed that corticosteroid insensitivity could be due to decreased GR-α expression, decreased affinity of ligands for GR-α, decreased ability of GR-α to bind DNA, increased expression of pro-inflammatory transcription factors such as NF-κB, AP-1, IRF-1, and decreased expression and activity of co-repressor proteins or inhibitory proteins such as HDAC-2 and Iκ-B. Recent evidence also showed that Th17 lymphocytes via production of IL-17 or up-regulation of GR-β in neutrophils may also contribute to the decrease in cell sensitivity to corticosteroids.
VI. CROSS TALK BETWEEN TNF-α AND IFNs IN THE PATHOGENESIS OF ASTHMA

1. TNF-α is key player in asthma

Tumour necrosis factor-alpha (TNF-α) is a relevant cytokine in the innate immune response and it is the most broadly studied cytokine member of TNF super families. It is a 17 kDa protein which can bind two receptors, TNFR1 or p55, and TNFR2 or p75. It is produced mostly by activated macrophages, but also T lymphocytes, mast cells, neutrophils, dendritic cells and eosinophils as well as some structural cells like fibroblasts, epithelial cells and smooth muscle cells [178, 208]. Secretion of TNF-α may be caused by physical, chemical stimuli or pathogens, and a variety of cytokines such as IL-1, GM-CSF and IFN-γ [178]. TNF-α plays role in controlling the growth, differentiation and proliferation of a variety of cell types as well as mediating apoptosis [209]. It also causes the expression of various other members of the cytokine network such as CXCL8, CCL5 and CCL11 in BSM cells and epithelial cells [178]. Several lines of evidences show that high levels of TNFα are directly linked to asthma pathogenesis; TNF-α is increased in the airways during allergic airway inflammation [210], evokes airway hyper-responsiveness [211]. BALF and bronchial biopsies from asthmatic patients showed an increase in TNF-α levels [130, 212]. Moreover, cells from BALF of asthmatic patients express significantly high amounts of TNF-α [213].

TNF-α can participate in asthma pathogenesis via the expression of multiple inflammatory mediators, chemokines and cytokines, which could
perpetuate and/or aggravate the ongoing inflammatory and remodelling processes [178]. For example, IL-33 an important cytokine in asthma [214] was found to be co-expressed with TNF-α within endobronchial biopsies of subjects with severe asthma. IL-33 can be induced by TNFα alone or with IFN-γ co-stimulation. Dexamethasone, however, fails to supress TNF-α stimulated IL-33 expression [215]. There is also indication for a direct role of TNF-α in airway remodelling via the release of MMP-9 and the ECM protein tenascin from bronchial fibroblasts [216]. IL-6, a cytokine which promotes BSM hyperplasia, and immunoglobulin secretion and modulates B- and T-cell proliferation is also induced by TNF-α in BSM cells and epithelial cells [217].

A considerable amount of literature has been published to prove that TNF-α promotes airway hyper-responsiveness in asthma; TNFα increased maximal isotonic contraction to methacholine of guinea pig tracheal preparations in vitro [218]. Previous studies have reported that human BSM cells derived from subjects with asthma showed an elevated level of CD38 induced by TNF-α compared to BSM cells derived from patients without asthma [219]. In animal studies, TNFα has been shown to mediate LPS and allergen-induced inflammation and airway hyper-responsiveness [220]. Additionally, in murine tracheal tissues, IFN-β and CD38 are essential for TNF-α induced airway hyper-responsiveness in response to carbachol [221]. Furthermore, TNFα inhalation augments airway responsiveness and induces sputum neutrophilia in normal controls [222] and asthmatic patients [211]. However the exact mechanism of TNF-α in the development of airway hyper-responsiveness has not been fully elucidated.
However, the most compelling evidence demonstrating a key role of TNF-α in the pathogenesis of asthma comes from preclinical studies in various animals of allergic asthma [223] and recent clinical trials in patients with asthma [178] that have used different anti-TNF-α strategies. All these studies using mostly various monoclonal antibodies, fusion proteins to target specifically TNF-α confirmed that TNF-α participates in the development of key asthmatic features such as airway inflammation, airway hyper-responsiveness and airway remodelling.
Figure 1.5: Role of TNF-α in the pathogenesis of asthma: TNF-α plays a key role in many of the features of the asthma disease by exerting effects on both inflammatory cells and structural cells that express TNF-α main receptor called TNFR1. Adapted from [178].
2. IFN-γ as a mediator in asthma

Interferon-γ (IFN-γ) is an important cytokine that orchestrates many distinct cellular programs through transcriptional control over large numbers of genes [224]. IFN-γ is produced primarily by T-lymphocytes and natural killer cells or in response to IL-12, IL-18 and IFNα, IFNβ. The role of IFN-γ in asthma is still controversial although some studies found a putative role [102]. Evidence suggests that IFN-γ may participate in both acute severe asthma and chronic asthma in response to certain pathogen, such as intracellular bacteria, viruses and fungi [225]. Other studies found that production of IFN-γ was significantly increased by T cells in the peripheral blood [226, 227] and in the bronchoalveolar lavage fluid (BALF) of patients with asthma [228]. Levels of IFN-γ correlated with disease severity [229]. As described for TNF-α, IFN-γ could participate in asthma pathogenesis via multiple mechanisms: IFN-γ has been shown to increase the cytotoxic function of lung macrophages and their secretion of an array of cytokines and chemokines like CXCL10, CXCL11, CXCL9 [217]. In addition, IFN-γ seems to be important in regulating both influx of neutrophils in the lungs and the increased airway responsiveness to methacholine [230]. This is not a surprising finding as considerable evidence supports the fact that Th1 cells and their cytokines, including IFN-γ, play a role to impaired lung function seen in asthma. For example, asthmatic children showed a consistent association between airway hyper-responsiveness and the enhanced IFN-γ production by peripheral blood mononuclear cells [231]. In experimental studies, using long-term challenge of systemically sensitised mice Foster's group demonstrated the anti-IFN-γ strategy prevented the development
of airway hyper-responsiveness [232] [233]. Furthermore, the same group showed that there is interaction between IFN-γ and pulmonary macrophages was essential in the prolongation of airway hyper-responsiveness in a mouse model in part through the production of IL-27 by the pulmonary macrophages [234]. Together these preclinical studies demonstrate the central role of IFN-γ and macrophages in driving abnormal lung function seen in asthmatic patients.

3. **TNF-α/IFN-γ combination leads to pro-asthmatic responses in BSM cells**

<table>
<thead>
<tr>
<th>Induced molecules</th>
<th>Relevance in Asthma</th>
<th>Mechanisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemokines: CX3CL1 CCL5 CXCL10</td>
<td>Airway inflammation</td>
<td>Chemoattractant for Mast cells, T cells, NK cells, and eosinophils</td>
</tr>
<tr>
<td>COX-2 TLR2 and 3</td>
<td>Airway inflammation</td>
<td>Secretion of inflammatory prostanoids and cytokines</td>
</tr>
<tr>
<td>The ectoenzyme CD38</td>
<td>Airway hyper-responsiveness</td>
<td>Modulation of agonist-evoked calcium signaling and contractility</td>
</tr>
<tr>
<td>The receptor isoform GRβ Transcription factor IRF-1</td>
<td>Steroid resistance</td>
<td>Impaired GRα activity</td>
</tr>
</tbody>
</table>

**Table 1.2:** Examples of pro-asthmatic genes synergistically induced by interferons and other cytokines in BSMC and their respective implication in asthma pathogenesis (data extracted from [166]).
Accumulating evidence support a role of interferons (IFNs) in airway allergic responses by their ability to synergize with other inflammatory conditions. Both *in vitro* and *in vivo* studies showed that a number of immunoregulatory proteins induced by either viruses [235] or pro-asthmatic cytokines such as IL-13 [236] or TNF-α [237, 238] can in fact be enhanced by the presence of IFN-γ. These synergistic actions can also be seen in vivo in mice exposed to both IL-13 and IFN-γ combination which show a greater inflammatory process in the airways [236]. These amplifying actions of IFN-γ may represent one potential mechanism explaining why viral infection, which increases production of IFNs, is an important trigger for allergic asthma and chronic obstructive pulmonary disease exacerbation [239]. A number of studies from Amrani’s group have showed that a combination of IFN-γ with TNFα leads to enhanced pro-asthmatic proteins in BSM cells (see Table 1.2), which in turn could play essential roles in driving key features in asthma including airway inflammation, airway hyper-responsiveness and steroid resistance (see Figure 1.6). Although the mechanisms of this synergy in BSM cells have not been investigated, IFN-γ has been shown to up-regulate TNF-α receptors or vice-versa [240, 241]. The presence of IFN-γ also leads to increased TNF-α receptor–associated signalling in murine macrophages and rat cell lines such as PC12 [242, 243]. Also both cytokines leads to increased transcription factors activation such as NF-kB and STAT1 shown to be important for the expression of ICAM-1 [244], CCL5 [245, 246].
This PhD thesis will investigate whether TNF-α/IFN-γ combination can induce a steroid resistant state in BSM cells as previously suggested by studies using cultured tracheal smooth muscle cells [187, 247].
Figure 1.6: Molecular mechanisms by which the combined action of TNFα/IFNγ in BSM cells could play a role of in pathogenesis of asthma: A variety of triggers, including viral or bacterial proteins, may induce the expression of TNF-α/IFN-γ from both BSM and infiltrated mast cells and T cells. These mediators may act directly on HBSMC in both autocrine and paracrine manners to synergistically induce i) expression of various chemokines (CXCL10, CCL5, CX3CL1, CXCL8), ii) over-activation of different transcription factors (IRF-1, STATs, NF-κB), and iii) up-regulation of cell surface adhesion molecules (ICAM-1, CD38). These pro-asthmatic responses in BSM could then amplify and/or orchestrate the inflammatory process present in the airways of asthmatic patients who are refractory to corticosteroid therapy. (Adapted from [166]).
VII. **K\textsubscript{Ca}3.1 CHANNELS AND ASTHMA**

1. **Ion channels and asthma**

Ion channels are the molecular units that cause electrical signalling in cells. Many physiological procedures are dependent upon ion channel signalling, as dysfunction often leads to severe pathophysiological consequences. Ionic channels are integral membrane proteins forming a single aqueous pores which use the ions electrochemical gradient to ensure the passage of inorganic ions mainly \( \text{Na}^+ \), \( \text{K}^+ \), \( \text{Cl}^- \) and \( \text{Ca}^{2+} \) across the plasma membrane [248].

Ion channels play a dynamic role in basic cell physiological functions including generation of electrical activity in nerves and muscle, the regulation of the membrane potential, hormone secretion, control of cardiac excitability, cell proliferation, adhesion, migration, secretion, apoptosis and intracellular signalling and many other biological processes. Ion channels therefore, have critical roles in the function of all cells, tissues and organs, and thus whole organisms [249]. Dysfunction of these ion channels lead to many pathophysiological conditions such as epilepsy, cystic fibrosis, arrhythmias and many others have been related to ion channel dysfunction that might be due to mutations in the genes encoding for ion channels are termed “Channelopathies” [249, 250].

Ion channels have been a centre of many investigations to understand the pathogenesis of asthma and aiming to identify therapeutic targets for better control of the disease. These channels regulate many key functions of inflammatory cells and structural cells implicated in asthma pathophysiology
Recent studies have focused on the potential role of potassium channels which have been reported to play an important role in respiratory conditions such as asthma and chronic obstructive pulmonary disease (COPD) [251].

2. **Potassium (K⁺) Channels**

   Potassium ion channels form the major superfamily of channels present in both procaryotic and eucaryotic cells [252], K⁺ channels are multimeric membrane-spanning proteins formed by an assembly of transmembrane helices. Apart from the selectivity filter in the central pore, K⁺ channels differ in membrane topology, subunit structure and mechanisms of activation and inactivation [253]. K⁺ channels can be classed according to the mechanism of activation or gating. There are K⁺ channels whose activation is due to variations in membrane potential. These channels are known as voltage-gated (Kᵥ), which are closed at rest and only open in response to depolarization of the membrane potential; examples of these voltage gated channels are the Na⁺ and K⁺ voltage-gated channels of nerve and muscle cells, and the Kᵥ1.3 channel which is essential for human lymphocyte function [248]. There are other types of K⁺ channels called Ligand-gated channels or ATP-sensitive K⁺ (KATP) channels which has been found in variety of tissue like cardiac muscles, skeletal muscles, smooth muscles and pancreas [254]. Ca²⁺ activated potassium channels (KᵈCa) represent the large family of K⁺ channels and will be described in details in the next section.

3. **Ca²⁺-activated K⁺ channels**

   A main family of Ca²⁺-sensitive K⁺ channels with six transmembrane segments and a single pore is divided in five subfamilies. They are called
These channels are present in a wide range of cells and are involved in intracellular calcium signalling cascades. These Ca$^{2+}$-sensitive K$^+$ channels vary between subfamilies in their single-channel conductance, mode of Ca$^{2+}$-activation, subunit composition, voltage-dependence, and pharmacology. Three families of Ca$^{2+}$-sensitive K$^+$ channels have been identified and are subdivided according to the size of their single channel conductance [256].

i. Large conductance K$_{Ca}$ channels (BK, K$_{Ca}$.1.1):
These channels have a single channel conductance in the range of 200-300 pico-siemens (pS). Under physiological conditions, K$_{Ca}$.1.1 channel gating is not only controlled by free intracellular calcium, but also by membrane voltage. The activity of K$_{Ca}$.1.1 channel can also be modulated by a wide variety of substances such as channel blockers (charybdotoxin) and channel openers (NS1619) [257]. K$_{Ca}$.1.1 channels are distributed in both excitable and non-excitable cells where they play a physiological role in controlling vascular tone [258]. Many studies showed that K$_{Ca}$.1.1 channels dysregulation have been implicated in the proliferation of numerous brain cancers, including gliomas and neuroblastomas [259] and breast cancer [260].

ii. Small conductance K$_{Ca}$ channels (SK2, K$_{Ca}$.2.1, K$_{Ca}$.2.2, and K$_{Ca}$.2.3): These channels have small unitary conductance between 4-14 pS and are activated solely by internal Ca$^{2+}$ ions. These channels are more sensitive to Ca$^{2+}$ than K$_{Ca}$.1.1 channels because of the calmodulin binding domain located on the C- terminus of the protein. SK2 channels
can be blocked by apamin, the bee venom neurotoxin peptide [248]. These channels have an important role in excitable cells mainly nerve cells, and have been identified to play a role in Parkinson disease [261].

iii. Intermediate conductance $K_{\text{Ca}}$ channels ($K_{\text{Ca}3.1}$): These channels are the main focus in this PhD thesis and channel properties and functional roles in different tissues and organs are presented in more details in the following sections.

4. The intermediate conductance $\text{Ca}^{2+}$-activated $K^+$ channel ($K_{\text{Ca}3.1}$ channel)

The intermediate conductance $\text{Ca}^{2+}$-activated $K^+$ channel ($K_{\text{Ca}3.1}$) (also known as IK1, SK4 or KCNN4) has a unitary conductance between 20-85 pS and are gated solely by the binding of intracellular $\text{Ca}^{2+}$ ions. $K_{\text{Ca}3.1}$ Channels play relevant roles in modulating calcium-signalling and membrane potential in both excitable and non-excitable cells [257]. $K_{\text{Ca}3.1}$ was first characterized in erythrocytes by Gardos in 1958 [262]. In 1997, $K_{\text{Ca}3.1}$ was first cloned from human pancreas by the Ishii’s group [263] and from placenta [264] who found about 40-44% homology with the small conductance family of $K^+$ channels [263]. The $K_{\text{Ca}3.1}$ channel is encoded in human by the KCNN4 gene located on chromosome 19q13.2 [265].

4.1 $K_{\text{Ca}3.1}$ channel structure

$K_{\text{Ca}3.1}$ channel is a tetrameric membrane protein composed of four identical subunits; each subunit has six transmembrane domains (S1-S6) with the pore composed of S5, the P-helix, and S6. $K_{\text{Ca}3.1}$ functions as a
heteromeric complex with calmodulin as a Ca$^{2+}$-binding protein which is the most important segment of the $K_{Ca}3.1$ channel structure as this acts as the Ca$^{2+}$ sensor; the calmodulin binding domain is located in the C-terminus, just distal to S6 [262]. Similar to other $K_{Ca}$ channels, $K_{Ca3.1}$ is activated by small increase of intracellular Ca$^{2+}$ ($\approx$0.1-0.3 μM) above resting levels that will evoke K$^{+}$ efflux due to the opening of the channel above resting levels [265].
Figure 1.7: Schematic representative of $K_{Ca3.1}$ structure: subunits contain six transmembrane segments (S1–S6) and one pore region (P) highly selective to $K^+$. Four of these structures are assembled tetramERICally to structure $K_{Ca3.1}$ ion channel with a central pore. Picture adapted from [257].

4.2 Pharmacology of $K_{Ca3.1}$ channels

Unlike the other $K^+$ channels, $K_{Ca3.1}$ has a relatively well-developed pharmacology and a number of different modulators (activators and inhibitors) are available to study the function of $K_{Ca3.1}$ channel in different tissues.
4.3. **KCa3.1 channel Activators**

Researchers have developed reliable KCa3.1 channel openers which include the benzimidazolone compound, 1-ethyl-2-benzimidazolinone (EBIO, EC50 ~74 μM) and its much more potent derivative 5, 6-dichloro-1-ethyl-2-benzimidazolinone (DC-EBIO). Both compounds activate KCa3.1 channels by increasing the apparent Ca^{2+} affinity of the channel keeping the channel in the open state [266]. There are other activators that have been developed such as SKA-20, SKA-31 [267] and NS309 [268]. These activators exert their effect by increasing the sensitivity of the calmodulin binding domain [269].

4.4. **KCa3.1 channel Blockers:**

There are many KCa3.1 blockers which have been discovered; Charybdotoxin (IC50 ~ 5 nM) is present in the venom of the scorpion which can bind to a site on the external surface of the channel. Charybdotoxin is not selective for KCa3.1 channels, as it can also block KCa1.1 with a similar potency [270]. KCa3.1 channel can also be directly blocked by the antifungal drug Clotrimazole (IC50 ~ 70 nM), which was thought to be therapeutically interesting for treating diseases where KCa3.1 blockers have been implicated. However, one of the major limitations of Clotrimazole is its non–selectivity because it can block mammalian cytochrome P450 enzymes and its serious side effects (liver damage and changes in cortisol level) [271]. Some groups have modified clotrimazole to design 2 novel drugs which specifically target the KCa3.1 channel and are free of cytochrome P450 inhibition these drugs are discussed below (Figure 1.8):
• **TRAM-34**: \((1-\{(2\text{-chlorophenyl})\text{diphenylmethyl}\}-1\text{H}-\text{pyrazole})\): The TRAMs are one of these groups of clotrimazole analogues that show high therapeutic ability [272]. The most promising of this group is TRAM-34 (IC50 ~ 20 nM) which exhibits high selectivity to bind to \(K_{Ca}3.1\) (200-500 fold) compared to the other potassium channels. \(K_{Ca}3.1\) binds to the cytoplasmic side of the S5-P-S6 region of the channel below the selectivity filter [271].

• **ICA-17043**: (4-fluoro-\(\alpha\)-(4-fluorophenyl)-\(\alpha\)-phenyl-benzene acetamide) or (Senicapoc) designed by Icagen Inc, is a potent and selective inhibitor of the \(K_{Ca}3.1\) channels which has been developed more recently with a greater potency when compared to TRAM-34 with an IC50 ~11 nM [273]. ICA-17043 was used by patients with sickle cell anaemia in phase 2 and phase 3 clinical trials where it was found to be safe and well tolerated at doses that effectively blocked \(K_{Ca}3.1\) channels [273, 274]. Both inhibitors will be used in our studies to investigate the role of \(K_{Ca}3.1\) channels.

![Figure 1.8: The chemical structures of selective \(K_{Ca}3.1\) channel blockers (TRAM-34 and ICA-17043)](image)
4.5 Roles of $K_{Ca}3.1$ channels in health and disease

$K_{Ca}3.1$ channels are widely expressed in many organs and tissues, but they are not expressed in certain excitable tissues such as skeletal muscle, the nervous system and cardiac myositis [263, 264].

In the following part we discuss the most well-understood roles and/or mechanisms by which $K_{Ca}3.1$ channels contribute to cell physiology and pathology; however the function of the channel varies according to the cell types.

4.5.1 $K_{Ca}3.1$ channel and cell proliferation

One of the first characterized roles of $K_{Ca}3.1$ channels was its implication in regulating proliferation. The proposed mechanism, although some have not been elucidated yet, is the ability of $K_{Ca}3.1$ channel to promote cell hyperpolarization, $Ca^{2+}$ influx, and thus, sustain an increase of intra-cellular $Ca^{2+}$ concentration essential for gene transcription required for the cell cycle [274]. $K_{Ca}3.1$ channels have been involved in T lymphocytes proliferation [275]. This observation could have some clinical implication as uncontrolled cells growth and proliferation of B cell can cause some autoimmune diseases and cancer such as chronic lymphocytic leukemia (CLL) [274]. $K_{Ca}3.1$ channel is expressed in endothelium of blood vessels from human and other species where it can regulate endothelial cell proliferation and vascular tone [276]. Moreover, induction of $K_{Ca}3.1$ on endothelial cells can be upregulated in response to basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) and is essential for their proliferative action [276]. Similar observation has been made in vascular smooth muscle cells (VSMCs) where
K\textsubscript{Ca}3.1 expression is significantly increased by other growth factors such as PDGF and EGF, a mechanism which could play a role in vascular diseases such as atherosclerosis and coronary artery restenosis [274, 277]. Proliferation of fibroblasts is a hallmark of progressive renal fibrosis. Animal studies showed that upregulation of K\textsubscript{Ca}3.1 was accompanied by a rise in collagen-1, FSP-1, collagen-III and TGFβ1 in unilateral ureteral obstruction (UOO, a model of kidney fibrosis) compared to wild type [278]. Recently Bradding group showed that K\textsubscript{Ca}3.1 can promote pro-fibrotic human lung myofibroblast function in response to TGFβ and bFGF and have suggested that blocking K\textsubscript{Ca}3.1 may offer a novel approach to treat IPF patients [279].

4.5.2 K\textsubscript{Ca}3.1 channel and migration

K\textsubscript{Ca}3.1 channels are required in migration of T lymphocytes [275], VSMC [277], macrophages [280], microglia [281] and mast cells [282].

4.5.3 K\textsubscript{Ca}3.1 channel and cell volume

In addition to the migration and proliferation K\textsubscript{Ca}3.1 channels play a predominant role in cell volume regulation in erythrocytes and T lymphocytes [274], which is important for the maintenance of cellular homeostasis.

4.5.4 K\textsubscript{Ca}3.1 channel and inflammatory cytokine expression

Studies have reported that K\textsubscript{Ca}3.1-mediated increase of intracellular calcium was essential for the production of inflammatory chemokines and cytokines by T cells, mast cells and macrophages [275, 282]. K\textsubscript{Ca}3.1 channels seem also to play a role in the nitric oxide production, and oxidative burst
expression in microglia cells [283]. **Figure 1.9** summarizes the role of K\(_{Ca3.1}\) in different cell types.

**Figure 1.9**: Role of K\(_{Ca3.1}\) channels in different cell types.

### 4.6 Pathophysiological roles of K\(_{Ca3.1}\) channels

As mentioned above, K\(_{Ca3.1}\) channels have also been implicated in many health disorders. **Table 1.3** summarizes some of the important diseases, tissue or cell expression localization and possible treatments that have been documented for K\(_{Ca3.1}\).
<table>
<thead>
<tr>
<th>Diseases</th>
<th>Tissue or cells implicated (expression)</th>
<th>$\text{K}_{\text{Ca}3.1}$ blockers or activators in clinical trials or under current investigation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sickle cell Anaemia</td>
<td>Blood red cells</td>
<td>ICA-17043</td>
<td>[284, 285]</td>
</tr>
<tr>
<td>Retinal Degeneration</td>
<td>Ganglion cells</td>
<td>TRAM-34</td>
<td>[283]</td>
</tr>
<tr>
<td>Cystic Fibrosis</td>
<td>Airway epithelial Cells</td>
<td>$\text{K}_{\text{Ca}3.1}$ Openers EBIO, CBIQ Chlorzoxazone</td>
<td>[266, 286]</td>
</tr>
<tr>
<td>Atherosclerotic vascular disease</td>
<td>VSMC, macrophages and white blood cells</td>
<td>TRAM-34, Clotrimazole</td>
<td>[287]</td>
</tr>
<tr>
<td>Hypertension and Restenosis</td>
<td>Vascular endothelial cells</td>
<td>Activators $\text{K}<em>{\text{Ca}3.1}/\text{K}</em>{\text{Ca}2.3}$ NS309/Aminopiridine</td>
<td>[267, 288]</td>
</tr>
</tbody>
</table>

Table 1.3: Important diseases associated with $\text{K}_{\text{Ca}3.1}$ dysfunction, localization and possible treatments.
VIII. EMERGING ROLE OF \( K_{Ca3.1} \) CHANNELS IN ALLERGIC ASTHMA

As mentioned earlier, \( K_{Ca3.1} \) channels are widely expressed in the human lung and are found in lung fibroblasts, BSM cells, airway epithelial cells, endothelial cells, mast cells, T cells and macrophages [269]. With respect to asthma, Bradding group provided the first evidence of role of \( K_{Ca3.1} \) channels in asthma by showing inhibition of \( K_{Ca3.1} \) channels using TRAM-34 affected both degranulation and migration responses in human lung mast cells [282]. This observation was further supported in a mouse model where \( K_{Ca3.1} \)-deficient mice had impaired mast cell degranulation [289]. Moreover, modulators of \( K_{Ca3.1} \) channels such as beta2-adrenoreceptor, prostaglandin and adenosine inhibit mast cell function [290]. Together these studies show that \( K_{Ca3.1} \) are essential in driving mast cell activation and represent therefore a novel class of anti-allergic drugs.

With regard to the role of \( K_{Ca3.1} \) channels in lung structural cells, very limited data are available. \( K_{Ca3.1} \) channels were shown to regulate the migration of human fibroblast [291] and lung dendritic cells [292]. In BSM cells, Bradding group showed that FGF and TGF-\( \beta \) stimulated the expression of \( K_{Ca3.1} \) at both mRNA and protein levels. The pharmacological blocker TRAM-34 showed that \( K_{Ca3.1} \) channels were essential in FGF and TGF-\( \beta \)-induced BSM proliferation [293]. Recent study confirmed that \( K_{Ca3.1} \) channels in BSM cells can also be increased by other growth factors such as PDGF and that this upregulation was required in modulating BSM responses including proliferation, migration and contractility [294].
These different observations suggest that activation of $K_{Ca}3.1$ channels on structural (smooth muscle/fibroblasts) airway cells and on inflammatory cells (mast cells, T cells) may represent an important pathway driving key features of allergic asthma. While doing this PhD, recent preclinical studies in animal models of asthma have indeed confirmed the implication of $K_{Ca}3.1$ channel in asthma pathogenesis. TRAM-34 inhibited allergen-associated eosinophilic inflammation, airway hyper-responsiveness, and airway remodeling in a murine model of allergic asthma [284]. In a sheep model of chronic asthma $K_{Ca}3.1$ channel blocker reduced allergen-induced airway responses, inflammation and vascular remodelling and lung eosinophilia [295]. In human clinical trials, ICA-17043 (Senicapoc®) showed an encouraging results in allergen challenge in atopic Asthmatic subjects (phase II). In this trial, Senicapoc® treatment at 80 mg twice daily for 3 days followed by maintenance dose (40 mg daily for 11 days) led to a reduction in airway resistance and the inflammation [274]. However, in another clinical trial in which Senicapoc®’s effect on exercise-induced asthma was examined there were no improvement in lung function following 4 weeks of treatment (40 mg/day) [274]. These studies support the concept that $K_{Ca}3.1$ channels could represent a potential novel target in a subset of asthmatic patients.
CENTRAL HYPOTHESIS
There are still many questions regarding the role of $K_{Ca}3.1$ channels in asthma pathogenesis. Considering that $K_{Ca}3.1$ channel regulates proliferation of T lymphocytes and activation of mast cells, one can assume that targeting $K_{Ca}3.1$ channels could be beneficial in the treatment of allergic asthma, a disease driven by the dendritic cells-Th2 type lymphocyte-mast cell axis.

One important question is the contribution of $K_{Ca}3.1$ channels on structural cells such as BSM in the pathogenesis of asthma. Here, we tested whether $K_{Ca}3.1$ channels are involved in the regulation of the immunomodulatory function of BSM cells. We also tested whether $K_{Ca}3.1$ channels regulate BSM cells sensitivity to corticosteroid therapy in experimental conditions mimicking steroid sensitive and insensitive states in asthma.
The specific aims of this PhD are:

- To reproduce a cellular model of corticosteroid insensitivity in BSM treated with both TNFα/IFN-γ
- To examine the expression of \( K_{Ca}3.1 \) channels in BSM in both cultured and native conditions
- To examine whether \( K_{Ca}3.1 \) channels modulate the expression of chemokine expression in BSM cells under steroid-resistant conditions
- To examine whether \( K_{Ca}3.1 \) channels play any role in TNFα/IFNγ-induced steroid resistance in BSM cells by acting at the transcriptional levels
- To examine whether \( K_{Ca}3.1 \) channels play a role in TNFα/IFNγ-induced steroid resistance in BSM cells via the modulation of GRα expression and/or function
CHAPTER 2

MATERIALS AND METHODS
I. **BRONCHIAL SMOOTH MUSCLE (BSM):**

1. **Human subjects**

Healthy volunteers, Asthmatic subjects, and patients with chronic obstructive pulmonary disease (COPD) were recruited from Glenfield Hospital outpatients, staff and by local advertising. Asthma severity was defined by British Guideline on the Management of Asthma treatment steps. All COPD patients used for the study were classified as GOLD (Global Initiative for Chronic Obstructive Lung Disease) I and II [296]. The studies were approved by the Leicestershire, Northamptonshire, and Rutland Research Ethics Committee (references: 4977, 04/Q2502/74 and 08/H0406/189). Written informed consent was gained from all participants prior to their involvement. **Table 2.1** shows the demographics and clinical characteristics of the patients used in this study.
Table 2.1: Subjects demographics and clinical characteristics. Data are shown as *means ± SEMs. #Median (interquartile range). † P < 0.05, ††P < 0.01, and ††† P < 0.001 versus healthy control. §Defined as positive skin prick test responses to 1 or more common aeroallergens. BDP (Beclomethasone dipropionate), FEV1 (forced expiratory volume in 1 second), FVC (forced vital capacity); NA (Data not available).

2. BSM Isolation and Culture

Bronchial airway smooth muscle cells (BSMC) were isolated and prepared from lung resection and biopsy tissue as described previously [297]. Lung resection and biopsy tissue were dissected free of connective tissue under sterile conditions with the aid of a dissecting microscope. Small explants of airway smooth muscle were then bathed in media and incubated in humidified 5% CO$_2$-95% air at 37°C. When cells were approaching confluence, the explants were removed. Once confluent, cells were trypsinized with 0.25% trypsin and 0.02% EDTA in phosphate buffered saline (PBS), centrifuged, re-suspended in DMEM with Glutamax-1 supplemented with, 1% antibiotic-
antimycotic (Sigma; containing 100 units/ml penicillin, 100 mg/ml streptomycin sulphate, and 0.25 μg/ml amphotericin B), non-essential amino-acids (NEAA) (100 μM) (Invitrogen, Paisley, UK), sodium pyruvate (SP) (1 mM) and fetal bovine serum (FBS) (10%) (Sigma, Gillingham, UK) and plated out in 75 cm² flasks. Once cells had again reached confluence, the passaging procedure was repeated and cells re-suspended in a freezing media containing 90% FCS - 10% dimethyl sulphoxide (DMSO), frozen in liquid nitrogen and stored until required. BSM cell phenotype was determined by assessing the presence of α-smooth muscle (Sigma), and myosin by flow cytometry using antibodies coupled to fluorescein isothiocyanate (FITC) (Sigma) (> 90% of α-smooth muscle considered as pure BSM). BSMC passages 2-6 were used for all experiments. At the stage of confluence, BSMC demonstrate typical spindle morphology and hill-and–valley pattern observed under light microscopy (Figure 2.1) All the cells used in this study were serum-deprived for 24 hours using DMEM supplemented with 1% ITS+3 (Sigma, Gillingham, UK), 1% sodium pyruvate, 1% non-essential amino acid NEAA to synchronise cell growth, the stimulation were performed in this medium in BSMC at 37°C in the incubator to syn. All BSMC were routinely tested for mycoplasma infection prior each set of experiments.
Figure 2.1: Light Microscopy of BSMC in culture.

The number of cells in a cell suspension was determined using a haemocytometer and a light microscope. Cells were diluted 1:10 with Trypan blue to distinguish between live and dead cells. 10µl of cells was pipetted under the cover slip on the haemocytometer. The cells were observed under the microscope using the x40 magnification lens, once total cell counted, cell concentration was calculated using the following formula:

\[ \text{Total cells/ml} = \text{Total cells counted} \times \text{dilution factor} \times 10,000 \text{ cells/ml} \]
### II. Enzyme linked immune-sorbent assay [ELISA]:

ELISA method is one of the more practical ways to detect the presence of an antigen in a cell supernatant, which has been widely used either in clinical laboratory or research.

#### 1. Sample preparation

Confluent cells grown in 24 well-plates were serum-deprived for 24 hours in serum-free media as described above and then treated with TNF-α (0 – 100 ng/ml), IFN-γ (0 – 50 ng/ml) for 24 hours in concentration response experiments. In inhibitor studies, cells were pre-treated with fluticasone propionate (FP) (100nM) alone or in combination with effective concentrations of K\textsubscript{Ca}3.1 channel blockers ICA 17043 (100 nM) or TRAM-34 (200 nM) for 2 hours before being stimulated with a combination of 25 ng/ml of IFNγ and 10 ng/ml TNFα for an additional 24 hours. Every experimental condition was performed in triplicate and repeated in cells derived from at least 3 to 5 different subjects. Dimethylsulphoxide (DMSO) for both K\textsubscript{Ca}3.1 inhibitors and FP was also added to control wells at equivalent concentrations maximum concentration (0.1%).

---

<table>
<thead>
<tr>
<th>Number of the cells plated</th>
<th>75cm flasks</th>
<th>6 well plates</th>
<th>24 well plates</th>
<th>8 chamber slides</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5x10^5 cells</td>
<td>4.5 x10^4 cells</td>
<td>1.8x10^4 cells</td>
<td>0.8x10^4 cells</td>
<td></td>
</tr>
</tbody>
</table>
Cell culture supernatants were collected following stimulation of cells and stored at -20°C.

2. **Chemokine Immunoassays**

Measurement of chemokine expression: CXCL10, CCL5, CCL11 and CX3CL1 concentrations in cell culture supernatants were measured by ELISA using the commercially available Duoset ELISA kits (R&D Systems) according to the manufacturer’s instructions. 96 well plates were coated with the specific capture antibodies in phosphate buffered saline (PBS) and incubated overnight at room temperature. Plates were then washed 3 times in 0.05% Tween 20 in PBS and blocked with 1% bovine serum albumin (BSA)/PBS to prevent non-specific binding for 1 h at room temperature. An eight point standard curve was generated by serial two-fold dilution of the different human recombinant chemokines supplied in the kit in 1% BSA in PBS with concentrations ranging from 2000-15.625 pg/ml. Standards or samples (100 μl) were then added to wells in triplicate and incubated at room temperature for 2 hours. After washing 100 μl of detection antibodies in 1% BSA/PBS was added to all wells, and incubated for 2 hours at room temperature. After a further wash, samples were incubated for 20 min at room temperature with 100 μl streptavidin-conjugated horseradish peroxidase streptavidin, diluted 1:200 in 1% BSA in PBS. Plates were then washed again before addition of 100 μl per well of substrate solution for 20 min at room temperature. The resulting colorimetric reaction was stopped by addition of 50 μl per well of 1 M sulphuric acid. The optical density of the wells was read at 450 nm using a microplate reader (Vector palate reader), the
concentrations of CXCL10, CCL5, CCL11 and CX3CL1 in samples were determined using a standard curve.

<table>
<thead>
<tr>
<th>Chemokines</th>
<th>CCL5</th>
<th>CXCL10</th>
<th>CCL11</th>
<th>CX3CL10</th>
</tr>
</thead>
<tbody>
<tr>
<td>capture antibody</td>
<td>1.0μg/ml</td>
<td>2.0μg/ml</td>
<td>2.0μg/ml</td>
<td>4.0μg/ml</td>
</tr>
<tr>
<td>Detection antibody</td>
<td>10ng/ml</td>
<td>100ng/ml</td>
<td>100ng/ml</td>
<td>500ng/ml</td>
</tr>
<tr>
<td>standards</td>
<td>1000pg/ml</td>
<td>2000pg/ml</td>
<td>2000pg/ml</td>
<td>2000pg/ml</td>
</tr>
</tbody>
</table>

Table 2.3: shows the working concentrations for the different chemokines used.

### III. WESTERN BLOTTING

Western blot is commonly used to detect the protein presence and measure changes in protein expression it utilises gel electrophoresis to separate proteins within a sample of mixed proteins according to their molecular weight.

1. **Protein preparation**

BSM cells were grown to confluence in 6 well plate and growth arrested for 24 hours. Two wells were used for each condition. The cells were treated with IFN-γ 25ng/ml and TNF-α 10ng/ml in the presence or absence of pharmacological inhibitors ICA-17034 (100nM) or TRAM 34 (200nM) alone or in combination with FP (100nM) for 6 hours. Following removal of culture medium the cells were washed with ice-cold PBS. and lysed on ice with 120μl RIPA lysis Buffer containing 1x PBS, 1% Nonidet P-40, 0.5% Sodium Deoxycholate, 0.1% SDS, phenylmethanesulphonylfluoride (PMSF) 200μM, Sodium
orthovanadate (NA3VO4) 2mM, sodium fluoride (NaF) 10mM, 2mM Ethylenediaminetetraacetic acid (EDTA) and various protease inhibitors: 10μg/ml Leupeptin, 10μg/ml (Aprotinin). The cells were scraped and the samples were collected into prechilled eppendorfs and placed in the ice for 25 min. The lysates were centrifuged at 12000 g for 20 min at 4°C and the supernatants that contain total proteins were collected and stored at -20°C until used.

2. **Protein Concentration**

Protein concentrations were determined using the Bio-Rad detergent compatible protein assay kit (Bio-Rad); a commercial preparation based on the Bradford method which is a colorimetric assay which binds the Coomassie Brilliant Blue G-250 dye to the amino acids in experimental samples.

Protein concentrations are estimated by reference to absorbance obtained for a series of standard protein dilutions assayed alongside the samples. In this study, bovine serum albumin (BSA) was used as standard protein and the assay was performed at different concentrations ranging from 0 mg/ml to 0.7 mg/ml. Cell extracts were diluted 1/10 in the distilled water then 100μl reagent A and 800μl reagent B were added for each sample and standard, left for 15 min in room temperature the protein concentrations were calculated using the spectrometry where the absorbance was measured at 750 nm.

3. **Protein electrophoresis**

The samples were then diluted 1:5 in Laemmli buffer containing (0.125 M Tris-HCl, 20% vol/vol glycerol, 0.2% wt/vol SDS, 6% vol/vol β-mercaptoethanol
and 0.2% wt/vol bromophenol blue) and heated for 5 min at 100°C. β-mercaptoethanol reduced disulfide bonds and subsequent boiling at 100 °C denatured the protein. Samples then were collected by brief centrifugation and placed on ice.

Denatured proteins ~ 50μg were separated by Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis (SDS-PAGE) using 8% or 10% SDS gel depend on molecular weight of the protein of interest and were transferred by semi-wet electro-transfer at room temperature onto a nitrocellulose membrane (GeneFlow), soaked in transfer buffer for a total of 1 hour at 250 mA for 20 minutes, 270 mA for 20 minutes and 310 mA for 20 minutes. The membranes were blocked for 1hr in TBS-T with 5% non-fat dry milk powder and then incubated at the correct dilution for the primary antibodies directed against a protein of choice (see table 3.3) overnight at 4°C in TBS-Tween containing 5% wt/vol BSA for phosphokinase or in 5% milk for all other primary antibodies. Blots were washed 3 times 10min each with 1x TBS-0.1% tween and incubated with 1:5000 dilution of the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (table 3.3) in 5% milk /TBS-T was left on a rocker for 1 h at room temperature. The blot was washed in TBS-T and staining was achieved with enhanced chemiluminescence western blotting detection reagent. Equal volumes of the two ECL™ detection reagents were mixed together and poured over the membrane ensuring the whole surface of the membrane was covered and the membrane was left for 2 minutes and finally exposed to high performance chemiluminescence film (Fisher scientific) for appropriate time. The position and molecular weight of the protein of interest
was validated by reference to coloured molecular weight markers (Spectra Multicolor Broad Range Protein Ladder Fermentas). Densitometric image analysis was performed using (ImageJ software, National Institute of Mental Health, Bethesda, Maryland, USA).

4. Stripping western membranes

The membranes were re-probed to confirm equal loading using either antibody against β-actin or against the total of the activated protein by stripping the membrane in 100 ml of stripping buffer (20 ml SDS 10%, 12.5 ml TrisHCl pH 6.8 0.5M, 67.5 ml ultra-pure water) and 0.8 ml β-mercaptoethanol added under the fumehood. The membranes were incubated with a stripping buffer for 15 min at 70°C then washed extensively for 5 minutes in TBS-Tween. The membranes were then blocked for 1 h at room temperature in 5% milk then following the above steps to detect (β-actin or total GR).
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human anti KCa3.1</td>
<td>biorbyt</td>
<td>1:500</td>
</tr>
<tr>
<td>Human anti Phospho GR (ser211)</td>
<td>Cell signalling Technology</td>
<td>1:1000</td>
</tr>
<tr>
<td>Human anti total GR</td>
<td>Santa Cruz</td>
<td>1:200</td>
</tr>
<tr>
<td>Human anti β-actin</td>
<td>Santa Cruz</td>
<td>1:200</td>
</tr>
<tr>
<td>Donkey anti-rabbit IgG-HRP</td>
<td>Santa Cruz</td>
<td>1:5000</td>
</tr>
<tr>
<td>Goat anti mouse IgG-HRP</td>
<td>Santa Cruz</td>
<td>1:5000</td>
</tr>
</tbody>
</table>

Table 2.4: Primary antibodies for western blot

IV. Isolation of Nuclear and Cytoplasmic Protein Fractions

Isolation of nuclear and cytoplasmic proteins was performed using Nuclear and Cytoplasmic Extraction Reagent Kit (NE-PER) (Fisher scientific). According to the manufacturer’s instructions, BSM cells were harvested using trypsin-EDTA and then centrifuged at 500 x g for 5 minutes. The supernatant was discarded, leaving the cell pellet as dry as possible. To proceed cytoplasmic and nuclear protein extraction ice cold CER I was added to the cell pellet which was vortexed vigorously on the highest setting for 15 seconds to fully suspend the cell pellet. Then the tube was incubated on ice for 10 minutes. Ice-cold CER II was added to the tubes, incubate tube on ice for 1 minute then centrifuged at maximum speed in a microcentrifuge (~16,000×g) for 5 minutes, cytoplasmic extract was transferred to a clean pre-chilled tube and stored in -20°C. Ice-cold NER was added to fraction produced from the previous step.
(pellet) which contains nuclei, which was vortexed vigorously on the highest setting for 15 seconds every 10 minutes, for a total of 40 minutes, the tubes were centrifuged at maximum speed (~16,000×g) in a microcentrifuge for 10 minutes. Then (nuclear extract) fraction were transferred to a clean pre chilled tube and stored in -80°C. Samples were normalized for protein concentration using Bradford Protein Assay. 30µg of each cytosolic and nuclear extract sample was analyzed by 10% SDS-PAGE and Western blotted as described above using K_{Ca}3.1 antibody diluted 1:500, anti-rabbit HRP (Santa Cruz) diluted 1:5000 was used as the secondary antibody with ECL (Thermo Scientific) for detection.

V. Quantitative Real Time PCR [qRT-PCR]

Quantitative polymerase chain reaction (Q-PCR), also known as real-time polymerase chain reaction, is used to amplify and quantify DNA within a tissue or cell sample using fluorescent DNA binding dyes such as SYBR Green. The intensity of the fluorescent signal is directly proportional to the mass of DNA present and is determined after each cycle of amplification. At the end of the reaction, the PCR products are subjected to dissociation curve analysis to ensure that a single amplification product has been produced and no primer dimers have formed.

1. Sample Preparation

Confluent, growth arrested HBSMC in 6 well plates were pre-treated with fluticasone (FP) (100nM) alone or in combination with effective concentrations of K_{Ca}3.1 channel blockers ICA-17043 (100nM) or TRAM-34 (200nM) for 2
hours before being stimulated with a combination of 25ng/ml of IFNγ and 10ng/ml TNFα for an additional 4 hours, The vehicle was dimethylsulphoxide (DMSO) for both K_{Ca3.1} inhibitors vehicle was added to control wells at equivalent concentrations maximum concentration DMSO [0.1%].

2. RNA Extraction

Following aspiration of cell culture supernatants, cells were washed with phosphate buffered saline [PBS]. PBS was removed and discarded and RNA was extracted and purified using the PureLink® RNA Mini Kit according to the manufacturer’s instructions. The concentration of RNA in the purified samples was measured by NanoVue-Plus Spectrophotometer to ascertain the purity of samples containing high-quality total RNA with ratios between 1.8 and 2.1, the absorbance is measured at 260 and 280 nm. 1μl sample was used in this analysis. Measurement of absorbance at 260nm (A260) and at 280nm (A280) wavelength was taken, and the A260/A280 ratio determined. An A260/A280 ratio of between 1.8 and 2.1 was accepted as being of sufficient quality for subsequent use.

3. Reverse transcription to cDNA

Reverse transcription using 2μg of total RNA was performed using first strand cDNA synthesis Kit from (Fermentas, UK). Briefly xμl nuclease free water mix and 1μl Random Hexamer primers were added to the 2μg sample of RNA (total volume 12μl), mixed and incubated at 65°C for 5 min. Microtubes were then placed on ice before adding: 4μl 5x reaction buffer, 1μl Ribolock RNase Inhibitor, 2μl dNTP mix and 1μl RevertAid M-MuLVRT. Microtubes were
incubated for 5 min at 25°C followed by 60 min at 42°C then terminate the reaction by heating at 70°C for 5min.

4. Quantitative Real Time PCR

All reagents, samples and enzymes were kept on ice throughout the experiment. Quantitative real time RT-PCR was performed using the human CCL5, CX3CL1, CCL11 primers and β-actin as housekeeping gene (Eurofins MWG Operon). Please see Table 4.1. Primers were reconstituted in molecular grade water. For each 20μl reaction, 10μl mastermix Fast SYBR® Green Master Mix (fisher scientific), 250-400nM of both sense and antisense primers were used, 2μl cDNA and H2O to a final volume of 20μl. Reactions were pipetted into a 96 well plate (stratagene) in duplicate, alongside with negative controls (water and non-transcribed RNA). Plates were sealed with Microseal sealer film (Bio-Rad) and PCR performed on thermocycler that monitored sample fluorescence. PCR cycling conditions included incubation at 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 minute. Integration of the fluorescent SYBR Green into the PCR product was monitored after each annealing step. Amplification of one specific product was confirmed by melt curve analysis where a single melting peak eliminated the possibility of primer-dimer association. All QPCR samples were run in Stratagene Mx3000P QPCR system. The cycle threshold (Ct), which is the point at which PCR product is detectable above a fixed threshold, was determined for both the target gene and the housekeeping gene (β-actin).Expression of target gene was calculated using the $2^{-\Delta\Delta Ct}$ method [298].Data was normalised to the housekeeping gene β-actin by subtracting the Ct for β-actin from the Ct for the target gene. Data for
control samples (Basal) was then subtracted from all data sets resulting in values for \( \Delta\Delta C_t \). The relative expression of the target gene compared with control was then calculated using the following equation:

\[
\text{Relative expression (Fold Increase)} = 2^{-\Delta\Delta C_t}
\]

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Product size (bp)</th>
<th>Gene ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human CCL5</td>
<td>F 5′-TCTGCCTCTGCATCTG-3′ R 5′-GGGCAATGTAGGCAAAGCA-3′</td>
<td>69</td>
<td>NM_002985.2</td>
</tr>
<tr>
<td>Human CCL11</td>
<td>F 5′-AATGTCCCCAGAAAGCTCTG-3′ R 5′-TCCTGCACCCACTTCTTCTT-3′</td>
<td>82</td>
<td>NM_002986.2</td>
</tr>
<tr>
<td>Human CX3CL1</td>
<td>F 5′-GCTGAGGAACCCATCAT-3′ R 5′-GAGGCTCTTGGTAGTGAACA-3′</td>
<td>165</td>
<td>NM_002996.3</td>
</tr>
<tr>
<td>Human GILZ</td>
<td>F 5′-CCTCCCCCTTCTCCTGC-3′ R 5′-GAACCTTAAGCAGTCATCC-3′</td>
<td>294</td>
<td>NM_004089.3</td>
</tr>
<tr>
<td>Human MKP-1</td>
<td>F 5′-GACGCTCTCTCAGTCAC-3′ R 5′-GGCGGTGTCCGAAGAAAG-3′</td>
<td>82</td>
<td>NM_004417.3</td>
</tr>
<tr>
<td>Human β-actin</td>
<td>F 5′-CCAAGGCAACCGGAGAAGAT-3′ R 5′-GTCCCGGCCAGCCAGGTCC-3′</td>
<td>559</td>
<td>NM_001101.3</td>
</tr>
</tbody>
</table>

Table 2.6: Primer sequences for quantitative real-time PCR (F, forward primer and R, reverse primer. bp, base pairs)

PCR products were visualized by electrophoresis on a 2% agarose gel (2 g agarose was added to Tris-acetate-EDTA (TAE) buffer) that was heated until the agarose had dissolved. The solution was cooled down until it turned opaque, after which ethidium bromide (4 μl) was then added to the solution. To construct the gel, the solution was poured into an appropriately sized tray to which a comb was added to leave space for the sample loading, and left to set for 20 min. A 100bp DNA ladder (Promega) was also used to verify sizes of the PCR products. The gel was run at 100 volts and 55 mA for approximately 45
minutes. The separated products in the gel were then visualized with a UV transilluminator and photographed.

**VI. IMMUNOFLOUORESCENCE**

Immunofluorescence is a technique permitting the visualization of a specific protein or antigen in cells or tissue sections via the use of specific antibody chemically conjugated with a fluorescent dye. There are two main types of immunofluorescence staining methods: 1) direct immunofluorescence staining in which the primary antibody is labelled with fluorescence dye, and 2) indirect immunofluorescence staining in which primary antibody is recognized by a secondary antibody labelled with fluorochrome. In our study indirect immunofluorescence was used.

1. **Sample preparation**

BSMC were seeded at $8 \times 10^3$ cells per well into Lab-TekTM II 8 well chamber slides (Nunc, ThermoFisher). They were placed in an incubator at 37°C in 5% CO2/95% air and grown for 24 hours prior the staining.

2. **Method**

Cells were then rinsed with 1x PBS (Sigma-Aldrich) and fixed with 100 µl/well of 10% neutral buffered formalin for 15 mins at room temperature, washed 3 times with 1x PBS, then permeabilized with ice-cold 90% methanol for 15 min at room temperature. To block nonspecific binding sites, cells were incubated with 100 µl/well 3% BSA/PBS for 1 hour at room temperature. Primary antibodies and Isotype controls were diluted in 3% BSA/PBS and
incubated overnight at 4 °C; the chamber slides were placed on moistened tissue in order to ensure they remained humidified during the primary incubation. Following incubation with the primary antibody, cells were washed with 1x Phosphate Buffered Saline (PBS). Alexa Fluor® 488 secondary antibody was diluted in 3% BSA/PBS and incubated for 45 min at room temperature. Cells were washed again following incubation with secondary antibody. Cells were mounted using VectaMount with DAPI (Vector Laboratories) and coverslip (Fisher Scientific). Immunofluorescence microscopy was performed with an inverted microscope (Carl Zeiss Inc). Data acquisition and analysis was performed by the Zen 2012 lite software (Carl Zeiss Inc). Summary of Primary and secondary antibodies used for immunofluorescence can be found in Table 2.7.

<table>
<thead>
<tr>
<th></th>
<th>Host species</th>
<th>Dilution</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>K_Ca3.1</td>
<td>Rabbit</td>
<td>1:50</td>
<td>biorbyt</td>
</tr>
<tr>
<td>HDAC-1</td>
<td>Rabbit</td>
<td>1:100</td>
<td>Cell signalling</td>
</tr>
<tr>
<td>Alexa Fluor® 488</td>
<td>Goat</td>
<td>1:300</td>
<td>Life technology</td>
</tr>
<tr>
<td>Isotype Control</td>
<td>normal rabbit IgG</td>
<td></td>
<td>Santa Cruz</td>
</tr>
</tbody>
</table>

**Table 2.7**: Primary and Secondary antibodies used for immunofluorescence.
VII. FLOW CYTOMETRY

Flow cytometry is another powerful technique used to accurately quantify the expression (both intracellular or cell surface) of a particular protein in a cell population using fluorochrome labelled antibodies. This method allows characterisation of labelled individual cells suspended in a focused stream of fluid, which passes through different laser beams. FACScan flow cytometer contains various colour detectors to evaluate the different colours of light emitted by the fluorochromes attached to the antibodies against the protein of interest expressed by BSMC. The flow cytometer has also light detector to allow the forward light scatter measurement (for relative cell size) and side scatter measurement (for cell granularity).

1. Sample Preparation

In order to assess the expression of Protein phosphatase 5 (PP5) within the BSM bundle as well as the effect of $K_{Ca3.1}$ inhibitors the cells were plated in 6 well plate serum deprived and time course was performed by stimulating the cells with 25ng/ml of IFNγ and 10ng/ml TNFα for 2, 4, 6, 24 hours. In the inhibitory effect experiments the cells were stimulated with $K_{Ca3.1}$ channel blockers ICA-17043 (100nM) or TRAM-34 (200nM) for 2 hours before being stimulated with a combination of 25ng/ml of IFNγ and 10ng/ml TNFα for an additional 24 hours. The vehicle dimethylsulphoxide (DMSO) for both $K_{Ca3.1}$ inhibitors vehicle was added to control wells at equivalent concentrations maximum concentration DMSO [0.1%].
2. **Method**

BSMC were harvested using Accutase (Sigma Aldrich), centrifuged at 227 g for 10 minutes. The supernatants were removed and the pellet washed in 0.5% BSA/PBS, centrifuged again before the supernatant discarded. BSMC in the pellet were then fixed by re-suspending it in 1 ml of 4% paraformaldehyde (PFA)/0.1% saponin left 15 minutes on ice. Following washing the cells were then permeabilized in 1x PBS/0.5% BSA and 0.1% saponin (Sigma-Aldrich). Cells were split equally between FACS tubes, and centrifuged at 227 g for 8 minutes, washed and block in 200 µl of 3% BSA/PBS for 20 minutes at 4°C. BSMC were washed again, and cells were re-suspended in 100 µl of 1x PBS/0.5% BSA containing either 2 μg/ml anti- PP5 antibody (Santa Cruz) or isotype matched control antibody. The cells were left with the antibodies overnight at 4°C. After removal of the primary antibody by centrifugation the cells and washing, BSMC were incubated with the secondary FITC labelled antibody 1:10 dilution and left on ice for 1 hour. BSMC were washed once with 1 ml 1 x PBS/0.5% BSA and 0.1% saponin and then centrifuged at 227 x g for 8 minutes. The supernatant was removed and the BSMC re-suspended in 350 µl of 1 x PBS/0.5% BSA and mixed thoroughly. PP5 expression was assessed by flow cytometry (FACScan, CellQuest software: BD Biosciences) and quantified as fold difference in geometric mean fluorescent intensity (GMFI) over the isotype matched control antibody.
VIII. IMMUNOHISTOCHEMISTRY

Bronchial biopsies from subjects with asthma obtained through bronchoscopy were fixed in acetone and embedded in glycomethacrylate (GMA). Antibody titrations were performed to determine the optimal antibody dilution for the section staining the antibody was diluted (1:50, 1:100, 1:200, 1:400 and 1:500) from the stock.) Sequential 2μm sections were cut from GMA embedded sections and floated on 0.2% ammonia water. Sections were collected onto positively charged slides and left to dry overnight at room temperature in glass dish. The staining was performed using EnVision™ FLEX Mini Kit (DAKO) as followed: the slides were incubated with Peroxidase Block for 10 min at room temperature in humid chamber before being washed using EnVision™ FLEX Wash Buffer. The slides were then incubated with diluted anti-KCa3.1 (biorbyt) 2.5μg/ml or α-smooth muscle actin (Merch Millipore) 1μg/ml, the isotype controls used are same isotype and same concentration, all the antibodies and the isotype controls were diluted in EnVision™ FLEX Antibody Diluent buffer. The slides were incubated for 1 hour at room temperature washed 3x5 min in EnVision™ FLEX Wash Buffer, incubated with EnVision™ FLEX/HRP for 30 min at RT, washed 3 x 5 min before being developed using ready to use EnVision™ FLEX DAB + Chromogen and substrate (1drop in 1ml of substrate) for 5-10min. The slides were then washed with water then counterstained using Mayer’s Haematoxylin for 10 min, washed in running tap water and dried before mounting the slides using DPX mounting solution (Sigma Aldrich).
IX. SMALL HAIRPIN RNA GENE KNOCKDOWN OF K\textsubscript{Ca}3.1 CHANNELS

1. Determination of the transduction efficiency of the recombinant adenoviral vector using a control GFP-tagged shRNA adenovirus:

   All transient transductions were conducted using (shRNA) adenovirus of K\textsubscript{Ca}3.1 channels expressing adenoviruses (Ad5C20Att01) were purchased from BioFocusDPI (Leden, the Netherlands).

   Cultured BSM at 50–70% confluence were exposed to different multiplicity of infection (MOI, MOI is calculated as PFU/cell numbers) at 1, 5, 10, 30, 50 MOI per cell and then the transduction were monitored after 24, 48, and 72 hours. Transduction efficiency was visualised using immunofluorescence microscope. Photographs were taken at 24, 48 and 72 hours post infection with 10× objective.

2. K\textsubscript{Ca}3.1 Knockdown in BSMC using different shRNA adenoviruses:

   BSMC seeded into 6-well plates at a concentration 2 x 10\textsuperscript{5} cells were plated for 24 hours until they reached 50 to 60% confluence; the media was changed with antibiotic free DMEM and they were transducted using 30 MOI of different shRNA adenoviruses expressing adenoviruses (Ad5C20Att01) targeting K\textsubscript{Ca}3.1 channel called V1, V2, V3 and V8 purchased from BioFocus DPI (Leden, the Netherlands). shRNA adenovirus-GFP used as controls and for monitoring transduction efficiency. The cells were serum deprived for 3
hours and then stimulated by fluticasone propionate (100 nM) for 2 hours alone or in combination with TNFα/IFNγ for an additional 24h. After 24 hours, proteins supernatants were collected for ELISA assays of CCL5, CX3CL1 and CCL11 while cells were lysed for total cell extract preparation as described above.

X. CELL VIABILITY (MTT ASSAY)

Confluent BSMC plated in 96-well plates at 5000 cells per well were incubated with ICA-17043 (10 nM, 100 nM, and 1 μM) or with TRAM-34 (20 nM, 200 nM, and 2 μM) for 2 hours and then followed by TNFα/IFNγ for an additional 24 hours. MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed by adding 20 μL of MTT solution (5 mg/ml in PBS) to each well, and incubated for three hours at 37°C. The media was removed carefully and 100 μl of MTT solvent (4mM HCL, 0.1% Nondet-P-40 all in isopropanol) was added to each well and incubated for 15min to allow the solubilization the blue-coloured tetrazolium. Optical density was then read at 590 nm on EnSpire Multimode (Perkin Elmer) plate reader. Viability was compared to that of control cells, with viability of controls defined as 100%.

XI. ANNEXIN V AND PROPIDIUM IODIDE APOPTOSIS ASSAY

Apoptosis was detected using the annexin V-FITC apoptosis detection kit I (BD Biosciences). BSMC were washed twice with cold PBS and then resuspended in 1X binding buffer at a concentration of 1 x 106 cells/ml. 100 μl of the solution (1 x 105 cells) was then transferred to a 5 ml culture tube along
with the addition of annexin V-FITC (5 μl) and propidium iodide (PI) (5 μl). The cells were vortexed and incubated for 15 minutes at room temperature in the dark. 1X binding buffer (400 μl) was added to each tube, and analysed by flow cytometry as described in section 2.9. Controls such as unstained cells, or cells stained with either annexin V-FITC alone (no PI), or PI alone (no annexin V-FITC) were used to set up compensation and quadrants.

XII. PATCH-CLAMP ELECTROPHYSIOLOGY

The whole-cell variant of the patch-clamp technique was used as previously described [293]. Briefly, BSMC were cultured until confluent and then maintained in ITS media. Cells were then incubated overnight with TNF-α and IFN-γ for 24 hours, with control cells being maintained under identical conditions without added cytokines. Prior to electrophysiological recording, the cells were trypsinized and resuspended in BSMC culture media complete DMEM. Cells were pipetted into a heated (30°C) recording chamber ready for patching. Individual (nonadhered) cells were voltage clamped using the whole-cell variant of the patch-clamp technique. Briefly, voltage commands were applied to potentials ranging from 2100 to +100mV from a holding potential of 220mV. Membrane currents were recorded and subsequently plotted against command potential. Solutions used were as follows: external (mM)—NaCl (140), KCl (5), MgCl2 (1), and CaCl2 (2) (pH was adjusted to 7.4 using NaOH); internal (mM)—KCl (140), MgCl2 (2), HEPES (10), ATP (2), and GTP (0.1) (pH was adjusted to 7.3 using KOH).
These experiments were done by Dr Mark Duffy (senior research associate from National Institute for Health Research. Leicester. Respiratory Biomedical Research Unit Glenfield Hospital) part of our collaboration.

XIII. DATA ANALYSIS AND STATISTICS

Data is expressed as Means ± SEM for all experiments unless otherwise stated. Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, USA). When a comparison of only two data sets was needed an unpaired Student's t test was performed. When comparing more than 2 data sets a one-way analysis of variance (ANOVA) was performed, with either a Bonferroni's post-test to compare between the two specific data sets, or a Dunnet post-test to compare all data sets with a control data set. P values less than 0.05 were accepted as significant.
CHAPTER 3

Establishing a cellular model of corticosteroid resistance using Human Bronchial Smooth Muscle Cells (HBSMC)
I. RATIONALE

Although many patients with asthma are well controlled with inhaled glucocorticoid (GC) therapy, roughly 5 to 10% of patients are poorly responsive to this treatment. These patients are responsible for a disproportionate share of asthma-related health care costs and morbidity [163].

Despite significant progress in the field, the precise molecular mechanisms mediating GC insensitivity in severe asthma are poorly understood. Several potential mechanisms have been postulated, identified mostly from studies in immune cells including alveolar macrophages and circulating peripheral blood mononuclear cells (PBMCs) obtained from patients with GC insensitivity [121, 168]. In addition to infiltrating inflammatory cells, the bronchial smooth muscle (BSM) is another key player in severe asthma pathophysiology, as these cells contribute to airway hyperresponsiveness (AHR) and airway obstruction. Furthermore, BSM has been attributed a role in the inflammatory progression of this disease by producing various inflammatory mediators such as chemokines like CXCL10, CCL5, CX1CL3 and CCL11. These chemokines are involved in the recruitment of inflammatory cells into the lung [44]. The need to understand the underlying mechanisms driving these steroid-resistant pathways in BSM could then lead to novel therapeutic approaches.

Studies from Amrani and colleagues were the first who reported a novel \textit{in vitro} model of inflammation-associated steroid resistance by showing that
tracheal smooth muscle cells exposed to a cytokine combination consisting of both TNF-α/IFN-γ become insensitive to the anti-inflammatory actions of fluticasone propionate (FP) [166, 187, 247, 299]. Indeed, the expression of a number of “pro-asthmatic” mediators including chemokines, transcription factors and ectoenzymes by TNF-α/IFN-γ combination are resistant to the inhibitory action of fluticasone when compared to responses induced by TNF-α alone [300]. As described in the introduction (section III. 1.4), these BSM-derived pro-asthmatic mediators could participate in the pathogenesis of asthma via different mechanisms including lymphocyte chemoattraction (by the chemokines CX3CL1, CCL5) or expression of additional inflammatory mediators (by the transcription factor IRF-1), impairment of corticosteroid signalling (by the dominant negative isoform GRβ). As well that activation of the transcription factor interferon regulatory factor 1 (IRF-1) not only regulates the transcriptional induction of CD38, but is also responsible for cytokine-induced steroid resistance in part via the suppression of GRα activities as well via the reduction of GRIP-1 from the GR transcriptional regulatory complexes [187, 247, 299]. Induction by TNF-α/IFN-γ of CD38, a cell surface ectoenzyme that regulates calcium signalling, could also play a role in airway hyperresponsiveness by increasing BSM sensitivity to contractile agonists [221]. These different studies raise the novel concept that corticosteroid insensitivity seen in severe asthma could also result from structural components within the airways being less responsive to the anti-inflammatory action of corticosteroid therapy. Studies from Amrani’s group strongly suggest that corticosteroid insensitivity could also develop in the smooth muscle layers.
II. AIM

Because previous studies describing the existence of corticosteroid resistance in airway smooth muscle were performed using tracheal smooth muscle cells, the main aim of this chapter was to investigate whether impaired steroid sensitivity could also develop in bronchial smooth muscle (defined thereafter as HBSMC), a clinically more relevant muscle tissue in asthma. I determined the profile of chemokine production (CCL5, CXCL10 and CCL11) by cultured human bronchial smooth muscle cells isolated from both healthy and asthmatic subjects (Table 2.1) exposed to i) TNF-α alone, ii) TNF-α/IFN-γ combination and iii) TNF-α/IFN-γ combination plus fluticasone.

III. RESULTS

1. TNF-α dose-dependently induced chemokine expression by HBSM Cells:

We first determine the production of CCL5, CXCL10 and CCL11 from cultured HBSMC isolated from healthy controls and asthmatics in response to increasing concentrations of TNF-α (0.5-100 ng/ml, 24 hours). All conditions used sub-confluent, growth arrested cultured HBSMC (passage 3-5) and were performed in triplicate in at least n=3 subjects. Cell culture supernatants were collected for each condition and used to quantify the levels of these chemokines by ELISA assays as described in method section.

The studies show that TNF-α stimulation increased production of CCL5, CXCL10 and CCL11 in a dose-dependent manner. The maximum chemokine
induction took place at a concentration of 10ng/ml TNF-α while higher concentrations led to surprising decrease in all chemokine expression. Therefore, in all subsequent experiments a concentration of 10ng/ml was used to treat HBSMC. For example, in cells exposed to 10ng/ml TNF-α, levels of CCL11 were 614 ± 135.5pg/ml in healthy HBSMC (Figure 3.1A) and (1693 ± 139.9pg/ml) in asthmatic HBSMC (Figure 3.1B). Levels of CCL5 were 3872 ± 366.8pg/ml in healthy HBSMC (Figure 3.2A) and 10220 ± 1282pg/ml in asthmatic HBSMC (Figure 3.2B); Levels CXCL10 were 32817 ± 2100pg/ml in healthy control (Figure 3.3A) and 61567 ± 5787pg/ml in asthmatic patients (Figure 3.3B). These findings correlate well with previous findings from different groups [301]. It is interesting to note that at higher concentrations (>10 ng/ml), levels of chemokines did not increase with TNF-α concentrations. In addition, chemokine production was seen in cells exposed to TNF-α at 0.5 ng/ml, showing that HBSMC are particularly sensitive to low concentrations of TNF-α. Another important observation is the fact that, with the exception of CCL11, production of CCL5 and CXCL10 induced by TNF-α were found to be significantly higher in asthmatic HBSMC than in healthy HBSMC. Figures 3.2 and 3.3 showed that the production of CCL5 and CXCL10 were much higher at low concentrations of TNF-α. For example, TNF-α-induced CCL5 at 0.1 and 0.5 ng/ml were 149.2± 62.30 and 934.7 ± 173.8 in asthmatic HBSMC and none in healthy controls. TNF-α-induced CXCL10 at 0.1 and 0.5ng/ml were 3060±272.8and 6227 ± 522.4 in healthy HBSMC while in asthmatic HBSMC CXCL10 levels were 7213 ± 2557and 15178 ± 2274. Unpaired T-test analyses
revealed that induced chemokine levels between asthmatic and healthy HBSMC were statistically different (p<0.05).

These studies show that TNF-α is a potent inducer of three important pro-asthmatic chemokines in HBSMC, with induction being visible acting at the picogram/ml range. More importantly, these data show that asthmatic HBSMC produced significantly more chemokines than healthy HBSMC in response to TNF-α stimulation raising the possibility of abnormal HBSMC phenotype in asthma.
Figure 3.1 Dose-dependent induction of CCL11 by TNF-α: HBSMC (from healthy controls and asthmatic subjects) were exposed for 24 hours to the indicated concentrations of TNF-α, after which levels of CCL11 expression was measured in the supernatants by ELISA. Results presented are the means ± SEM of n=4 healthy controls (A) and n=3 asthmatic subjects (B) with each condition performed in triplicate. ***P < 0.001 compared with Basal.
Figure 3.2: Dose-dependent induction of CCL5 by TNF-α: HBSMC (from healthy controls and asthmatic subjects) were exposed for 24 hours to the indicated concentrations of TNF-α, after which levels of CCL5 expression was measured in the supernatants by ELISA. Results presented are the means ± SEM of n=4 healthy controls (A) and n=3 asthmatic subjects (B) with each condition performed in triplicate. ***P < 0.001 compared with Basal.
Figure 3.3: Dose-dependent induction of CXCL10 by TNF-α: HBSMC (from healthy controls and asthmatic subjects) were exposed for 24 hours to the indicated concentrations of TNF-α, after which levels of CXCL10 expression was measured in the supernatants by ELISA. Results presented are the means ± SEM of n=4 healthy controls (A) and n=3 asthmatic subjects (B) with each condition performed in triplicate. ***P < 0.001 compared with Basal.
2. IFN-γ augments TNF-α-induced chemokine expression by HBSM Cells:

The next set of experiments was to determine whether IFN-γ modulates chemokine production induced by TNF-α in HBSMC. Sub-confluent, growth arrested cultured HBSMC from healthy subjects were incubated with 10ng/ml TNF-α alone or in the presence of different concentrations of IFN-γ (0-100 ng/ml) for 24 hours. Cell culture supernatants were collected and ELISA assays were used to quantify the levels of CCL11, CCL5, CXCL10 and CX3CL1.

With the exception of CCL11 induction, the production of all other three chemokines by TNF-α was significantly enhanced by the presence of IFN-γ. This enhancing effect was not dose-dependent (with the exception of CXCL10, Figure 3.4C), and was more pronounced in cells co-treated with TNF-α and IFN-γ at 25ng/ml. As shown in the figures, HBSMC co-treated with 25ng/ml IFN-γ significantly enhanced TNF-α-induced CCL5 from 2261 ± 302.4pg/ml to 4145 ± 224.8pg/ml in cytokine combination (Figure 3.4B). CXCL10 was also increased by cytokine combination from 22005 ± 1252pg/ml to 45762 ± 2860pg/ml (Figure 3.4C). As mentioned previously, there were no significant differences in CCL11 expression by cells treated with TNF-α alone (190.7 ± 16.35 pg/ml) or co-treated with 25 ng/ml IFN-γ (169.4 ± 6.964 pg/ml) (Figure 3.4A). With regard to CX3CL1 production, TNF-α (10ng/ml) alone failed to induce any detectable release compared to basal levels (Figure 3.4D). Interestingly, HBSMC treated with a combination of IFN-γ and TNF-α led to a significant induction of CX3CL1 reaching 1126 ± 71.38pg/ml.
These data suggest that IFN-γ significantly enhanced the ability of TNF-α to induce the production to CX3CL1, CXCL10 and CCL5 but not CCL11 which in contrast appears to be inhibited by IFN-γ. This shows that the enhancing effects of IFN-γ are gene specific but not concentration dependent.
Figure 3.4: Potentiating effect of IFN-γ on TNF-α-induced chemokine induction:
HBSM cells were either unstimulated or stimulated with increasing concentration of
IFN-γ (1–100 ng/ml) in combination with TNF-α 10 ng/ml for 24 hours (A) CCL11 (B)
CCL5 (C) CXCL10 and (D) CX3CL1 were released into the supernatant measured by
ELISA. Results presented are the means ± SEM within each experiment, values were
determined in triplicate. ***P < 0.001 compared with Basal. (n = 3) from independent
donors.
3. Production of CCL11, CCL5, CXCL10 and CX3CL1 by TNF-α and IFN-γ is resistant to fluticasone treatment

To determine whether the production of cytokine-induced CCL11, CCL5, CXCL10 and CX3CL1 release was sensitive or insensitive to the suppressive action of corticosteroids, HBSMC were incubated with fluticasone propionate (FP) at 100 nM, a concentration known to suppress chemokine production in steroid sensitive states, i.e., cells exposed to TNF-α alone (see Figure 3.5). Sub-confluent, growth arrested cultured HBSMC were pre-treated with FP (100nM) for 2 hours then stimulated with TNF-α (10ng/ml) alone or with TNF-α (10ng/ml) and IFN-γ (25ng/ml) for 24 hours. Cell culture supernatants were collected and used to quantify the levels of CCL11, CCL5, CXCL10 and CX3CL1 using ELISA as described in method section.

The studies show that production of CCL11 by TNF-α alone (1156 ± 63.44 pg/ml) was completely suppressed by fluticasone (Figure. 3.5A). Although presence of IFN-γ CCL11 led to a smaller CCL11 production (1052 ± 56.31 pg/ml), fluticasone only manage to reduce by 50% the CCL11 production induced by cytokine combination (900.3 ± 78.93 pg/ml) (Figure. 3.5A). Similarly, levels of CCL5 by TNF-α alone (5835 ± 415.1 pg/ml) were dramatically reduced to 1978 ± 246.5 pg/ml by FP with almost a 66.10 ± 4.52% inhibition Figure. 3.5B). By contrast, in HBSMC treated with both TNF-α and IFN-γ, CCL5 production was completely resistant to FP treatment with levels being 10212 ± 440.3 pg/ml and 11222 ± 495 pg/ml in the absence and presence of FP, respectively (Figure. 3.5B). Production of CXCL10 induced by TNF-α alone was also reduced by FP with levels varying from 104765 ± 900
pg/ml to 48235 ± 631 pg/ml in the absence and presence of FP, respectively giving an approximate 53.9 ± 3.02% inhibition (Figure. 3.6B). By contrast, in HBSMC treated with both TNF-α and IFN-γ, CXCL10 production was completely resistant to FP treatment with levels being 142065 ± 1837 pg/ml and 154102 ± 3657 pg/ml in the absence and presence of FP, respectively (Figure. 3.6B). Whereas TNF-α alone has no effect on CX3CL1 expression, only the combination of TNF-α and IFN-γ significantly increased CX3CL1 secretion levels in HBSMC; Interestingly, FP significantly enhanced cytokine-induced CX3CL1 production from 5939 ± 564.3 pg/ml to 8436 ± 1074 pg/ml; Figure. 3.6A).

These studies shows that HBSMC exposed to TNF-α and IFN-γ become resistant to the anti-inflammatory action of fluticasone as previously described in tracheal smooth muscle cells [300].
Figure 3.5: Effect of the fluticasone on cytokine-induced CCL11 and CCL5 production: HBSMC cells were growth arrested for 24 hours and pretreated for 2 hours with fluticasone (100nM) before stimulation with TNF-α (10ng/ml) alone or in combination with IFN-γ (25ng/ml) for 24 hours. CCL11 (A) or CCL5 (B) released into the supernatants was measured by ELISA. Results presented are the means ± SEM in cells from n = 4 from independent donors, each point being done in triplicate. **P<0.05 Significant difference from non-stimulated control cells; *** P<0.05, significant difference from TNF-α stimulated control group.
Figure 3.6: Effect of the steroid fluticasone on cytokine-induced CX3CL1 and CXCL10 production. HBSMC were growth arrested for 24 hours and pretreated for 2 hours with fluticasone (100nM) before stimulation with TNF-α (10ng/ml) alone or in combination with IFN-γ (25ng/ml) for 24 hours. Levels of CXCL3 (A) or CXCL10 (B) in the supernatants were measured by ELISA. Results presented are the means ± SEM of cells from n=4 independent donors, each condition performed in triplicate. *P<0.05 significant difference from non-stimulated control (basal); ***P<0.05 significant difference from stimulated control group (TNF-α).
IV. DISCUSSION

A proportion of asthmatic patients (5–10%) still experiences uncontrolled asthma despite receiving appropriate or even high doses of corticosteroid therapy. These patients often referred as having “severe” or “refractory” asthma, have the highest morbidity and mortality, and account for more than 50% of the total healthcare costs in the UK with a striking estimate of 2 billion pounds/annum (AsthmaUK). Alternative therapeutic interventions are urgently needed to address this unmet clinical need [14, 15]. Developing an experimental model either In vivo or In vitro that can simulate steroid insensitivity in asthmatic patients will certainly provide the possibility of understanding the causes of corticosteroid insensitivity seen in severe asthmatics. This chapter shows that HBSMC exposed to a combination of two “pro-asthmatic cytokines” become refractory to the inhibitory action of fluticasone, thus providing a unique model to investigate the underlying molecular mechanisms of corticosteroid insensitivity.

The initial aim of the present chapter was to determine whether smooth muscle cells isolated from bronchial tissues would reproduce the same sensitivity profile to corticosteroids compared to previous studies that used smooth muscle cells isolated from tracheal tissues. We first found that HBSMC were responding to TNF-α in a dose dependent manner by producing various “pro-asthmatic” chemokines at concentrations starting at the picogram/ml range and reaching a maximum induction at 10 ng/ml. Zhang and colleagues who used HBSMC as a cellular model also confirmed that these cells responded to
similar TNF-α concentrations in the production of Thymic Stromal Lymphopoietin (TSLP) [302]. In human tracheal smooth muscle cells a similar observation was made where levels of different inflammatory chemokines such as CCL11 [301] and IL-8 [303] were also dose-dependently increased by TNF-α with a significant response also starting at 0.1 ng/ml and reaching a plateau at 10 ng/ml. A more recent report published in 2012 showed that indeed cultured human tracheal smooth muscle cells produced high levels of pentraxin 3 in response to 0.1 ng/ml TNF-α [304]. Taken together studies show that smooth muscle cells from bronchial and tracheal tissues share somewhat an equal sensitivity to TNF-stimulation at least with regard to the induction of pro-inflammatory mediators. Our studies showing an increased production of chemokines by asthmatic versus healthy HBSMC do support the suggestion raised by Knox's group that asthmatic cells have an abnormal phenotype with respect to chemokine secretion [305, 306]. Although we did not investigate the mechanisms behind the hypersecretion of chemokines by asthmatic HBSMC, others showed an increased NF-kB pathways in these cells [305]. Future studies will address this important observation.

The other important conclusion of this chapter relates to sensitivity of HBSMC to corticosteroids. Our studies here found that HBSMC treated with TNF-α/IFN-γ lost their sensitivity to fluticasone when compared to cells treated with TNF-α (Figures 3.5 and 3.6). It is interesting to note that in TNF-α treated HBSMC, fluticasone appears to suppress chemokine induction with different efficiencies ranging from a complete (CCL11) to ~60% inhibition (CCL5 and CXCL10). Our findings are somewhat in agreement with Seidel and colleagues’
work who found that TNF-α-induced CXCL10 was similarly reduced by ~50% by fluticasone [307] as reported in our study. Other studies revealed that dexamethasone almost completely repressed TNF-α-induced IL-6 or CCL5 expression in human tracheal smooth muscle cells [308] while Pang and colleagues found that both dexamethasone and fluticasone reduced only by ~50-70% the production of CCL11 [301] and CXCL8 [303] induced by TNF-α. Together, these data suggest that the anti-inflammatory actions of corticosteroids in HBSMC are highly gene-specific and show the complexity of the mechanisms of action used by corticosteroids to repress expression of different inflammatory mediators.

Another important observation we made was that HBSMC incubated with both TNF-α/IFN-γ completely lost their sensitivity to corticosteroids. The production of CX3CL1, CXCL10 and CCL5 by TNF-α/IFN-γ were unaffected by fluticasone treatment (Figures 3.5 and 3.6). Previous reports from Amrani’s groups revealed that a similar phenomenon happens with human tracheal smooth muscle cells exposed to TNF-α/IFN-γ which resulted in the production of an array of “pro-asthmatic” proteins ranging from adhesion molecules, chemokines, and transcription factors [166, 247, 309, 310]. More importantly, TNF-α/IFN-γ responses in tracheal smooth muscle were completely insensitive to fluticasone treatment even when used at high concentrations (>100nM) [166, 247]. These studies reinforce the concept that corticosteroid resistance may develop in the structural components of the airways including the bronchial smooth muscle cells [311].
The aim of following chapters is to exploit this unique HBSMC model of corticosteroid resistance with the goal of dissecting the molecular mechanisms that impair corticosteroid pathways.
K_{Ca}3.1 channels in HBSMC in Health and Asthma
I. **RATIONALE:**

Ion channels are the molecular units that regulate electrical signalling in cells. Many physiological processes are reliant on this signalling mechanism, as a dysregulation often has severe pathophysiological consequences [248]. Potassium (K\(^+\)) ion channels are transmembrane proteins from the major superfamily of ion channels present in both prokaryotic and eukaryotic cells. These channels have the ability to selectively facilitate the permeation of K\(^+\) between intracellular and extracellular Environments. Calcium-activated K\(^+\) (K\(_{Ca}\)) channels form the main family of these channels [284]. There are few reports that have reported that human bronchial smooth muscle cells and tissues express the large conductance K\(_{Ca}\)1.1 (BK\(_{Ca}\)) channel, the low conductance SKCa family (K\(_{Ca}\)2.1–2.3) and the intermediate conductance channel K\(_{Ca}\)3.1 (KCNN4, also known previously as IK\(_{Ca}\)1) [272, 293, 312-314]. Their implications in asthma pathogenesis still remain to be investigated.

The intermediate-conductance Ca\(^{2+}\)-activated K\(^+\) channel K\(_{Ca}\)3.1 channel (also referred as IK1, SK4, or KCNN4) belongs to the small and intermediate-conductance-calcium-activated potassium channel subfamily which gated by the binding of intracellular Ca\(^{2+}\) to calmodulin (CaM) [272]. The K\(_{Ca}\)3.1 channel controls the activity of numerous structural and inflammatory cells, including lymphocytes [275], mast cells [282], and dedifferentiated smooth muscle cells [293], through the regulation of cell proliferation, activation [293], migration [10] and mediator release [300, 315].

K\(_{Ca}\)3.1 channel has been show to closely associate with the progression of number of human diseases. Inhibitors of K\(_{Ca}\)3.1 channels, such as TRAM-34,
are effective in treating various inflammatory diseases in preclinical models, including atherosclerosis [274, 277], neurodegenerative disorders [283], autoimmune encephalomyelitis [316], and coronary vasculoproliferative diseases [317]. With respect to asthma, the initial evidence came from a study which showed that TRAM-34 prevented the development of eosinophilic inflammation, airway hyperresponsiveness, and airway remodeling in a murine model of allergic asthma [318]. Subsequent preclinical studies confirmed the role of $K_{Ca}3.1$ channels in asthma using different models of allergic asthma [295, 319]. The mechanisms by which $K_{Ca}3.1$ channels contribute to the pathogenesis of allergic asthma have not yet be defined, but Bradding’s group provided evidence that $K_{Ca}3.1$ channels are key in regulating mast cell degranulation and migration [282, 293] as well as fibrocyte migration [291]. Others have implicated $K_{Ca}3.1$ channels in the migration of lung dendritic cells to CCL19 and CCL21 [292]. These observations suggested that activation of $K_{Ca}3.1$ channels on structural (smooth muscle/fibroblasts) airway cells may represent an important pathway driving key features of allergic asthma.

II. AIM

The main aim of this chapter was to provide additional evidence for the existence of $K_{Ca}3.1$ channels \textit{in vivo} in the bronchial smooth muscle bundles and cultured HBSMC and whether $K_{Ca}3.1$ expression is changed in corticosteroid insensitive state in our experimental HBSMC model reported in chapter 3.
III. RESULTS

1. Expression of $K_{Ca}3.1$ in BSMC bundles:

Sequential 2-µm sections were cut from glycol methacrylate embedded bronchial biopsies and immunostained as described previously in the method section, stained sequentially using a rabbit polyclonal antibody anti-human $K_{Ca}3.1$ (2.5µg/ml) or α-SMA (1 µg/ml) or isotype control rabbit IgG. The staining was performed using EnVision™ FLEX Mini Kit (DAKO) as described in the method section.

Immunohistochemistry assays reveal that bronchial smooth muscle bundles express $K_{Ca}3.1$ channels in vivo as evidenced by the marked DAB staining in the area that was also positive for α-smooth muscle actin (Figures 4.1 and 4.2). We did not see any intensity differences between $K_{Ca}3.1$ staining in the HBSM bundles between healthy controls and asthma patients.

No immunostaining was observed when a consecutive biopsy section of the same field was incubated with the isotype control antibody increasing the confidence in the specificity of the staining; α-smooth muscle actin staining was used as positive control of smooth muscle bundles.

Of interest, analysis of immunohistochemistry sections not only revealed $K_{Ca}3.1$ localized to the plasma membrane and cytoplasm (consistent with channel trafficking), but it was also localized to the nuclear membrane (Figures 4.1 and 4.2).
Figure 4.1: *In vivo* $K_{Ca}3.1$ expression in HBSM bundles from a healthy control:
Representative photomicrographs of GMA embedded bronchial biopsy specimens at optimal primary antibodies dilution. Smooth muscle bundle in bronchial sections from a healthy control has a strong immunoreactivity (brown colour) for $K_{Ca}3.1$ (magnification, $\times200$; 2 Upper left panel), whereas no immunoreactivity was seen with the Isotype control antibody (IC) (magnification, $\times200$; 2 Upper right panel). HBSM bundle is positive for $\alpha$-smooth muscle actin staining (magnification $\times200$, lower left panel). Results have been confirmed in $n=2$ healthy Controls.
**Asthma**

**Figure 4.2: In vivo $K_{Ca3.1}$ expression in HBSM bundles from an asthmatic subject:** Representative photomicrographs of GMA embedded bronchial biopsy specimens at optimal primary antibodies dilution. Smooth muscle bundle in bronchial sections from asthmatic subjects has a strong immunoreactivity (brown colour) for $K_{Ca3.1}$ (magnification, ×200; 2 Upper left panel), whereas no immunoreactivity was seen with the Isotype control antibody (IC) (magnification, ×200; 2 Upper right panel). HBSM bundle is positive for $\alpha$-smooth muscle actin staining (magnification ×200, lower left panel). Results have been confirmed in n=3 asthmatic subjects.
2. Nuclear expression of $K_{Ca}3.1$ channel in HBSM tissues and cells

It was surprising to see that in the HBSM bundles, $K_{Ca}3.1$ channels had a nuclear localisation as this channel has been reported in the plasma membrane [272]. The immunostaining when examined at a higher magnification clearly shows a marked expression of $K_{Ca}3.1$ channel in the nucleus (Figure 4.3). To further confirm whether $K_{Ca}3.1$ channel was indeed present in the nuclear compartment, we performed additional expression studies in cultured HBSMC.

$K_{Ca}3.1$ expression in the nucleus was confirmed by assaying nuclear fractions from HBSMC for $K_{Ca}3.1$ by immunoblot analysis as described in method section. Nuclear fractions were isolated from unstimulated healthy and asthmatic HBSMC. Following separation of the proteins by SDS-PAGE the membrane was probed with the anti-$K_{Ca}3.1$ antibody. The nuclear fraction contained discreet bands recognized by an anti-$K_{Ca}3.1$ antibody ~50 kDa, (Figure 4.4A). The quantitative analysis of $K_{Ca}3.1$ expression in the nuclear fractions by immunoblot assay revealed no significant changes between healthy controls and asthmatic subjects (Figure 4.4B).

Immunofluorescence staining in permeabilized cells derived from healthy controls and asthmatic patients was also performed to provide additional evidence for the nuclear expression of $K_{Ca}3.1$ channel in HBSMC as shown in (Figure 4.5A). $K_{Ca}3.1$ channel was identified within the nucleus in HBSMC. Staining for HDAC1 (Figure 4.5B), a protein exclusively found in the nucleus, was used as a positive control for the immunostaining procedure. These data obtained using different experimental procedures confirm the nuclear expression of intracellular $K_{Ca}3.1$ in HBSMC.
Figure 4.3: Representative Immunohistochemistry staining at higher magnification (X1000) showing the nuclear expression of KCa3.1 channel in the HBSM bundles *In vivo* when compared to isotype control (IC) antibody staining (left panel).

Figure 4.4. **KCa3.1 expression in nuclear extracts:** (A) HBSMC were lysed using Pierce Nuclear and Cytoplasmic Extraction Reagent Kit (NE-PER). 30µg of nuclear extract sample was analyzed by Western blot using antibodies against KCa3.1 and β-actin. The western blot image is representative of 6 independent experiments (3 asthmatics, 3 normal). (B) Scanning densitometric evaluation of KCa3.1 channel normalized over the corresponding β-actin. Data are presented as Means ± SEM of blots performed in 6 subjects (3 healthy and 3 asthmatics). Comparison between groups was made using unpaired *t*-test (NS, not significant).
Figure 4.5: Nuclear expression of KCa3.1 channel in cultured HBSMC. Permeabilized HBSMC were stained for (A) KCa3.1 (original magnification; X200) or (B) HDAC1 as positive control for nuclear staining (original magnification; X200). Cells were also incubated with the corresponding isotype-matched antibodies used as negative controls. DAPI counterstaining was performed on the same cell population to localize the nucleus. The immunofluorescence image is representative of 6 independent experiments performed in HBSMC from n=3 healthy controls and n=3 asthmatic subjects.
3. K\textsubscript{Ca}3.1 expression in HBSMC is not changed between healthy control and asthmatic patients:

Because we showed that localized nuclear K\textsubscript{Ca}3.1 expression was not altered in asthmatic HBSMC, it was important to examine whether there was an overall general change in K\textsubscript{Ca}3.1 expression between health and disease states. To this end, we repeated the experiments described in Figure 4.4B with HBSMC whole cell lysates in cells from n=3 healthy controls and n=3 asthmatic subjects. Cell lysates were subjected to SDS-PAGE and Western Blot as described in method section. The predicted weight of the K\textsubscript{Ca}3.1 channel protein is ~50 kDa. Relative intensities of the K\textsubscript{Ca}3.1 immunoreactivity were normalized to the corresponding β-actin level, and densitometry was performed using Image J Software to quantify K\textsubscript{Ca}3.1 expression.

Figure 4.6 shows that K\textsubscript{Ca}3.1 channels were expressed in cell extracts from asthmatic subjects and healthy controls (Figure 4.6A). The densitometry value using Image J software also showed that there was no statistical difference between healthy and asthmatic subjects, although a slight non-significant increase in K\textsubscript{Ca}3.1 was noticed in asthmatics (Figure 4.6B).

RT-PCR analysis were also conducted and demonstrated no changes in levels of K\textsubscript{Ca}3.1 mRNA expression between asthmatic and healthy HBSMC (Figure 4.6C), the overall expression is similar to previous results obtained with conventional RT-PCR [293].
Figure 4.6: $K_{Ca3.1}$ expression in HBSMC in healthy and asthmatic subjects: (A) Immunoblot of $K_{Ca3.1}$ channel expression and $\beta$-actin protein in unstimulated HBSMC. (B) Quantitative analysis of $K_{Ca3.1}$ channel protein using densitometric analysis. The results represent the normalized values over the corresponding $\beta$-Actin and are expressed as Means ± SEM. (C) Products from quantitative real-time PCR for $K_{Ca3.1}$ were visualized on a 1.5% agarose gel showing the presence of only one amplified product of the expected size (130 bp). $\beta$-Actin was used as the normalizing control.
4. **K\textsubscript{Ca}3.1** expression and activity are not altered in corticosteroid insensitive conditions

4.1 **K\textsubscript{Ca}3.1** expression

We assessed whether K\textsubscript{Ca}3.1 channel/function expression was affected in steroid-resistant HBSMC, i.e., cells treated with TNF-\(\alpha\)/IFN-\(\gamma\). A previous report by our group showed that increased K\textsubscript{Ca}3.1 expression was required for the regulation of HBSMC proliferation induced by TGF\(\beta\) [293]. Confluent HBSMC grown in 6 well plates were growth arrested for 24 hour in serum free medium and then stimulated with 10ng/ml TNF-\(\alpha\) and 25 ng/ml IFN-\(\gamma\) for 1, 2, 4, 6 and 24 hours before flow cytometry was performed to examine change in K\textsubscript{Ca}3.1 channel expression. Healthy controls and asthmatic HBSMC were permeabilised with 0.1% Saponin/0.5 %BSA/PBS then stained with 2.5 µg/ml anti-K\textsubscript{Ca}3.1 antibody followed by a fluorescein isothiocyanate (FITC) labelled anti-rabbit secondary antibody (1:300 dilution). The fluorescence intensity of the cells was determined using the Becton Dickinson FACScan (Oxford, UK). Geometric Mean fluorescence intensity (GMFI) was used to quantify the shift in fluorescence intensity of the K\textsubscript{Ca}3.1 positive cell population. The reason we switch to flow cytometry is because this is a much better method in term of sensitivity for quantifying K\textsubscript{Ca}3.1 levels in HBSMC than the previously used immunoblot assays.

As shown in **Figure 4.7A**, HBSMC demonstrated high levels of K\textsubscript{Ca}3.1 in HBSMC under unstimulated conditions when compared with isotype control staining but no significant difference was noticed in K\textsubscript{Ca}3.1 staining in HBSMC.
between n=3 healthy controls (42.6±4.063) and n=3 asthmatic subjects (61.49±4.655).

Because we found no difference in \(K_{Ca}3.1\) expression between asthmatic and healthy conditions, flow cytometry data from normal and asthmatic HBSMC were combined in the following experiments assessing the effect of TNF-\(\alpha\)/IFN-\(\gamma\) on \(K_{Ca}3.1\) expression. **Figure 4.7B** shows that the average expression of \(K_{Ca}3.1\) in HBSMC from n=6 subjects was not affected by stimulation with TNF-\(\alpha\)/IFN-\(\gamma\) at different time points when compared to unstimulated HASM cells.
Figure 4.7: Effect of TNF-α/IFN-γ on K_{Ca3.1} expression in HBSMC. (A), example fluorescent histograms representing K_{Ca3.1} staining in healthy HBSMC (blue) and asthmatic HBSMC (pink) or cells incubated with the corresponding isotype control antibody (dashes line). (B) Quantitative analysis of K_{Ca3.1} staining in HBSMC treated with TNFα/IFN-γ for different time points. K_{Ca3.1} staining was calculated according to the formula: (GMFI K_{Ca3.1} – GMFI Isotype) and expressed as fold change as Means ± SEM in HBSMC from n=6 subjects (3 healthy controls, 3 asthmatics). US, Unstimulated HBSMC.
4.2 $K_{Ca3.1}$ channel activity

We next investigated whether activity of $K_{Ca3.1}$ channel in HBSMC. To this end, we measured $K_{Ca3.1}$ activity using patch-clamp electrophysiology, a technique well established in our lab and described in method section [279, 282, 291, 293]. Figure 4.8 compares the average current-voltage (I/V) curves performed in 16 healthy isolated HBSMC from diluent or cytokine-treated conditions for 24 h. In agreement with our previous report [293] we found no evidence of $K_{Ca3.1}$ currents in un-stimulated HBSMC. Of note, $K_{Ca3.1}$ currents were also not noticeable in cells treated with TNF-α/IFN-γ, suggesting that cytokines do not stimulate plasma membrane $K_{Ca3.1}$ channel activity in HBSMC cells. This experiment was done in 16 cells from one healthy donor without the positive control (EBIO) to measure plasma membrane potential not nuclear membrane potential.

![Figure 4.8](image-url)

**Figure 4.8:** Current-voltage curves showing the lack of detectable $K_{Ca3.1}$ currents in HBSMC at baseline and after cytokine treatment as indicated. Data are expressed as Means ± SEM of measurement performed in $n = 16$ cells per condition taken from one healthy subject.
IV.  DISCUSSION:

\( \text{K}_{\text{Ca}}3.1 \) is widely expressed in endothelial cells, cells of hematopoietic origin and epithelial cells [284]. In these tissues \( \text{K}_{\text{Ca}}3.1 \) channels contribute to cell volume regulation, proliferation and migration and thus can modulate immune responses, blood pressure, restenosis disease, fluid secretion and fibrosis [256, 284]. Airway remodelling is believed to result from the activation of different airway cells, including smooth muscle which are known to regulate key asthmatic features such as airway hyper-responsiveness (AHR), airway obstruction and the local inflammatory process via the producing inflammatory mediators including cytokines and chemokines as found in the present thesis [123]. Because of the mounting preclinical evidence showing a role of \( \text{K}_{\text{Ca}}3.1 \) channel in the pathogenesis of asthma [295, 316, 319], it was essential to investigate the expression of \( \text{K}_{\text{Ca}}3.1 \) in HBSM in both the native tissues and in cultured cells.

Immunohistochemistry performed on bronchial biopsies demonstrated that \( \text{K}_{\text{Ca}}3.1 \) channels are indeed expressed \textit{in vivo} in HBSM bundles in both healthy controls and asthmatic patients and interestingly, this included a nuclear localization. This nuclear distribution of \( \text{K}_{\text{Ca}}3.1 \) was further confirmed by various
approaches assessing $K_{Ca3.1}$ expression in cultured HBSMC including immunoblot analysis directly on nuclear extracts (Figure 4.4), and immunofluorescence staining (Figure 4.5). The intracellular expression of $K_{Ca3.1}$ in BSM cells is highly novel, although one report identified $K_{Ca3.1}$ channels in mitochondrial membranes in human colon carcinoma 116 cells [320]. The functional role of nuclear $K_{Ca3.1}$ channels remains to be further determined.

$K_{Ca3.1}$ channel mRNA has been shown to be expressed in human lung and trachea cDNA libraries [321]. Cell lines derived from human lung mast cells and human airway epithelial cells have been shown to contain $K_{Ca3.1}$ channel mRNA and activity [290, 322, 323]. Shepherd and colleagues were the first to have detected the expression of both $K_{Ca3.1}$ channel mRNA and protein in BSMC [293]. We also confirmed the presence of KCa3.1 channel in HBSMC but we reported the first evidence of that $K_{Ca3.1}$ channel expression is not altered in cells from asthmatic when compared to cells from healthy subjects (Figure 4.6A). We also fail to detect any apparent changes in $K_{Ca3.1}$ channels in vivo in HBSM bundles between asthmatic and healthy controls, although we did not perform a comparison between the intensities of staining between health and asthma (Figures 4.1 and 4.2). Our expression studies contrast with one recent report that showed increased levels of $K_{Ca3.1}$ mRNA and protein in bronchial smooth muscle tissues in murine asthmatic of allergic model [319]. Differences in species could explain the discrepancy seen between our data and this animal study.
Assuming that $K_{\text{Ca}3.1}$ channels in HBSMC indeed play a role in asthma, our data suggest that changes in channel activity rather than protein expression within HBSMC bundles could explain their contribution to the disease. In contrast to two studies showing that expression of $K_{\text{Ca}3.1}$ channels can be significantly increased by pro-fibrotic factors such as TGF-β [293] or PDGF [294] in HBSMC, our study shows that expression $K_{\text{Ca}3.1}$ channel was not affected by pro-asthmatic cytokines TNF-α/IFN-γ (Figure 4.7). This observation suggests that modulation of $K_{\text{Ca}3.1}$ expression or function in HBSMC is highly stimulus dependent (growth factors versus cytokines). The fact that no change in $K_{\text{Ca}3.1}$ activity occurs when measured at the cell surface supports the concept that compartmental changes in $\text{Ca}^{2+}$ levels rather than cytoplasmic changes could explain the activation of $K_{\text{Ca}3.1}$ in GC sensitivity.

Our data indicate that $K_{\text{Ca}3.1}$ channels are expressed in bronchial smooth muscle both in vivo and in vitro. Their expression is not affected in asthma (at least in the studied patient population) and by inflammatory cytokines known to impair corticosteroid insensitivity in HBSMC. The next chapter will provide novel evidence for a role of $K_{\text{Ca}3.1}$ channels in promoting corticosteroid resistance in HBSMC.
CHAPTER 5

$K_{\text{Ca}3.1}$ Channels and Corticosteroid Insensitivity in HBSMC
I. RATIONALE

Asthma is a multifactorial chronic disease that has shown noticeable increase prevalence over the past decades both in UK and worldwide [324]. Present treatments are either unsuccessful or have intolerable side effects for patients suffering from severe asthma; new management and treatment approach are needed for patients with severe and difficult asthma. Although corticosteroid therapy is very efficient in managing asthma and other inflammatory diseases but still small percentage of patients with asthma fail to react even to high doses of oral glucocorticoids; understanding the mechanisms behind corticosteroid insensitivity will likely lead to more effective therapies [168]. Numerous studies have provided strong evidence that bronchial smooth muscle could play a significant role in the pathogenesis of asthma, not only by triggering the acute episodes of airway obstruction, but also by producing pro-asthmatic mediators such as chemokines, pro-fibrotic factors, cytokines, and growth factors [100, 121, 325]. A number of cytokines and chemokines produced by HBSMC could participate in the pathogenesis of asthma via the recruitment and/or activation of inflammatory cells in the lungs, thus leading to the activation of other lung-resident cells indirectly contributing to airway remodelling [114, 121]. Whether the pathogenesis of severe asthma is driven by the steroid-resistant production of proteins from HBSMC represents an interesting hypothesis. Indeed, previous reports convincingly showed that despite high doses of inhaled or oral GC taken by asthmatic patients, expression of different “proasthmatic” proteins is still visible in HBSM bundles, including the following chemokines: chemokine (C-X3-C motif) ligand (CX3CL)
CCL11 [40], CCL15 [327], and CCL19 [31], and a disintegrin and metalloprotease domain (ADAM) 33 and ADAM8 [328]. More importantly, evidence mostly from Amrani group showed that under inflammatory conditions, the production of a variety of pro-asthmatic mediators by smooth muscle cells from tracheal tissues is insensitive to GC treatment [166, 247]. Our present report also showed that smooth muscle cells derived from bronchial tissues become less sensitive to corticosteroids when exposed to TNFα/IFNγ (see chapter 3). These studies provide indisputable in vivo and in vitro evidence for the existence of steroid resistant pathways in HBSM that are potentially driving inflammatory processes and HBSM contractile dysfunction in asthmatic airways. A better understanding of the underlying mechanisms driving these steroid-resistant pathways in BSM could therefore lead to novel therapeutic approaches.

It is remarkable to note that blockade of K_{Ca}3.1 channels is effective in treating various human inflammatory conditions where cytokines are thought to play an important role. First, K_{Ca}3.1 channels are known to be expressed in key cells involved in the pathogenesis of asthma due to their high pro-inflammatory potential; these include T cells, mast cells, macrophages, HBSMC, fibroblasts and epithelial cells [275, 279, 282, 291, 293]. Second, in vitro studies showed that K_{Ca}3.1 channels regulate various cell function also susceptible to regulate asthmatic responses including T cell activation, migration and proliferation of HBSMC and lung fibroblasts, mast cell degranulation[269, 282]. Third, emerging pre-clinical evidence shows the contributions of K_{Ca}3.1 in key features of asthma such as airway inflammation and remodelling in animal studies [294,
K\textsubscript{Ca}3.1 channels have also been proposed as possible therapeutic targets for many inflammatory disease models such as cardiovascular diseases, kidney fibrosis and atherosclerosis [269, 274, 295]. Whether K\textsubscript{Ca}3.1 blockers may represent a promising therapeutic strategy for patients with severe asthma remains to be further investigated.

II. **AIM:**

As discussed above, there is no doubt that K\textsubscript{Ca}3.1 channels appear to be essential in driving various inflammatory conditions where the pathogenesis involving cytokines acting on different structural tissues (cardiac, kidney, lungs, and brain) are playing a key role in the pathogenesis. In this chapter we asked whether K\textsubscript{Ca}3.1 channels could represent a therapeutic target in severe asthma via the regulation of cytokine-induced corticosteroid insensitivity. We therefore used our newly described model of HBSMC exposed to TNF-\textalpha/IFN-\gamma to investigate i) whether chemokine production was differentially regulated in cells from healthy, asthmatic and COPD subjects, ii) whether K\textsubscript{Ca}3.1 blockade using different experimental strategies modulates the expression of fluticasone-resistant chemokine expression and iii) whether this latter occurs via transcriptional or post-transcriptional pathways.
III. RESULTS

1. TNF-α/IFN-γ combination differentially regulates the production of CXCL10, CCL5, CCL11 and CX3CL1 in HBSMC from healthy controls and patients with asthma and COPD

Before studying the implication of Kv3.1 channels in cytokine-induced corticosteroid insensitivity in cells from healthy, asthmatic and COPD, it was important to determine whether differences in chemokine production were present in these different subjects. Previous studies indeed showed the existence of phenotypic differences in HBSMC between healthy and asthma subjects [133, 134, 310, 329].

We first determined whether production of four different chemokines (CCL5, CCL11, CX3CL1 and CXCL10) in response to TNF-α/IFN-γ was affected by disease status. HBSMC grown at 90% confluency, were serum-starved and stimulated with TNF-α/IFN-γ (10 ng/ml/25 ng/ml) for 24 hours before the supernatants were collected. The concentrations of CCL5, CCL11, CXCL10 and CX3CL1 in the supernatants were measured using an ELISA assay as described in the method section.

Compared to healthy cells, we found levels of CCL5, CCL11, CXCL10 and CX1CL3 produced by HBSMC were highly affected by disease status under basal and stimulated conditions. Basal levels of both CCL5 and CXCL10 were significantly higher in asthmatic HBSMC compared with healthy HBSMC. In stimulated conditions, CCL5 induced by TNF-α/IFN-γ was also higher in...
asthmatic and COPD HBSMC with levels (11356±2235pg/ml), (9131±1013pg/ml), compared to 4506± 842.0 pg/ml measured in healthy HBSMC (Figure 5.1A). In stimulated conditions, CXCL10 induced by TNF-α/IFN-γ was also higher in asthmatic and COPD HBSMC with levels of (88867 ± 5162 pg/ml) and (91050± 10152 pg/ml) compared to (69056± 5578pg/ml) measured in healthy HBSMC (Figure 5.1B). In stimulated conditions, there was also no difference in CCL11 levels induced by TNF-α/IFN-γ between asthmatic and COPD HBSMC with levels of 848.7±95.5pg/ml 634.5±71.4pg/ml compared to 669.7±50.88pg/ml measured in healthy HBSMC (Figure 5.2A) The only difference was in the basal levels between asthmatic and healthy HBSMC (Figure 5.2A). In stimulated conditions, there was also no difference in CX3CL1 levels induced by TNF-α/IFN-γ between asthmatic and COPD HBSMC with levels of 2422±288.3pg/ml and 2261±384.8pg/ml compared to 1873±249.7pg/ml measured in healthy HBSMC (Figure 5.2B).
Figure 5.1: TNF-α/IFN-γ-induced CCL5 and CXCL10 in HBSMC from healthy control and patients with asthma and COPD. Growth-arrested HBSMC treated with cytokines for 24 hours before levels of CCL5 (A) and CXCL10 (B) in the supernatants were assessed by ELISA assays. Horizontal lines represent medians. ##P < 0.001 versus basal (unstimulated). †P < 0.05, ††P < .01 versus baseline of healthy control; **P < 0.01 versus TNF-α/IFN-γ stimulated healthy HBSMC. The number of cell lines used is also indicated.
Figure 5.2: TNF-α/IFN-γ-induced CCL11 and CX3CL1 in HBSMC in healthy control and patients with asthma and COPD Growth-arrested HBSMC treated with cytokines for 24 hours before levels of CCL11 (A) and CX3CL1 (B) in the supernatants were assessed by using ELISA assays. Horizontal lines represent medians. ##P <0.001 versus basal (unstimulated), ††P < .01 versus unstimulated healthy HBSMC.
2. \( K_{\text{Ca}3.1} \) channel blockade differentially suppresses the production of steroid-resistant chemokines induced by TNF-\( \alpha \)/IFN-\( \gamma \)

2.1 Effect of \( K_{\text{Ca}3.1} \) inhibitors on HBSMC viability

We first tested whether any of conditions involving the \( K_{\text{Ca}3.1} \) inhibitors had any effect on HBSMC cell viability using the well described 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT assay was performed on cells stimulated with different concentrations of ICA-17043 (10nM, 100nM, 1\( \mu \)M) or with TRAM-34 (20nM, 200nM, 2\( \mu \)M) for 2 hours then followed by TNF\( \alpha \)/IFN\( \gamma \) an additional 24 hours. MTT protocol was performed as describe in method section. The optical density of each well was evaluated at 590 nm on EnSpire Multimode (Perkin Elmer) plate reader. The results were presented as Means ± SEM of \( n=2 \). Viability was compared to that of control cells, with viability of controls expressed as 100% (Figure 5.3).

Measuring cell viability using the MTT assay did not show any signs of cytotoxicity in cells treated with the different concentrations of \( K_{\text{Ca}3.1} \) inhibitors used in the literature (< 100 nM for ICA-17043 and <1 \( \mu \)M for TRAM-34). We did observe a significant drop in cell viability at 1 \( \mu \)M ICA-17043 (44.44±17.47\%) and 2 \( \mu \)M TRAM-34 27.22±1.092\%. As used in other studies we used the safe concentration of 100nM for ICA-1743 [285], and 200nM for TRAM-34 [272] in our following experiments.
Figure 5.3: Effect of TRAM-34 or ICA-1743 on HBSMC viability: HBSMC cells were pre-treated with ICA 17043 (10nM, 100nM, 1µM) or with TRAM-34 (20nM, 200nM, 2µM) for 2 hours before TNFα/IFNγ was added for 24 hours. HBSMC viability was measured using the MTT assay as described in Methods section. Data are expressed as Means ± SEM for n=2.
2.2 $K_{Ca3.1}$ channel blockers (TRAM-34 and ICA-17043) modulate TNFα/IFN-γ induced chemokines production

We next asked the question of whether $K_{Ca3.1}$ channels were involved in the development of cytokine-induced corticosteroid insensitivity in HBSMC described in chapter 3. We therefore assessed the effect of $K_{Ca3.1}$ channel blockers on the expression of four different chemokines (CCL5, CCL11, CX3CL1, CXCL10) in HBSMC derived from healthy ($n = 4$), asthmatic ($n = 6$), and COPD subjects ($n = 4$). Growth-arrested HBSMC were first exposed $K_{Ca3.1}$ channel inhibitors TRAM-34 (200nM) or ICA-17043 (100nM) alone or in combination with fluticasone (100nM) for 2 hours followed by TNF-α/IFN-γ treatment for 24 hours. Supernatants were collected and ELISA was performed as described previously.

As shown in Chapter 3, the production of CX3CL1, CCL5, CCL11, and CXCL10 by TNF-α/IFN-γ was completely resistant to fluticasone treatment in HBSMC (Figures 5.4 to 5.7). Chemokine production was not affected in cells treated with 0.1% DMSO, the final concentration of the inhibitor solvent. The specific $K_{Ca3.1}$ blockers TRAM-34 (200 nM) and ICA-17043 (100 nM) alone did not affect TNF-α/IFN-γ–induced production of CCL5 (Figure 5.4) or CCL11 (Figure 5.5), with the exception of cells derived from asthmatic patients (Figure 5.5B) where the inhibitory effect of $K_{Ca3.1}$ blockade was further enhanced by 90% in the presence of fluticasone (Figure 5.5B). It was interesting to note that TNF-α/IFN-γ–induced production of CCL5 in the three tested groups (Figure 5.4) or CCL11 in healthy and COPD subjects (Figure 5.5 A–C) was inhibited
only when fluticasone was combined with either $K_{Ca}3.1$ channel blocker. Fluticasone and ICA-17043 in combination reduced TNF-α/IFN-γ–induced CCL5 levels to 57 ± 12%, 61 ± 14.5%, and 52 ± 14% in HBSMC from healthy, asthmatic, and COPD subjects, respectively (Figure 5.4). Fluticasone and TRAM-34 reduced TNF-α/IFN-γ–induced CCL5 levels to 57 ± 11%, 51 ± 21%, and 54 ± 9.6% in cells from healthy, asthmatic, and COPD subjects, respectively (Figure 5.4).

The production of CCL11 by TNF-α/IFN-γ was reduced to 36 ±14%, 12±14%, and 28 ± 12% of control by the fluticasone and ICA-17043 combination in cells from healthy, asthmatic, and COPD subjects, respectively. TNF-α/IFN-γ–induced CCL11 production was reduced to 38.5 ± 14.2%, 9 ± 9%, and 31 ±15% by the fluticasone and TRAM-34 combination in cells from healthy, asthmatic, and COPD subjects, respectively (Figure 5.5). Of note, induction of CX3CL1 by TNF-α/IFN-γ in cells from healthy, COPD, and asthmatic subjects was reduced to 31.4 ± 12.1%, 8.8 ± 3.4%, and 33.2 ± 8.9% by ICA-17043 alone (100nM) and to 28.9 ± 10.8%, 21.1 ±10.9%, and 26.4 ± 14.7% by TRAM-34 alone (200nM) (Figure 5.6A–C). The degree of CX3CL1 inhibition by KCa3.1 blockade was not changed by the presence of fluticasone (Figure 5.6A–C, last two columns). In contrast, production of CXCL10 by TNF-α/IFN-γ was not affected by either $K_{Ca}3.1$ inhibitors used alone or in combination with fluticasone (Figure 5.7).
Figure 5.4: Effect of K\textsubscript{Ca}3.1 blockers alone or in combination with fluticasone on corticosteroid-resistant CCL5 expression. HBSMC pre-treated with TRAM-34 (200nM) or ICA-17043 (100nM), with or without fluticasone (100 nM, 2 hours), were stimulated with TNF-α/IFN-γ for 24 hours. CCL5 levels in the supernatants were assessed by ELISA. Data are expressed as means ± SEM % of TNF-α/IFN-γ-induced CCL5 levels in HBSMC from n = 4 healthy controls (A), n = 6 asthmatic patients (B), and n = 4 COPD patients (C). *P<0.05, **P<0.01 compared with TNF-α/IFN-γ control.
Figure 5.5: Effect of $K_{Ca}$3.1 blockers alone or in combination with fluticasone on corticosteroid-resistant CCL11 expression. HBSMC pre-treated with TRAM-34 (200nM) or ICA-17043 (100nM), with or without fluticasone (100 nM, 2 hours), were stimulated with TNF-α/IFN-γ for 24 hours. CCL11 levels in the supernatants were assessed by ELISA. Data are expressed as means ± SEM % of TNF-α/IFN-γ–induced CCL5 levels in HBSMC from n = 4 healthy controls (A), n = 6 asthmatic patients (B), and n = 4 COPD patients (C). *P<0.05, ***P<0.001 compared with TNF-α/IFN-γ control.
Figure 5.6: Effect of KCa3.1 blockers alone or in combination with fluticasone on corticosteroid-resistant CX3CL1 expression. HBSMC pre-treated with TRAM-34 (200nM) or ICA-17043 (100nM), with or without fluticasone (100 nM, 2 hours), were stimulated with TNF-α/IFN-γ for 24 hours. CX3CL1 levels in the supernatants were assessed by ELISA. Data are expressed as means ± SEM % of TNF-α/IFN-γ-induced CCL5 levels in HBSMC from n = 4 healthy controls (A), n = 6 asthmatic patients (B), and n = 4 COPD patients (C). *P<0.05, **P<0.01, ***P<0.001 compared with TNF-α/IFN-γ control.
Figure 5.7: Effect of KCa3.1 blockers alone or in combination with fluticasone on corticosteroid-resistant CXCL10 expression. HBSMC pre-treated with TRAM-34 (200nM) or ICA-17043 (100nM), with or without fluticasone (100 nM, 2 hours), were stimulated with TNF-α/IFN-γ for 24 hours. CXCL10 levels in the supernatants were assessed by ELISA. Data are expressed as means ± SEM % of TNF-α/IFN-γ–induced CCL5 levels in HBSMC from n = 4 healthy controls (A), n = 6 asthmatic patients (B), and n = 4 COPD patients (C). None of the values were significantly different when compared with TNF-α/IFN-γ control.
3. **K$_{Ca3.1}$ downregulation attenuates TNF-α/IFN-γ induced expression of steroid-resistant chemokines**

3.1 **Optimization of adenoviral transduction efficiency for silencing studies**

We next used an additional K$_{Ca3.1}$ blockade strategy by silencing K$_{Ca3.1}$ channels using small hairpin RNA (shRNA) expressing adenoviruses (Ad5C20Att01) purchased from BioFocus DPI (Leden, the Netherlands). Two K$_{Ca3.1}$ specific shRNA adenoviruses called V1 and V2 and one shRNA control adenovirus called V5 were used for the following experiments. The GFP–shRNA adenovirus was also used i) to verify if the adenovirus vectors had successfully integrated into HBSMC and ii) to determine transduction efficiency using different multiplicity of infection (MOI, ratio of virus to target cells) of 1, 5, 10, 30, and 50 MOI incubated for the different time points 24, 48 and 72 hours. Following adenoviral infection, the transduction efficiency was assessed by the number of GFP-positive cells determined by two independent observers using the fluorescence microscope. Compared to all other tested MOIs, it was found that adenovirus at 30 MOI gave the best transduction efficiency with >85% of GFP-positive cells at 24 hr post-infection compared to nontransduced cells (Figure 5.8, n=3). Therefore all K$_{Ca3.1}$shRNA were used at a concentration of 30 MOI incubated for 24 hr for the silencing experiments.
**Figure 5.8: Transduction efficiency of GFP-shRNA adenovirus in HBSMC:** Pictures of living HBSMC by light (panels a and c) or fluorescence (panels b and d) microscopy photographed 24 hours post adenoviral infection with either control shRNA adenovirus (upper panels a and b), or EGFP-shRNA adenovirus (lower panels c and d) at 30 MOI. This figure is representative of 3 independent infection experiments with both control and control adenovirus, while HBSM transduced with the shRNA-EGFP adenoviruses.
3.2 Silencing of \( K_{\text{Ca}3.1} \) Channels using shRNA adenoviruses

**Apoptosis assay**

In order to assess whether shRNA adenoviruses cause any cell death, an Apoptosis assay was performed using a combination of propidium iodide (PI) and Annexin V staining as described in method section. HBSMC were treated with \( K_{\text{Ca}3.1} \) shRNA adenovirus \textbf{V1} (MOI 30) and \( K_{\text{Ca}3.1} \) shRNA adenovirus \textbf{V2} (MOI 30) for 24 hours. The cells then were stained with both Annexin V and propidium iodide, and staining was followed by flow cytometric analysis as described in Materials and methods. Data were analysed using the Flowing software version 2.5.1 using Becton Dickinson FACScan (Oxford, UK). Flow cytometry analysis of annexin V-FITC- and propidium iodide-stained HBSMC demonstrated that either \( K_{\text{Ca}3.1} \) shRNA V1 or \( K_{\text{Ca}3.1} \) shRNAs V2 infection did not affect cells viability. The percentage of untreated live cells in the lower left quadrant (unstained) (80.70±4.866%, **Figure 5.9b**) were not affected after treatment with \( K_{\text{Ca}3.1} \) shRNAs V1 (78.43±5.117%, **Figure 5.9c**) and \( K_{\text{Ca}3.1} \) shRNA V2 (80.07±7.41%, **Figure 5.9d**). The distribution of cells in the absence of dyes (control) (94.12±2.15%, **Figure 5.9a**).

The average percentage of apoptotic cells (early apoptosis) that were positively stained to Annexin V-FITC in lower right quadrant are 13.1±5.1%, 9.94±2.37% and 9.37±3.37% in untreated cells, cells infected with \( K_{\text{Ca}3.1} \) shRNAs V1 and \( K_{\text{Ca}3.1} \) shRNAs V2, respectively. The average percentage of late apoptotic and/or necrotic cells that were Annexin V-FITC and propidium iodide positive upper right quadrant were 4.50±0.42%, 9.17±4.46% and 9.80±4.44% in untreated cells, cells infected with \( K_{\text{Ca}3.1} \) shRNAs V1 and \( K_{\text{Ca}3.1} \) shRNAs V2, respectively.
shRNAs V2, respectively (Figure 5.10). These data confirm that shRNAs V1 and K_{Ca3.1} shRNAs V2 adenovirus can be used safely without causing any cell death when knockdown K_{Ca3.1} channels.

Figure 5.9: Effect of K_{Ca3.1} shRNA adenoviruses on HBSMC apoptosis. Representative flow cytometric quadrant plots for HBSMC staining with PI and FITC-labelled annexin-V assessed by FACScan analysis, untreated cells (a), cells infected with K_{Ca3.1} shRNA V1 (b) and K_{Ca3.1} shRNA V2 (c). Numbers inside the quadrants represent the percentage of live cells (lower left quadrant), early apoptotic cells (lower right quadrant) and dead or necrotic cells (upper right quadrant) in each condition.
Figure 5.10: Quantitative analysis of percentage of live, apoptotic, or dead HBSMC from the flow cytometry analyses of cells staining with annexin V-PI. Data are represented as Means ± SEM of three independent infection experiments.
3.3  **shRNA adenovirus-mediated silencing of K\textsubscript{Ca}3.1 channels**

To confirm the previous findings with pharmacological blockers of K\textsubscript{Ca}3.1 channels, we knockdown of K\textsubscript{Ca}3.1 channels using shRNA delivered by adenoviruses. For the experiments, HBSMC cells were transducted for 24 hours (transduction condition optimised above), serum-deprived for 3 hours and then exposed to fluticasone (100nM) for 2 hours, followed by addition of control media or TNF\textalpha/-IFN-\gamma combination for an additional 22 hours. Cells supernatants were then collected for ELISA assays (CCL5, CX3CL1, CCL11), and lysed to prepare total cell extracts for protein knockdown confirmation by immunoblot assays as described in method section.

Compared to cells infected with shRNA control adenovirus (V5) where K\textsubscript{Ca}3.1 channel protein expression was not affected, K\textsubscript{Ca}3.1 channel were completely knockdown by the two K\textsubscript{Ca}3.1 shRNA adenoviruses V1 and V2 (Figure 5.11A-B). None of the shRNA adenoviruses affected levels of β-actin expression (Figure 5.11A). In addition, K\textsubscript{Ca}3.1 knockdown was not affected in cells treated with TNF-α/IFN-γ treatment alone or in the presence of fluticasone (Fig. 5.11 B). These data show that K\textsubscript{Ca}3.1 shRNA adenoviruses can be used to safely down-regulate K\textsubscript{Ca}3.1 channel expression in HBSMC.
Figure 5.11: Silencing of $K_{Ca}3.1$ channel expression using $K_{Ca}3.1$ shRNA adenoviruses in HBSMC. (A) Representative immunoblot of $K_{Ca}3.1$ protein expression in cells transduced with $K_{Ca}3.1$ shRNA (V1 and V2) and control (V5) adenoviruses in the presence or absence of cytokines and fluticasone (FP) (B) Scanning densitometric measurement of $K_{Ca}3.1$ expression by immunoblot assays normalized over the corresponding β-actin showed complete knockdown of $K_{Ca}3.1$ expression in BSM cells, using $K_{Ca}3.1$ shRNA V1 and V2, when compared with control adenovirus. Results are shown as means ± SEM of blots performed in three healthy donors.
3.4 Downregulation of K\textsubscript{Ca}3.1 channel in HBSMC attenuates TNF-\(\alpha/\)IFN-\(\gamma\)-induced expression of steroid-resistant chemokines

As reported in un-transduced cells (Chapter 1), cells transduced with the shRNA control adenovirus TNF-\(\alpha/\)IFN-\(\gamma\) stimulated the production of CX3CL1 (Figure 5.12A) and CCL5 (Figure 5.12B) and CCL11 (Figure 5.12C) that was unaffected by fluticasone. Interestingly, CCL5 production was inhibited by \(\sim 30\%\) by K\textsubscript{Ca}3.1 shRNA V1 and V2 adenoviruses, an effect that was further increased to almost 50\% in the presence of fluticasone (Figure 5.12B). In agreement with the data obtained with the soluble inhibitors, cytokine-induced CX3CL1 expression was dramatically inhibited by K\textsubscript{Ca}3.1 shRNAs V1 and V2 irrespective of fluticasone treatment (Figure 5.12A). Compared to CX3CL1 levels produced by HBSMC infected with control shRNA, the percent inhibition of CX3CL1 levels by shRNA V1 was 68\% and 49\% in the absence or presence of fluticasone, respectively. The percent inhibition of CX3CL1 levels by shRNA V2 was 45\% or 30\% in the absence or presence of fluticasone, respectively. Cytokine-induced CCL11 production was inhibited by \(\sim 35\%\) by K\textsubscript{Ca}3.1 shRNAs V1 and V2, an effect that was further increased to almost 50\% in the presence of fluticasone (Figure 5.12C). These data confirm that K\textsubscript{Ca}3.1 channels are involved in the regulation of fluticasone-resistant chemokines induced by TNF-\(\alpha/\)IFN-\(\gamma\) in HBSMC.
Figure 5.12: KCa3.1 shRNA adenoviruses modulate TNF-α/IFN-γ–induced chemokine expression: Effect of the KCa3.1 shRNA (V1) and KCa3.1 shRNA (V2) adenoviruses on TNF-α/IFN-γ–induced expression of CX3CL1 (A), CCL5 (B) and CCL11 (C) assessed by ELISA. Results are shown as Means ±SEM of experiments performed in triplicate in three healthy donors. * p<0.05, **p<0.01 compared with respective control shRNA.
4. Inhibition of $K_{Ca}3.1$ channel suppresses TNF-α/IFN-γ induced steroid-resistant chemokine at the mRNA expression

To determine whether $K_{Ca}3.1$ channel blockers modulate TNF-α/IFN-γ–induced chemokine expression at the transcriptional level, HBSMC were pre-treated with TRAM-34 (200nM) or ICA-17043 (100nM), with or without fluticasone (100nM, 2 hours), then stimulated with TNFα/ IFN-γ for an additional 4 hours. Total RNA was extracted, and real-time quantitative PCR was performed, as described in Materials.

In HBSMC cells exposed to TNF-α/IFN-γ for 4 hours, CCL5 (Figure 5.13A), CX3CL1 (Figure 5.13B), and CCL11 (Figure 5.13C) mRNA expression was increased significantly by 19.91 ± 6.92, 2.12 ± 0.6, and 185.3 ± 24 fold over basal, respectively (P< 0.05). Fluticasone did not alter the induction of CCL5, CX3CL1 or CCL11 mRNA, while non-significant trends were noted for a reduced expression of CCL5 and CCL11 mRNA with $K_{Ca}3.1$ blockade alone (Figures 5.14A, 5.15A, and 5.16A). Combining fluticasone with TRAM-34 or ICA-17043 led to a drastic inhibition of both CCL5 (Figures 5.14A) and CCL11 (Figures 5.16A) expression (p<0.05, n=4 HBSMC combined from n=2 asthmatic patients, 1 control, and 1 COPD patient). By contrast, fluticasone alone had no significant effect on TNF-α/IFN-γ-induced CX3CL1 expression, but this expression was almost completely reduced in cells treated with either $K_{Ca}3.1$ blocker alone, irrespective of fluticasone treatment (Figure 5.15A).

PCR products of all reactions were run on agarose gel (1.5 or 2%) to confirm the presence of a single product with the predicted size and the results
obtained by qPCR. β-Actin was used as the normalizing control (Figures 5.14B, 5.15B and 5.16B).

These data demonstrate that $K_{Ca}3.1$ channel blockade regulates the expression of corticosteroid-resistant chemokines in HBSMC by acting at the transcriptional level.
Figure 5.13: TNF-α/IFN-γ induced mRNA expression of inflammatory chemokines in HBSMC cells. After exposure to cytokines for 4 hours, CCL5 (A), CX3CL1 (B), and CCL11 (C) expression was assessed using qRT-PCR. Data are presented as fold change over basal (Means ± SEM) of experiments performed in duplicate in 4 different subjects. *P < 0.05. **P < 0.01.
Figure 5.14: K$_{Ca}$3.1 blockers modulate TNF-α/IFN-γ induced CCL5 expression at the mRNA level: (A) Results are expressed as fold induction of chemokine expression by calculating the negative inverse of the $2^{-\Delta\Delta Ct}$ value for each condition. Expression levels of CCL5 were expressed as % of TNF-α/IFN-γ response. Means ±SEM of experiments performed in duplicate in 4 different subjects (**p< 0.01 compared with TNF-α/IFN-γ/DMSO control). (B) Electrophoresis of PCR products of CCL5 and β-Actin on 2% agarose gel electrophoresis and visualized with ethidium bromide staining. Similar results were obtained in all 4 donors; β-Actin was used as a loading control.
Figure 5.15: \(K_{Ca^{3.1}}\) blockers modulate TNF-\(\alpha\)/IFN-\(\gamma\) induced CX3CL1 expression at the mRNA level: (A) Results are expressed as fold induction of chemokine expression by calculating the negative inverse of the \(2^{-\Delta \Delta CT}\) value for each condition. Expression levels of CX3CL1 were expressed as % of TNF-\(\alpha\)/IFN-\(\gamma\) response. Means±SEM of experiments performed in duplicate in 4 different subjects (**p < 0.01 compared with TNF-\(\alpha\)/IFN-\(\gamma\)/DMSO control). (B) Electrophoresis of PCR products of CX3CL1 and \(\beta\)-Actin on 2% agarose gel electrophoresis and visualized with ethidium bromide staining. Similar results were obtained in all 4 donors; \(\beta\)-Actin was used as a loading control.
Figure 5.16: K\textsubscript{Ca}3.1 blockers modulate TNF-\textalpha/IFN-\gamma induced CCL11 expression at the mRNA level. (A) Results are expressed as fold induction of chemokine expression by calculating the negative inverse of the $2^{-\Delta\Delta CT}$ value for each condition. Expression levels of CCL11 were expressed as \% of TNF-\textalpha/IFN-\gamma response. Means ± SEM of experiments performed in duplicate in 4 different subjects (*p < 0.05 compared with TNF-\textalpha/IFN-\gamma/DMSO control). (B) Electrophoresis of PCR products of CCL11 and \textbeta-Actin on 2\% agarose gel electrophoresis and visualized with ethidium bromide staining. Similar results were obtained in all 4 donors; \textbeta-Actin was used as a loading control.
IV. DISCUSSION

The molecular mechanisms driving corticosteroid resistance in asthma have not been characterized [168]. This is an unmet medical need as corticosteroid insensitivity represents a serious management problem, particularly in patients with severe asthma because conventional therapies failed to treat these patients. Studies from Amrani’s group have provided evidence of the existence of corticosteroid resistant pathways in tracheal smooth muscle cells [166, 221, 247]. We now provided additional evidence that corticosteroid resistance can also develop in bronchial smooth muscle cells (Chapter 3), although the underlying mechanisms have not been completely elucidated. We here provide the first evidence that $\text{K}_\text{Ca}3.1$ channels regulate corticosteroid insensitivity.

There is accumulating evidence that $\text{K}_\text{Ca}3.1$ plays a major role in chronic diseases characterized by excessive cell activation and proliferation, such as pulmonary fibrosis [279], renal fibrosis [278], and asthma [319]. The role of bronchial smooth muscle in asthma has been largely investigated and most evidence shows its potential role in driving many asthmatic features via its ability to secrete an array of different inflammatory mediators [330]. We found here that production of TNF-α/IFN-γ–induced CCL5 and CXCL10 was significantly increased in HBSMC from asthmatics and COPD patients compared to levels induced in cells from healthy controls (Figure 5.1). This “hypersecretory” phenotype of HBSMC in disease states was previously reported by Knox’s group showing an increased production CXCL8 in response to TNF-α alone [305] or VEGF following serum deprivation [306]. Although the mechanisms responsible for this “hypersecretory” phenotype of HBSMC in
asthma have not been completely defined yet, Knox's studies showed the existence of abnormal epigenetic pathways or increased activation of transcription factors including NF-κB or CAAT/enhancer binding protein (C/EBP)beta. Whether these pathways explain the changes seen in our results remain to be determined in future studies.

Our data showed that blockade or knockdown of $K_{Ca}3.1$ was effective in reducing CCL5, CCL11 and CX3CL1 expression at the protein and mRNA level. Our data showing that $K_{Ca}3.1$ inhibition does not affect CXCL10 induction strongly suggest that the inhibitory effect of $K_{Ca}3.1$ blockade is gene-specific and not due to a generalized non-specific or toxic effects linked to overall changes in Ca$^{2+}$ levels inside the cells. The two selective blockers of $K_{Ca}3.1$ called TRAM-34 and ICA-17043 have been used in various cell processes at physiologically relevant concentrations [282]. In addition, we did confirm that concentrations used here to block $K_{Ca}3.1$ channels were not associated with any cytotoxic effects ([Figure 5.3]) and these blockers were used at high concentration in relation to IC50. The ability to reproduce the same results when channels were knocked down by shRNA ([Figure 5.12]) shows unambiguously that $K_{Ca}3.1$ channel regulates, at least in part, production of steroid-resistant chemokines induced TNF-α/IFN-γ. We also saw some discrepancy between the effects of the two types of inhibitory strategies. For example, we found that cytokine-induced CCL5 and CCL11 expression was significantly affected by $K_{Ca}3.1$ shRNA ([Figure 5.12]), but not by the pharmacological blockers (TRAM-34 and ICA-17043) ([Figure 5.4]) and the degree of CX3CL1 inhibition was somewhat greater when combining fluticasone with pharmacological blockers
(Figure 5.6) and not with shRNA vectors (Figure 5.12). In contrast to soluble inhibitors, channel knockdown could indirectly have an impact on other cellular signaling pathways through the loss of interactions between the target protein (in this case, the \( \text{K}_{\text{Ca}3.1} \) channel) and other key binding partners. In the case of \( \text{K}_{\text{Ca}3.1} \), very little is known about the nature of proteins that associate with the channel. The direct binding of 5'-AMP-activated protein kinase (AMPK) [331] mammalian protein histidine phosphatase (PHPT-1) [332], or nucleoside diphosphate kinase B (NDPK-B) to \( \text{K}_{\text{Ca}3.1} \) was found to be essential in regulating channel activity [333]. Considering the fact that these proteins have multifunctional properties it is therefore plausible that reducing \( \text{K}_{\text{Ca}3.1} \) levels could lead to downstream effects not evoked by channel blockers alone. The apparent differences observed between the data obtained with the soluble inhibitors and silencing adenoviruses could also be due to the heterogeneity in patients’ responses because different subjects were used with the two inhibitory strategies.

With the exception of CX3CL1, the inhibitory action of \( \text{K}_{\text{Ca}3.1} \) blockers on CCL5 and CCL11 was dependent on the presence of fluticasone (Figures 5.4 and 5.5). This suggests the existence of a functional interaction between \( \text{K}_{\text{Ca}3.1} \) and corticosteroid receptor GR\( \alpha \) signalling pathways. This interesting point will be specifically addressed and discussed in Chapter 6. Regarding findings with CX3CL1 findings, it was interesting to see that TNF-\( \alpha \)/IFN-\( \gamma \) induced CX3CL1 expression, at both protein and mRNA levels, was inhibited by \( \text{K}_{\text{Ca}3.1} \) inhibitors (TRAM-34 and ICA-17043) irrespective of corticosteroid treatment (Figure 5.6). This unique observation shows that \( \text{K}_{\text{Ca}3.1} \) channel activity is required for the
transcriptional expression of CX3CL1. The fact that TNF-α/IFN-γ-dependent CX3CL1 expression in HBSMC was inhibited by two selective blockers of the $K_{Ca}3.1$ pore suggests that the ionic conductance of $K^+$, and not a regulatory channel domain, is a key in the mediation of this effect. Interestingly, TNF-α/IFN-γ alters $Ca^{2+}$ handling in HBSMC via the up-regulation of CD38 an ectoenzyme that converts NAD(+) to cyclic ADP-ribose (cADPr) [247]. It is possible that $K_{Ca}3.1$ channels regulate CX3CL1 via activation of $Ca^{2+}$-dependent signalling pathways. However, it seems unlikely that this is due to channel activity located in the plasma membrane and because $K_{Ca}3.1$ channels were found to be expressed in the nuclear compartment (Chapter 3) and we failed to detect any changes in channel activity in cells treated with TNF-α/IFN-γ (Chapter 4). Our study raises the possibility that nuclear modulation of $K_{Ca}3.1$ function by TNF-α/IFN-γ could explain their involvement in driving corticosteroid insensitivity in HBSMC. Of interest, the expression of $K_{Ca}3.1$ channels has been reported in cytoplasmic organelles such as mitochondria in a human colon tumor cell line [320]. Additional studies are clearly needed to define how intracellular $K_{Ca}3.1$ channels regulate cellular function in HBSMC.
Corticosteroid insensitivity in HBSMC involved a $K_{Ca3.1}$ channel-dependent PP5 modulation of GRα function
I. RATIONALE

Inhaled Corticosteroids (ICS) are very effective in the vast majority of patients with asthma as they decrease asthma exacerbations and mortality and improve lung function and patient quality of life [185]. A small proportion of patients referred as “severe asthma” cannot be properly managed even when using high-dose ICS or oral corticosteroid [334]. Several studies have tried to understand the mechanisms implicated in the patients’ poor sensitivity to corticosteroid therapy. Proposed mechanisms derived from studies using immune cells include “over-activation” of pro-inflammatory transcription factors (NF-κB, AP-1) and decreased expression and activity of co-repressor proteins (HDAC2), decreased GR-α expression, decreased affinity of ligands for GR-α, decreased ability of GR-α to bind DNA (reviewed [168]. The mechanisms involved in driving corticosteroid insensitivity in HBSMC have not been elucidated but studies using tracheal smooth muscle cells (HTSMC) showed multiple mechanisms mostly affecting GR-α function [300]. Indeed, cytokine-induced impairment of GR-α function in HTSMC includes upregulation of the dominant negative isoform GRβ [247], up-regulation of the transcription factor IRF-1 [166], activation of the protein phosphatase PP5 which dephosphorylates GRα on serine 211, which has been shown to be essential for its transcriptional activity [335]. Whether these mechanisms also participate in the impaired corticosteroid sensitivity induced in HBSMC (Chapter 3) remain to be explored and this was the aim of this final chapter of my thesis.
II. AIM

In previous chapters, we provided compelling and novel evidence for a role of $K_{Ca3.1}$ channels as potential players in mediating cytokine-induced steroid resistance in HBSMC. Here we tested whether GR-$\alpha$ function was impaired in cytokine-induced corticosteroid insensitivity in HBSMC by involving $K_{Ca3.1}$ channel-dependent mechanisms leading to decreased GR-$\alpha$ phosphorylation and transcription activity.
III. RESULTS

1. Fluticasone-induced GRα phosphorylation at Ser$^{211}$ is impaired in steroid-resistant states but restored in the presence of $K_{Ca3.1}$ channel inhibitors:

1.1 Fluticasone induced a dose-dependent GR phosphorylation at ser$^{211}$:

We first determine the kinetic of fluticasone induced GR-$\alpha$ phosphorylation at serine 211 in HBSMC in n=3 healthy donors. Serum-deprived HBSMC were incubated with fluticasone propionate (FP) (100nM) for different time points (0, 1, 2, 4, 6, and 24 hours) and total cell lysates were prepared and assayed for phospho-GR-$\alpha$, and $\beta$-actin by immunoblot analysis as described in Material and Methods. The data were normalised to $\beta$-actin and expressed as fold increase over un-stimulated cells (referred as basal).

We found that compared to basal condition, fluticasone induced a time-dependent increase in GR-$\alpha$ phosphorylation at Ser$^{211}$ (Figure 6.1A). Densitometric analyses of the blot revealed that the GR-$\alpha$ phosphorylation at Ser$^{211}$ after fluticasone treatment was significant at 2 hours and maximum at 6 hours (Figure 6.1B). The next following experiments were performed in cells treated with fluticasone for 6 hours.
Figure 6.1: Fluticasone Propionate increases GR-α phosphorylation at Ser\textsuperscript{211} in a time-dependent manner. HBSMC were treated with fluticasone (FP) (100 nM) for different times (0, 1, 2, 4, 6, and 24 hours), lysed and cell extracts were used for assessing phospho-GR (ser\textsuperscript{211}) by western blot. (A) Representative immunoblot of phospho-GR (ser\textsuperscript{211}) in HBSMC stimulated with FP for different time points. (B) Scanning densitometry of three representative immunoblots with each condition normalized over the area density of the corresponding β-actin content. The results are expressed as the fold increase over basal values. * P <0.05 compared with Basal/DMSO.
1.2 $K_{Ca}3.1$ blockers prevent cytokine-induced impairment of GR$\alpha$-phosphorylation induced by fluticasone

We next performed immunoblot to explore whether i) GR$\alpha$ phosphorylation was altered in cytokine-induced corticosteroid insensitive states and ii) $K_{Ca}3.1$ channels blockers (using TRAM-34 and ICA-17043) would affect this cytokines response. Immunoblots on total cell extracts were prepared from HBSMC treated with pharmacological inhibitors ICA-17034 (100 nM) and TRAM-34 (200nM) alone or in combination with fluticasone propionate 100nM for 2 hours followed by treatment TNF-$\alpha$/IFN-$\gamma$ for 4 hours. Cell extracts were assessed for phospho-GR$\alpha$-Ser$^{211}$ and $\beta$-actin to ensure equal loading after striping and reblotting the same membranes. The data were normalised to the corresponding $\beta$-actin and expressed as percentage increase of fluticasone (FP) treatment.

As shown in Figure 6.2A-B, fluticasone-induced phosphorylation of GR-$\alpha$ on serine 211 residue was reduced by 76.1 $\pm$ 5.7% in the presence of TNF$\alpha$/IFN-$\gamma$. Interestingly, the impairment of fluticasone-induced phosphorylation of GR$\alpha$ induced by cytokines was completely prevented by the presence of either ICA-17043 or TRAM-34. Importantly, $K_{Ca}3.1$ blockers (TRAM-34, ICA-17043) alone had no effect on basal GR-$\alpha$ phosphorylation in the absence of fluticasone.
Figure 6.2: Impaired fluticasone-induced GRα phosphorylation expression by cytokines is prevented by KCa3.1 inhibitors: Cells pretreated with TRAM-34 (200 nM) or ICA-17043 (100 nM), with or without fluticasone (100 nM, 2 hours), were stimulated with TNF-α/IFN-γ for 4 hours. (A) Total cell lysates were prepared and assayed for total GR, phospho-serine 211 GR Abs, and β-actin for loading by immunoblot analysis. (B) Means ±SEM of scanning densitometric analyses of blots from n = 3 healthy patients, with each value normalized over the mean density of the corresponding total GR bands. * p <0.05 compared with fluticasone. # p<0.05 compared with TNF-α/IFN-γ/FP.
2. **K\textsubscript{Ca}3.1** blockers prevent cytokine-induced impairment of GR\textalpha-dependent expression of GILZ induced by fluticasone

We next performed immunoblot to explore whether i) expression of GR\textalpha inducible genes such as glucocorticoid induced leucine zipper (GILZ) and MAPK phosphatase-1 (MKP-1) was altered in cytokine-induced corticosteroid insensitive states and ii) K\textsubscript{Ca}3.1 channels blockers (using TRAM-34 and ICA-17043) would affect this cytokines response. Total cell RNA was collected after stimulating HBSMC with fluticasone (2, 4, 6, 12, and 24hr) to determine the kinetic of GILZ and MKP-1 expression. WE also prepared RNA from HBSMC treated with K\textsubscript{Ca}3.1 blockers ICA-17043 or TRAM-34 alone or in combination with fluticasone for 2 hours followed by TNF\textalpha/IFN\textgamma for 4 hours. After reverse transcription (see Methods section) the resulting cDNA was subjected to q-PCR analyses for GILZ, MKP-1 and the housekeeping gene \beta-actin.

In HBSMC treated with fluticasone at different time points, levels of GILZ and MKP-1 mRNA were significantly increased compared to untreated cells which were 0.86 ± 0.2 and 11.06 ± 3.0 for GILZ (Figure 6.3) and MKP-1 (Figure 6.4), respectively. Fold increases of GILZ at 4hr and 6hr were 81.63 ± 38 and 105.3 ± 47.2 (Figure 6.3) while MKP-1 levels were increased by 99.46 ± 9.0 and 102 ± 41.1 (Figure 6.4), respectively. The 6 hr time point was found to be most optimal time for GILZ and MKP-1 detection and used therefore in the parallel experiments using K\textsubscript{Ca}3.1 blockers.

As shown in the Figure 6.2 fluticasone-induced GR\textalpha phosphorylation on Ser\textsuperscript{211} was almost completely inhibited in the presence of TNF-\alpha/IFN-\gamma.
Interestingly, we also found that HBSMC treated with TNF-α/IFN-γ also led to an impaired expression of GRα-inducible gene GⅡLZ (Figure 6.3B) and MKP-1 (Figure 6.4B). This inhibition of fluticasone-induced GRα-inducible GⅡLZ by cytokines was fully restored by the presence of K_{Ca}3.1 inhibitors (TRAM-3, ICA-17034) (Figure 6.3B). However both K_{Ca}3.1 inhibitors fail to prevent the full restoration of MKP-1 expression seen in steroid-resistant state (Figure 6.4B).
Figure 6.3: Effect of K<sub>Ca</sub>3.1 blockers on fluticasone-induced GILZ expression in steroid resistant state. Time course of GILZ expression (A), effects of K<sub>Ca</sub>3.1 inhibitors on fluticasone-induced GILZ expression by TNFα/IFNγ (B). Cells were harvested at the times indicated and Q-PCR analyses were performed for GILZ and β-actin expression. Data present as Means ± SEM of experiments performed in duplicate in 3 different subjects (*p<0.05 compared with Basal/DMSO. **P<0.01 and ***P<0.001 compared with TNFα/IFN-γ/FP). (C) Representative Q-PCR products of GILZ ran on 2% agarose gel showed one single product amplified with the correct size. β-Actin was used as loading control.
Figure 6.4: Effect of K<sub>Ca</sub>3.1 blockers on fluticasone-induced MKP-1 expression in steroid resistant state. Time course of MKP-1 expression (A), effects of K<sub>Ca</sub>3.1 inhibitors on fluticasone-induced MKP-1 expression by TNFα/IFNγ (B). Cells were harvested at the times indicated and Q-PCR analyses were performed for MKP-1 and β-actin expression. Data present as Means ± SEM of experiments performed in duplicate in 3 different subjects (*p<0.05 compared with Basal/DMSO. *P<0.05 and compared with TNF-α/IFN-γ/FP). (C) Representative Q-PCR products of MKP-1 ran on 2% agarose gel showed one single product amplified with the correct size. β-Actin was used as loading control.
3. **PP5 expression is induced by TNF-α/IFN-γ via K_{Ca}3.1 channel dependent pathways**

3.1 **Time course of PP5 expression induced by TNF-α/IFN-γ**

We next investigated whether up-regulation of PP5 could explain the impairment of fluticasone-induced GRα phosphorylation and GRα-dependent gene expression (Figures 6.3-6.4). The link between PP5 and steroid insensitivity has been suggested by Goleva and colleagues who found that PP5 knockdown restored GC responsiveness in oestrogen-treated breast cancer cells [206]. We first assessed the kinetic of PP5 expression following HBSMC treated with TNF-α /IFN-γ. Flow cytometry was performed on confluent HBSMC stimulated with TNF-α /IFN-γ for, 1, 2, 4, 6 and 24 hours. HBSM cells were fixed and permeabilized in 4% paraformaldehyde/0.1% saponin for 15 minutes on ice before being stained with 2 µg/ml of rabbit anti-human PP5 antibody (Cell Signaling, Danvers, MA USA) followed by fluorescein isothiocyanate (FITC) anti-mouse secondary antibody (1:10 dilution). The data were analysed using flowing Software (version 2.5.1).

As shown in Figure 6.5A, HBSMC had significant levels of PP5 in unstimulated conditions compared with isotype control staining. (Data present as mean ± SEM of 3 different donors). This basal level of PP5 was significantly increased upon stimulation with TNF-α/IFN-γ with a significantly increased seen at 6 h with 1.5 ± 0.15 fold and at 24 hours with 1.87 ± 0.51 fold compared to unstimulated BSM cells Figure 6.5B.
Figure 6.5: Effect of TNF-α/IFN-γ on PP5 expression: HBSMC were stimulated with TNF-α/IFN-γ for 1, 2, 4, 6 and 24 hours. (A) Example of fluorescent histograms for HBSMC showing populations of PP5 positive cells 24 hours post-cytokine stimulation. (B) Quantitative analysis of PP5 protein expression in HBSMC treated with TNFα/IFN-γ for different time points. Protein expression was calculated according to the formula: (GMFI-PP5–GMFI Isotype) and expressed as fold change to unstimulated condition (GMFI # Geometric mean fluorescence intensity). Data present as Means ± SEM of experiments performed in 3 different subjects (*p<0.05 compared with Unstimulated (US)).
3.2 $K_{Ca}3.1$ channel inhibition suppresses cytokine-induced PP5 expression

We next asked the question of whether $K_{Ca}3.1$ channel would play any role in the up-regulation of PP5 induced by cytokines by assessing the effect of $K_{Ca}3.1$ channel inhibitors. Confluent HBSMC were serum deprived for 24 hours and incubated with TRAM-34 or ICA-17043 for 2 hours followed by TNF-α/IFN-γ for an additional 24 hours. Cells were then stained with either 2 µg/ml mouse anti-human PP5 antibody or isotype-matched control followed by secondary rabbit anti-mouse FITC as described in the previous result section. Staining was again examined by flow cytometry.

Flow cytometry assays demonstrated that PP5 was indeed up-regulated following treatment with TNF-α/IFN-γ in HBSMC from nine additional subjects (four asthmatic subjects and five healthy subjects) (1.57± 0.2 fold), but interestingly, this response was significantly inhibited by ICA-17043 and TRAM-34 (0.8±0.15 fold), (1.06±0.165 fold) respectively (Figure 6.6). This finding suggests that $K_{Ca}3.1$ channel activity directly regulates the expression PP5.
Figure 6.6: PP5 upregulation by TNF-\(\alpha\)/IFN-\(\gamma\) is inhibited by \(K_{\text{Ca}}3.1\) inhibitors.

Cells pre-treated with TRAM-34 (200 nM) or ICA-17043 (100 nM) for 2 hours then were stimulated with TNF-\(\alpha\)/IFN-\(\gamma\) for 24 hours. PP5 levels assessed by flow cytometry. PP5 expression were expressed as the fold increase in geometric mean fluorescence intensity over basal data present as Means ± SEM of experiments performed in 9 subjects (\(n = 4\) asthmatic patients and \(n = 5\) healthy controls), *\(p <0.05\), **\(p <0.01\).
3.3 PP5 expression within BSM

Based on the reported role of PP5 in driving corticosteroid resistance in both tracheal and bronchial smooth muscle cells (Figure 6.6), it was important to determine whether PP5 was expressed in the bronchial smooth muscle tissues in vivo. Sequential 2-µm sections were cut from glycol methacrylate (GMA) embedded bronchial biopsies and immunostained using a mouse monoclonal anti-human PP5 (1.5 µg/ml) antibody or α-SMA (1 µg/ml) and isotype control mouse IgG (1 µg/ml). The staining was performed using EnVision™ FLEX Mini Kit (DAKO) as described in the method section.

Immunohistochemistry assays reveal that HBSM bundles express PP5 in vivo as evidenced by the marked DAB staining in the area that is also stained with the antibody against α-smooth muscle actin, used as positive control of smooth muscle bundles (Figure, 6.7). No immunohistochemical reaction was observed when a consecutive biopsy section of the same field was incubated with isotype control increasing the confidence in the specificity of the staining; α-smooth muscle actin staining was used as positive control of smooth muscle bundles (Figure, 6.7).
Figure 6.7: Expression of PP5 in bronchial smooth muscle bundles. The bronchial biopsies were stained with a mouse monoclonal antibody (1.5μg/mL) in acetone fixed GMA embedded sections. Positive staining was visualised with DAB detection and hematoxylin counter stain. (A) Magnification X200, (B) magnification X400. HBSM bundles were positively stained using α-smooth muscle actin. (C) No staining is seen in sections probed with Isotype controls (IC) (200X).
VI. DISCUSSION

There is considerable evidence from the literature speculating about the potential mechanisms causing steroid resistance in patients with severe asthma. Changes in post-translational modifications of GR-α have been proposed as possible mechanisms impairing GR-α function [334]. One of the major finding in our study was to show the functional interaction between K_{Ca}3.1 channels and GRα phosphorylation.

The first major finding of our study is the demonstration that cell sensitivity to fluticasone was restored by K_{Ca}3.1 blockade, an effect that was associated with a reinstatement of the GRα phosphorylation at Ser^{211} (Figure 6.2) and GR-α transactivation activities (Figures 6.3-6.4). Evidence shows that GR-α phosphorylation appears on three major residues located on its N terminus (Ser^{203}, Ser^{211}, and Ser^{226}) [334]. Although the modulation of site-specific GR phosphorylation regulates its function, expression and the overall GC responsiveness it is phosphorylation on Ser^{211} that is vital for optimal GRα transcriptional activity [188]. Indeed, our data show that TNF-α/IFN-γ effectively suppressed both fluticasone-induced expression of two anti-inflammatory proteins called GILZ-1 and MKP-1 (Figures 6.3-6.4). Ammit's group showed that induction of MKP-1 was playing an essential role in inhibiting expression of pro-inflammatory mediators in HBSMC [336-339]. This raises the possibility that restoration of corticosteroid anti-inflammatory properties by K_{Ca}3.1 blockers in HBSMC could be due to increased MKP-1 expression.
The second major finding of this chapter is the observation that the protein phosphatase PP5 was induced by TNF-α/IFN-γ (Figure 6.5) via a K_{Ca3.1}-associated mechanism (Figure 6.6). A previous study in tracheal smooth muscle cells by TLIBA’s group provided the first evidence of the involvement of PP5 in the impaired fluticasone-induced GRα phosphorylation on Ser^{211} in corticosteroid resistant state. Importantly, PP5 silencing was able to restore GR function and anti-inflammatory activity [335]. Our present data demonstrate that expression of PP5 is induced in a dose-dependent manner in HBSMC and sustained up to 24 hours. More importantly, cytokine-induced PP5 induction was associated with functional K_{Ca3.1} channels (Figure 6.6). Although a direct link between PP5 and steroid insensitivity has been proposed by Goleva and colleagues [340], who found that PP5 knockdown restored GC responsiveness in oestrogen-treated breast cancer cells, our finding, to our knowledge, is the first to show that this functional link between proasthmatic cytokines and PP5 occurs via K_{Ca3.1}-associated pathways.

Our present chapter supports the novel concept that TNF-α/IFN-γ impairs corticosteroid sensitivity in HBSMC by promoting GRα de-phosphorylation via K_{Ca3.1}-associate up-regulation of PP5 expression. The clinical importance of PP5 axis was supported by the observation that PP5 is expressed in vivo in bronchial smooth muscle bundles (Figure 6.7).
Chapter 7

Conclusions and Future directions
I. CONCLUSION

1. Growing evidence showing the existence of corticosteroid resistance in bronchial smooth muscle in asthma

The mechanisms underlying corticosteroid resistance in asthma have not been completely elucidated [185]. A number of in vitro and in vivo studies have demonstrated the existence of corticosteroid-resistant pathways in bronchial smooth muscle within asthmatic airways [341]. The strongest evidence comes from immunohistochemistry studies looking at whether inflammatory mediators were present in vivo in the smooth muscle bundles isolated from bronchial biopsies. The authors convincingly showed that in asthmatics, the bronchial smooth muscle positively stained for different inflammatory mediators that include CX3CL1 [326], CCL11 [342], CCL15 [291], CCL19 [296], ADAM33 and ADAM8 [59, 343], despite patients being treated with either high dose inhaled or oral GC. These studies suggest that in asthma bronchial smooth muscle develops a corticosteroid-resistant phenotype that in turn leads to the production of different inflammatory mediators. The mediators could then participate in the progression of the disease via different pro-inflammatory activities. The therapeutic benefit experienced by severe asthmatics treated with a therapy that attenuates bronchoconstriction via reduction of BSM mass called bronchial thermoplasty is also supporting the notion that BSM is playing a central role in the pathogenesis of the severe disease [107, 344-346]. In addition to its central role in bronchoconstriction, the role of BSM in the pathogenesis of severe asthma could derive also from its immunomodulatory
function as it secretes a variety of pro-inflammatory mediators which in turn could play in asthma pathogenesis [300].

2. **Use of cultured bronchial smooth muscle cells to establish an in vitro model of corticosteroid insensitivity seen in the muscle bundles of asthmatic patients**

   In previous studies, Amrani’s group modeled this *in vivo* corticosteroid-resistant state *in vitro* by exposing cultured human tracheal airway smooth muscle cells to a combination of TNF-α/IFN-γ. When compared to cells treated with TNF-α, cells treated with both cytokines resulted in the production of an array of inflammatory proteins including CD38, CCL5, CX3CL1 and IRF-1 that were completely resistant to fluticasone treatment [166, 187, 221, 247, 335]. Because these studies were performed in tracheal smooth muscle cells, it was clinically important to confirm these observations using smooth muscle cells isolated from bronchial tissues. Here, we confirmed that HBSMC treated with cytokine combination become steroid resistant (**Fig.3.5 and Fig 3.6**). More importantly, we found that in addition to CCL5 and CX3CL1, production of other key chemokines CCL11 and CXCL10 was also not affected by fluticasone treatment. In addition, we show for the first time that corticosteroid-resistant state also occurs *in primary HBSMC in health, asthma and COPD*. Our studies also showed an increased production of chemokines by asthmatic versus healthy HBSMC supporting the initial observation by Knox’s group that cultured asthmatic cells may still retain their “abnormal phenotype” with respect to chemokine secretion [305, 306], Although investigating the mechanisms behind the hypersecretion of chemokines by asthmatic HBSMC was not the
main goal of this PhD thesis, others found that NF-κB pathways was increased in these cells [305]. Further studies are clearly required to dissect the underlying mechanisms causing the “abnormal phenotype” seen in asthmatic HBSMC.

3. **KCa3.1 as a potential new target for the treatment of corticosteroid resistance in HBSMC**

   i) **Evidence for the expression of KCa3.1 channels both in vivo in bronchial smooth muscle bundles and in cultured cells**

Immunohistochemistry on bronchial biopsies demonstrated that KCa3.1 channels were expressed *in vivo* in HBSM bundles in asthmatic patients, and interestingly, this included a nuclear distribution. This observation was further confirmed by assessing KCa3.1 expression in cultured BSM cells using two different approaches, i.e., immunoblot analysis directly on nuclear extracts ([Fig. 4.4](#)) and immunofluorescence staining showing a nuclear distribution of KCa3.1 ([Fig. 4.5](#)). KCa3.1 expression in asthmatic HBSM bundles was not affected by disease severity and the associated intensity of anti-asthma treatment, and no differences in immunostaining intensity were evident between healthy subjects and asthmatic patients ([Fig. 4.6](#)). This suggests that changes in channel activity rather than in changes in protein expression within HBSMC bundles could explain their contribution to the pathogenesis in asthma. In contrast to the study showing that TGFβ increased both KCa3.1 expression and activity in HBSMC [293], we found that TNF-α/IFN-γ failed to significantly stimulate KCa3.1 protein levels ([Fig. 4.7](#)), or KCa3.1 channel activity ([Fig. 4.8](#)), suggesting that modulation of KCa3.1 expression/function in HBSMC is highly stimulus-
dependent (growth factors versus cytokines). The fact that there is no change in $K_{Ca3.1}$ activity when measured at the cell surface suggests the concept that compartmental changes in calcium levels rather than cytoplasmic changes could explain the role of $K_{Ca3.1}$ in HBSMC reported here (Chapter 5) and other [293]. Because expression of $K_{Ca3.1}$ channels was found to be expressed in the nuclear compartment (Fig. 4.5), our study raises the possibility that a nuclear $K_{Ca3.1}$ function also play a role in regulating HBSMC function. Interestingly, the expression of $K_{Ca3.1}$ channels has been reported in cytoplasmic organelles such as mitochondria in a human colon tumour cell line [320]. Additional studies are clearly needed to define how intracellular $K_{Ca3.1}$ channels regulate cellular function in HBSMC.

**ii) Evidence of a role of $K_{Ca3.1}$ channels in corticosteroid insensitivity in HBSMC**

We made the interesting observation that combining fluticasone and $K_{Ca3.1}$ blockers was effective in inhibiting the production of corticosteroid-resistant CCL5, CCL11 and CX3CL1 by HBSMC treated with both TNF-α/IFN-γ. CXCL10 in contrast was not affected by fluticasone and $K_{Ca3.1}$ blockade ruling out a non-specific effect of $K_{Ca3.1}$ inhibitors and defining a gene specific action of $K_{Ca3.1}$ in the regulation of chemokine expression. In addition we have confirmed our previous report [293], showing that $K_{Ca3.1}$ inhibitors had no cytotoxic action on BSM cells. This is the first evidence reporting a role of $K_{Ca3.1}$ channels in the regulation of cell sensitivity to corticosteroids in any given tissues. It is interesting to note that while $K_{Ca3.1}$ blockade had a strong inhibitory effect on cytokine-induced CCL5 expression at the mRNA level,
production of CCL5 protein was only reduced by 50%. We believe that in addition to transcriptional mechanisms, CCL5 induction by cytokines is also regulated at the post-transcriptional level. Previous reports performed in A549 cells [347] and in HBSMC [348] supports this hypothesis by showing that while IFNγ on its own did not stimulate CCL5 expression, it does enhance TNF-α-induced CCL5 expression via post-transcriptional mechanisms. We also have some preliminary evidence using a different pharmacological inhibitors demonstrating that the degree of CCL5 inhibition seen at the mRNA levels does not correlate with similar changes at the protein level (Chachi, unpublished observation). The ability to reproduce the same results when channels were downregulated by shRNA shows unequivocally that K_{Ca}3.1 regulates, at least in part, TNF-α/IFN-γ-dependent corticosteroid-resistant chemokine expression. In our study, we noted some expected discrepancy between the two inhibitory strategies. For example, cytokine-induced CCL5 expression was solely affected by shRNA but not by the pharmacological blockers or the degree of CX3CL1 inhibition was somewhat greater by combining fluticasone with pharmacological blockers and not with shRNA vectors. In contrast to soluble inhibitors, channel knockdown could indirectly impact on other cellular signalling pathways through the loss of interactions between the target protein (here the K_{Ca}3.1 channel) and other key binding partners. In the case of K_{Ca}3.1, very little is known about the nature of proteins that associate with the channel. The direct binding of 5'-AMP-activated protein kinase (AMPK) [331], mammalian protein histidine phosphatase (PHPT-1) [332], or nucleoside diphosphate kinase B (NDPK-B) to K_{Ca}3.1 was found to be essential in regulating channel activity [333].
Considering the fact that these proteins have multifunctional properties, it is therefore plausible that reducing $\text{K}_{\text{Ca}3.1}$ levels could lead to downstream effects not evoked by channel blockers alone. The apparent differences observed between the data obtained with the soluble inhibitors and silencing adenoviruses could also be due to the heterogeneity in patients’ responses since different subjects were used with the two inhibitory strategies. Interestingly, corticosteroid-resistant CX3CL1 expression, at both protein and mRNA levels, was inhibited by $\text{K}_{\text{Ca}3.1}$ inhibitors irrespective of corticosteroid treatment, suggesting that $\text{K}_{\text{Ca}3.1}$ channel activity is a key factor regulating the transcriptional expression of this chemokine in response to TNF-α/IFN-γ. The putative role of $\text{K}_{\text{Ca}3.1}$ in regulating expression of inflammatory mediators has been mostly described in T lymphocytes where $\text{K}_{\text{Ca}3.1}$ blockade (either via pharmacological inhibitors or the use of T cells deficient in the channel) significantly reduces T cell receptor-induced expression of IL-2, TNFα and IFNγ [349, 350]. In T cells it is likely that this results from the instrumental role of $\text{K}_{\text{Ca}3.1}$ in regulating Ca$^{2+}$ entry through the plasma membrane, a vital signal for optimal T cell activation and cytokine secretion. The fact that TNF-α/IFN-γ-dependent CX3CL1 expression in HBSMC is inhibited by two selective blockers of the $\text{K}_{\text{Ca}3.1}$ pore demonstrates that the ionic conductance of K$^+$ is key in the mediation of this effect, and not a regulatory channel domain. Interestingly, we and others have shown that TNFα alters Ca$^{2+}$ handling in HBSMC via the induction of ectoenzyme CD38 [351, 352] or transient receptor potential C3 channels [353]. It is therefore plausible that $\text{K}_{\text{Ca}3.1}$ regulates CX3CL1 via
activation of Ca\(^{2+}\)-dependent pathways. However, as discussed above, it seems unlikely that this is due to channel activity in the plasma membrane.

**iii) Evidence for role of K\textsubscript{Ca}3.1 channels in promoting corticosteroid insensitivity in HBSMC via the inhibition of corticosteroid receptor (GR\textsubscript{α}) function**

Another major observation from my PhD studies is the surprising functional interaction between K\textsubscript{Ca}3.1 channels and corticosteroid signalling pathways. Specifically, we found that K\textsubscript{Ca}3.1 inhibitors restored the ability of fluticasone to suppress the production of CCL5 and CCL11 in TNF-α/IFN-γ-induced GC-resistant conditions, irrespective of the K\textsubscript{Ca}3.1 inhibition strategy used. In addition, this restoration of cell sensitivity to fluticasone by K\textsubscript{Ca}3.1 blockade was concomitantly associated with a reinstatement of the GR\textsubscript{α} phosphorylation at ser\textsuperscript{211} that was impaired in TNF\textsubscript{α}/IFN\textsubscript{γ}-treated HBSMC. This shows that K\textsubscript{Ca}3.1 activity drives TNF\textsubscript{α}/IFN\textsubscript{γ}-induced corticosteroid insensitivity in HBSMC, in part by via the modulation of GR\textsubscript{α} phosphorylation status (Fig.6.1) and GR\textsubscript{α} transactivation activity (Fig.6.2). GR\textsubscript{α} is phosphorylated on three major residues located on its N terminus (ser\textsuperscript{203}, ser\textsuperscript{211}, and ser\textsuperscript{226}) which is an important event in regulating key functions of GR\textsubscript{α} including turnover and subcellular trafficking, although phosphorylation on ser\textsuperscript{211} is essential for optimal GR\textsubscript{α} transcriptional activity [188]. Indeed, in agreement with our previous reports [166, 187, 247] we did confirm that fluticasone-induced GR\textsubscript{α}-dependent transactivation was significantly impaired in TNF-α/IFN-γ-induced GC-insensitive state (Fig.6.2). More importantly, K\textsubscript{Ca}3.1 blockade was also able to
fully restore fluticasone-induced GRα-dependent transactivation, suggesting a role of KCa3.1 channel in impairing corticosteroid function in HBSMC. Our previous report showed that the impaired GRα transactivation was due to the up-regulation of the serine/threonine phosphatase PP5 which mediated cytokine-induced GRα dephosphorylation at ser211 [335]. Here, we not only confirm that PP5 levels are increased by TNFα/IFNγ in HBSMC independently of disease status (healthy and asthmatics), but more importantly, that PP5 induction was dependent on functional KCa3.1 channels. Although a direct link between PP5 and steroid insensitivity has been suggested by Goleva and colleagues who found that PP5 knockdown restored corticosteroid responsiveness in oestrogen-treated breast cancer cells [340], our report is the first to show that this functional link between pro-asthmatic cytokines and PP5 occurs via KCa3.1-dependent pathways. Our present study supports the novel model that TNF-α/IFN-γ impairs corticosteroid sensitivity in HBSMC by promoting GRα dephosphorylation via KCa3.1-dependent up-regulation of PP5 expression.

In summary, we have shown that KCa3.1 channels contribute to TNF-α/IFN-γ-associated GC-insensitivity (Figure 7.1). Transcription of some pro-asthmatic genes such as CX3CL1 is driven by KCa3.1-dependent pathways that are insensitive to corticosteroids. Other genes such as CCL5 or CCL11 are indirectly rendered resistant to corticosteroid via the induction of the KCa3.1-dependent serine/threonine phosphatase PP5 which interferes with GRα receptor function through decreased GRα phosphorylation and transactivation.
Our study uncovers the potential therapeutic value of targeting the $K_{Ca3.1}$-PP5 axis in the treatment of lung diseases such as severe asthma where relative GC resistance is evident. The availability of a well-tolerated orally bioavailable $K_{Ca3.1}$ blocker that has been used in phase III trials of sickle cell disease (ICA-17043 [Senicapoc] [274]) means that there is the potential for the rapid translation of these findings to the clinic. This is a likely possibility considering the benefit provided by $K_{Ca3.1}$ blockers in recent preclinical studies using animal models of asthma [295, 318, 319].
Figure 7.1: Role of K_{Ca}3.1 in mediating GC insensitivity in BSM cells: We uncovered two mechanisms by which K_{Ca}3.1 channels mediate TNF-α/IFN-γ–associated corticosteroid insensitivity. Transcription of some proasthmatic genes involves K_{Ca}3.1-dependent pathways that are insensitive to corticosteroids (right). Other genes are resistant to corticosteroids via the induction of the K_{Ca}3.1-dependent serine/threonine phosphatase PP5, which impairs GRα transcriptional function through decreased GRα phosphorylation (left).
II.  FUTURE DIRECTIONS

We show here that in addition to regulating airway inflammation and airway remodelling in asthma, HBSM may also regulate in the pathogenesis of severe asthma via the production of corticosteroid insensitive inflammatory mediators.

From the work presented here, we plan to focus our next experiments on:

- The implication of $K_{Ca}3.1$ channel in the regulation of other steroid-resistant genes in HBSMC also induced by TNF-$\alpha$/IFN-$\gamma$.
- The molecular mechanisms linking cytokines signalling and $K_{Ca}3.1$ channel activation (Calcium dependent mechanisms).
- The $K_{Ca}3.1$-dependent signalling pathways involved in the up-regulation of PP5 and CX3CL1
- The effect of TRAM-34 and ICA-17043 on other key responses in HBSMC such as contraction, migration, proliferation and remodelling.
- The ultimate goal would be to initiate a small pilot clinical trial to test whether $K_{Ca}3.1$ channel blockers have any protective effects in patients with severe asthma.
REFERENCES


196


Wulff, H. and N.A. Castle, Therapeutic potential of KCa3. 1 blockers: recent advances and promising trends. 2010.


209


