Novel insights into mechanisms of glucocorticoid actions and sensitivity in the airway epithelium

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ABSTRACT

Glucocorticoids (GCs) remain the frontline treatment in the management of chronic inflammatory diseases, as they are the most potent and effective anti-inflammatory agents available so far. However, impaired responses to glucocorticoid therapy in some patients with severe disease remain a challenging clinical problem.

The airway epithelial function influences inflammation in chronic respiratory diseases. Epithelium, as the site of deposition of inhaled glucocorticoids (ICS), is a key target of GC action. Synthetic GCs, including ICS, exert anti-inflammatory effects in airway epithelium by transactivation of genes and by inhibition of release of pro-inflammatory cytokines. Emerging evidence suggests that physiological GC, cortisol, might act as a partial agonist at the glucocorticoid receptor (GR) in the airway epithelium. However, whether cortisol can be a limiting factor to beneficial effects of synthetic GCs, remains to be established. Therefore, through a better understanding of the impact of cortisol on the effects of synthetic GCs in vitro and in vivo, as well as of novel individual mediators of GC actions in the airway epithelium, new strategies may arise for restoring GC responsiveness.

Data presented within this thesis has provided evidence that cortisol acts like a partial agonist at the glucocorticoid receptor, limiting GC-induced GC Receptor-dependent transcription in the BEAS-2B human bronchial epithelial cell line. Cortisol also limited the inhibition of granulocyte-macrophage colony-stimulating factor (GM-CSF) release by synthetic GCs in TNFα-activated BEAS-2B cells. The relevance of these findings is supported by observations on tracheal epithelium obtained from mice treated for 5 days with systemic GC, showing limitations in selected GC effects, including inhibition of pro-inflammatory cytokine IL-6. Moreover, gene transactivation by synthetic GCs was compromised by standard air-liquid interface (ALI) growth medium cortisol concentration of 1.4 μM in the ALI differentiated organotypic culture of primary human airway epithelial cells. These findings suggest that endogenous corticosteroids may limit certain actions of synthetic pharmacological GCs and contribute to GC insensitivity, particularly when corticosteroid levels are elevated by stress.
Data obtained during these thesis studies also highlight the potential of the transcriptional repressor, promyelocytic leukaemia zinc finger (PLZF) to mediate selected glucocorticoid effects in the airway epithelium, including the induction of targets important in mediating physiological effects on the normal lung development and of ones with relevance to the distinct glucocorticoid effects on the epithelial restitution following inflammation and injury.

This thesis has provided novel insights into mechanisms of glucocorticoid action and insensitivity in the airway epithelium, allowing the development of strategies for improved treatment of chronic airway inflammatory diseases.
DECLARATION

This is to certify that

a) This thesis compromise only my original work towards the PhD except where indicated in the preface.

b) This thesis contains no material previously published or written by other person.

c) The thesis is fewer than 100,000 words in length, exclusive of tables, graphs and bibliographies.

Danica Prodanovic, MPharm

Date: May 2018
PREFACE

Preparation of rat-tail collagen for establishing air-liquid interface cultures of primary human bronchial epithelial cells was performed by Ms Trudi Harris. The initial in vivo study investigating the effect of dexamethasone on the gene expression in the mouse tracheal epithelium and lung tissue was performed by Ms Shenna Langenbach. The initial preparation of human airway tissue sections (paraffin-embedding and cutting) for immunohistochemistry was performed by Ms Shenna Langenbach.
COMMUNICATIONS

PEER-REVIEWED PUBLICATIONS:


SELECTED CONFERENCE PRESENTATIONS:

   
   Submitted abstract

   
The role of glucocorticoid-inducible transcriptional repressor PLZF in the airway epithelium

2. Australia Society of Clinical and Experimental Pharmacologist and Toxicologists (ASCEPT) 2016, Melbourne, Australia
   
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3. Australia Society of Clinical and Experimental Pharmacologist and Toxicologists (ASCEPT) 2016, Melbourne, Australia
   
   Poster presentation

   
   Cortisol inhibits selected actions of inhaled corticosteroids (ICS) in human airway epithelium

   
   Poster presentation

   
   The physiological glucocorticoid (GC), hydrocortisone, limits selected actions of synthetic GCs in human airway epithelium
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Poster presentation


Transcriptional repressor ZBTB16 (PLZF) is strongly induced by glucocorticoids (GCs) in human bronchial epithelial cells
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ETHICS STATEMENT

All experiments using cells from animal or human origin were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, and the National Statement on Ethical Conduct in Human Research.
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Table 5-2 The effect of the non-silencing siRNA and PLZF siRNAs on the expression of cell-proliferation markers in the presence of dexamethasone (Dex) and/or 5% fetal calf serum (FCS)...

XIX
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotrophic hormone</td>
</tr>
<tr>
<td>ADX</td>
<td>Adrenalectomy</td>
</tr>
<tr>
<td>AHR</td>
<td>Airway hyperresponsiveness</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>ALD</td>
<td>Aldosterone</td>
</tr>
<tr>
<td>ALI</td>
<td>Air-liquid-interface</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>APL</td>
<td>Acute promyelocytic leukaemia</td>
</tr>
<tr>
<td>ASTH</td>
<td>Asthmatic</td>
</tr>
<tr>
<td>BEGM</td>
<td>Bronchial epithelial growth medium</td>
</tr>
<tr>
<td>BMI1</td>
<td>B cell-specific murine leukemia virus integration site 1</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
</tr>
<tr>
<td>BPE</td>
<td>Bovine pituitary extract</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Bud</td>
<td>Budesonide</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>Cas9</td>
<td>CRISPR associated protein 9</td>
</tr>
<tr>
<td>CBG</td>
<td>Cortisol-binding globulin</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB-binding protein</td>
</tr>
<tr>
<td>CDKN1A</td>
<td>Cyclin-dependent kinase inhibitor 1A</td>
</tr>
<tr>
<td>CDKN1C</td>
<td>Cyclin-dependent kinase inhibitor 1C</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>C/EBPα</td>
<td>CCAAT/enhancer-binding protein alpha</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclooxygenase-2</td>
</tr>
<tr>
<td>CRC</td>
<td>Concentration response curve</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Clustered regularly interspaced short palindromic repeats</td>
</tr>
<tr>
<td>CSF-2</td>
<td>Colony stimulating factor 2</td>
</tr>
<tr>
<td>Ct</td>
<td>Threshold cycle</td>
</tr>
<tr>
<td>CUX-1</td>
<td>Cut like homeobox 1</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3'-diaminobenzidine</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>Dex</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DUSP6</td>
<td>Dual specificity phosphatase 6</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</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>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>ENaCα</td>
<td>Epithelial sodium channel subunit alpha</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular-signal-regulated kinase</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FKBP5</td>
<td>FK506 binding protein 5</td>
</tr>
<tr>
<td>FOXN3</td>
<td>Forkhead box N3</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>FP</td>
<td>Fluticasone propionate</td>
</tr>
<tr>
<td>GA</td>
<td>Gentamicin</td>
</tr>
<tr>
<td>GC</td>
<td>Glucocorticoid</td>
</tr>
<tr>
<td>GFI-1</td>
<td>Growth factor independent 1</td>
</tr>
<tr>
<td>GILZ</td>
<td>Glucocorticoid-induced leucine zipper</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>HAT</td>
<td>Histone acetyltransferase</td>
</tr>
<tr>
<td>HBEC</td>
<td>Human bronchial epithelial cell</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>HC</td>
<td>Hydrocortisone</td>
</tr>
<tr>
<td>HCEC</td>
<td>Human corneal epithelial cell</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<tr>
<td>HGF</td>
<td>Human growth factor</td>
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<tr>
<td>HIFCS</td>
<td>Heat inactivated fetal calf serum</td>
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<tr>
<td>HPA</td>
<td>Hypothalamic-pituitary-adrenal axis</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat-shock protein</td>
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<tr>
<td>HSD</td>
<td>Hydroxysteroid dehydrogenase</td>
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<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>ICS</td>
<td>Inhaled corticosteroid</td>
</tr>
<tr>
<td>ID2</td>
<td>Inhibitor of DNA binding 2</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<td>IHC</td>
<td>Immunohistochemistry</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>IkB</td>
<td>Inhibitor of nuclear factor kappa B</td>
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<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IPF</td>
<td>Idiopathic pulmonary fibrosis</td>
</tr>
<tr>
<td>ISG</td>
<td>Interferon-stimulated gene</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>KC</td>
<td>(C-X-C motif) ligand 1 chemokine (CXCL1)</td>
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<tr>
<td>KCNB1</td>
<td>Potassium voltage-gated channel subfamily B member 1</td>
</tr>
<tr>
<td>LEF-1</td>
<td>Lymphoid enhancer binding factor 1</td>
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<tr>
<td>LHC-9</td>
<td>Laboratory of human carcinogenesis medium #9</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<tr>
<td>MESCA</td>
<td>Melbourne epidemiological study of childhood asthma</td>
</tr>
<tr>
<td>MKP-1</td>
<td>MAP kinase phosphatase 1</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MP</td>
<td>Methylprednisolone</td>
</tr>
<tr>
<td>MR</td>
<td>Mineralocorticoid receptor</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>NBF</td>
<td>Neutral buffered formalin</td>
</tr>
<tr>
<td>NCOR1</td>
<td>Nuclear receptor corepressor 1</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NHBE</td>
<td>Normal human bronchial epithelial cell</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>iNOS</td>
<td>(inducible) Nitric oxide synthase</td>
</tr>
<tr>
<td>NR3C1</td>
<td>Nuclear receptor subfamily 3 group C member 1</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PBX-1</td>
<td>Pre-B-cell leukaemia transcription factor 1</td>
</tr>
<tr>
<td>PCC</td>
<td>Pearson’s correlation coefficient</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
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</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PLA2</td>
<td>Phospholipase A2</td>
</tr>
<tr>
<td>PLZF</td>
<td>Promyelocytic leukaemia zinc finger</td>
</tr>
<tr>
<td>POZ</td>
<td>Poxvirus and zinc finger</td>
</tr>
<tr>
<td>RA</td>
<td>Retinoid acid</td>
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<tr>
<td>RARα</td>
<td>Retinoid acid receptor alpha</td>
</tr>
<tr>
<td>RFU</td>
<td>Relative fluorescence units</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory syncytial virus</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Real time quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>Sall4</td>
<td>Spalt like transcription factor 4</td>
</tr>
<tr>
<td>Sap</td>
<td>SLAM-associated protein</td>
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<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SMA</td>
<td>Smooth muscle actin</td>
</tr>
<tr>
<td>SMAD</td>
<td>SMA and MAD related proteins</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cell</td>
</tr>
<tr>
<td>SPL</td>
<td>Spironolactone</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
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<td>---------</td>
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</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>SUMO</td>
<td>Small ubiquitin-like modifier</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline containing</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TEER</td>
<td>Transepithelial electrical resistance</td>
</tr>
<tr>
<td>TF</td>
<td>Transcriptional factor</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper-1 type cell</td>
</tr>
<tr>
<td>Th2</td>
<td>T helper-2 type cell</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3',5,5'-tetramethylbenzidine</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TNS</td>
<td>Trypsin neutralising solution</td>
</tr>
<tr>
<td>Treg</td>
<td>T regulatory cell</td>
</tr>
<tr>
<td>TTP</td>
<td>Tristetraprolin</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Tight junction protein 1</td>
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CHAPTER 1:
GENERAL INTRODUCTION
1.1 Thesis statement

Glucocorticoids (GCs) remain central to treatment regimens for chronic airway inflammatory diseases. However, GC insensitivity remains a challenging clinical obstacle to successful treatment, particularly in patients with severe disease. This study firstly demonstrated that cortisol, the physiological GC, limits selected beneficial effects of synthetic, therapeutically used GCs in the airway epithelium and may contribute to the GC insensitivity in patients, particularly when its levels are elevated by stress. Subsequently, this study identified that the transcriptional repressor promyelocytic leukaemia zinc finger (PLZF) mediates selected GC effects in the airway epithelial cells. In elucidating novel mechanisms of GC action and insensitivity in the airway epithelium, we identify new potential strategies for improved treatment of chronic respiratory diseases.

1.2 Chronic respiratory diseases

Chronic respiratory diseases, including asthma and chronic obstructive respiratory disease (COPD), affect around 1 billion people of all ages worldwide and account for 7% (4.2 million) of all deaths annually (Navarro-Torné et al., 2015). In Australia particularly, according to National Health Survey (NHS), in recent years, around 7 million people were estimated to have a chronic respiratory condition, with approximately 2.5 million suffering from asthma and more than half a million people diagnosed with COPD (Australian_Bureau_of_Statistics, 2015). Therefore, due to the high prevalence and mortality rate, their complex nature and the fact their treatments demand a long-term and systematic approach, chronic respiratory diseases represent a significant health and economic burden worldwide.

1.2.1 Asthma – pathogenesis and phenotypes

Asthma is a complex, heterogeneous disorder of the airways which affects over 300 million people worldwide (Braman, 2006). It is characterized by the increased airway
hyperresponsiveness (AHR), reversible airway obstruction, chronic inflammation and hypersecretion of mucus (Kim et al., 2010).

The airway inflammation in asthma is predominantly driven by eosinophilic and/or neutrophilic activation, associated with different asthma phenotypes. The eosinophilic inflammation, coordinated by Th2 lymphocytes and their pro-inflammatory mediators is mainly associated with an early-onset, allergic asthma phenotype. This phenotype can present with mild to severe disease and it is generally corticosteroid-responsive. However, persistent sputum eosinophilia, despite corticosteroid treatment can also be associated with a late-onset, less allergic asthma phenotype (Wenzel, 2012). Certain individuals, particularly with severe, steroid-resistant asthma phenotype show mixed eosinophilia and neutrophilia in their sputum. This implies additional complex interactions of different immune pathways, including those triggered by the Th17 cells (Shannon et al., 2008, Doe et al., 2010).

Although airway inflammation is fundamental to asthma pathogenesis, structural changes that occur in all the layers of the airway wall also represent an important feature of asthma, connecting the airway inflammation and the airway obstruction (Saglani and Lloyd, 2015) (Figure 1.1).
Figure 1.1 Histological assessment of the normal and asthmatic human airway. From (Wadsworth et al., 2012). The asthmatic airway undergoes significant structural changes which contribute to the airway narrowing: epithelial (Ep) cell hyperplasia and hyperproduction of mucus; thickening of the basement membrane (Bm), as well as of the smooth muscle (Sm) layer. Bv – blood vessel. Airway sections were stained using Movat’s pentachrome stain. Scale bar = 100µM.

1.2.1.1 The immune responses and epithelial barrier dysfunction

A broadly accepted paradigm in the pathogenesis of the allergic asthma involves the captivation of allergenic antigens, such as dust mites or pollen by dendritic cells and subsequent activation of lymphocytes. These activated lymphocytes are predominantly Th2 cells, which further recruit secondary effector cells and orchestrate persistent chronic inflammation, ultimately leading to structural changes and airway remodelling (Barnes, 2008b). Increasing amounts of evidence in the past decade suggest that impaired epithelial barrier function and aberrant repair mechanisms have an important initial role in the abnormal response later observed in the persistent inflammatory phenotype (Loxham and Davies, 2017). Indeed, impaired barrier function of the airway epithelium in asthma has been reported both in vivo and in vitro, leaving the airways of asthmatics more susceptible to viral infections, allergens and other detrimental effects of the environment in early life (Georas and Rezaee, 2014). The exposure to the antigens then activates
dendritic cells, further leading to the complex interactions between inflammatory and structure cells, production of cytokines and other pro-inflammatory mediators, which repeatedly damage the epithelial barrier. This process finally results in the structural changes with deposition of extracellular proteins, goblet cell hyperplasia, smooth muscle hypertrophy and overall severe chronic disease (Holgate, 2011).

1.2.1.2 Chronic inflammation and pro-inflammatory mediators in asthma

Inflammation is an immune response to injury, which is beneficial under normal physiological conditions and essential for the clearance of the inflammatory stimuli and the protection of the tissue. The active process of resolution of inflammation involves many steps and a variety of different “stop” signals. Hence, any disruption in the mechanisms involved in the resolution might lead to persistent chronic inflammation and simultaneous destruction and repair of the tissue, as observed in asthma (Fullerton and Gilroy, 2016). Asthmatic inflammation implicates complex interactions between inflammatory and structural cell types, which are capable of producing dozens of different cytokines, chemokines, pro-inflammatory, anti-inflammatory and growth factors. Simplified scheme of cell interactions and the production of most important cytokines in the asthmatic inflammation is shown in Figure 1.2.

Briefly, following allergen exposure, the binding of antigen-specific IgE to FceRI sensitizes mast cells and other effector cells to release variety of mediators. Activated mast cells initiate bronchoconstriction, through the release of different mediators such as histamine, leukotrienes and prostaglandins (Galli and Tsai, 2012). The epithelial production of the stem cell factor (SCF) can also promote differentiation, recruitment and activation of mast cells (Wen et al., 1996), which can additionally release cytokines IL-13 and TNFα, promoting further airway epithelial damage and the inflammation process (Amin, 2012). The airway epithelium can release the thymic stromal lymphopoietin (TSLP), cytokine reported to be essential in allergic airway inflammation (Zhou et al., 2005, West et al., 2012), as it can stimulate dendritic cells to produce chemokines CCL17 and CCL22, leading to further activation of Th2 cells (Nakayama et al., 2004). The expression of TSLP is higher in asthmatics than in healthy individuals, and its levels
correlate with Th2 cytokine and chemokine expression and asthma severity (Shikotra et al., 2012, Ying et al., 2008). However, emerging clinical evidence suggests that TSLP may also activate other inflammatory cell types, including neutrophils, emphasising its potential role in different asthma phenotypes (Corren et al., 2017).

**Figure 1.2 Cell types and their mediators in the asthmatic inflammation.** Adapted from (Barnes, 2008b). Asthmatic inflammation implicates complex interactions between inflammatory and structural cell types, capable of producing the variety of different cytokines, chemokines, pro-inflammatory, anti-inflammatory and growth factors, ultimately leading to bronchoconstriction and airway narrowing.

Activated Th2 cells can produce a repertoire of cytokines and chemokines, acting on almost all cell types involved in the inflammation. Production of cytokines IL-4 and IL-13 promotes stimulation of B cells, initiating class switching, their maturation and production of allergen-specific IgE (Lambrecht and Hammad, 2015), which bind to FcεRI receptors on mast cells, leading to their sensitisation (Galli and Tsai, 2012). Th2 cells can also directly stimulate
proliferation and activation of mast cells through production of IL-3, IL-4 and IL-13 (Junttila et al., 2013, Burton et al., 2013). Finally, a number of cytokines, including GM-CSF, IL-3 and IL-5 can initiate maturation, proliferation and the recruitment of eosinophils, which further participate in the modulation of the immune response, induction of AHR and the airway remodelling (Holgate, 2012).

1.2.1.3 Airway remodelling

The airway remodelling is a feature of asthma that refers to the structural changes that occur in all of the layers of the airway wall around trachea, bronchi and bronchioles, following persistent inflammation (Stewart, 2012). These changes involve the epithelial shedding and metaplasia, goblet cell hyperplasia with hyper-secretion of mucus, fibrosis of the basement membrane, increased deposition of extracellular components and thickening of the muscle layer due to smooth muscle hyperplasia (Fahy, 2015).

Remodelling changes ultimately lead to increased AHR, airway obstruction and irreversible loss of lung function and are mostly regulated by different Th2 mediators (Tang et al., 2006). For example, cytokines IL-4, IL-9 and IL-13 can cause goblet cell hyperplasia and increased mucus production (Birchenough et al., 2015). IL-13 has been also reported to have pro-fibrotic effects, through direct TGF-β activation (Makinde et al., 2007). TGF-β is implicated as a key mediator in asthma, responsible for a number of remodelling events. This cytokine is mainly produced by the epithelium, fibroblasts, eosinophils and airway macrophages, contributing to tissue fibrosis through stimulation of myofibroblast differentiation, fibroblast proliferation and the release of matrix components, such as collagen and matrix metalloproteinases (MMPs) (Royce et al., 2012). Increased TGF-β expression is found in mucosal bronchial biopsies from asthmatic subjects compared with non-asthmatics (Kokturk et al., 2003). In addition, TGF-β expression in the asthmatic airways correlates with the severity of the disease (Al-Alawi et al., 2014). TGF-β also induces the production of vascular endothelial cell growth factor (VEGF), leading to the vascular remodelling in the asthmatic airway (Boxall et al., 2006).
Additionally, eosinophils can release a variety of cytokines, including IL-4, IL-13, fibroblast growth factor 2 (FGF2) and VEGF, contributing to structural changes in the airways (Kay et al., 2004). VEGF is particularly responsible for increased vascularity, through stimulation of the epithelial proliferation and endothelial cell survival (Green and Turner, 2017). Furthermore, VEGF was shown to increase antigen sensitization and contribute to Th2 inflammation in a murine model of asthma (Lee et al., 2004).

The airway remodelling is a common feature of chronic inflammatory lung diseases, including asthma and COPD (James and Wenzel, 2007), generally thought to be an inflammatory-driven process. However, the presence of the airway inflammation in patients with asthma does not always translate to airway remodelling (Benayoun et al., 2003). The airway remodelling was also shown to persist even upon the resolution of the airway inflammation (Leigh et al., 2002). Moreover, remodelling features such as collagen deposition and goblet cell metaplasia can be induced by bronchoconstriction in the absence of airway inflammation (Grainge et al., 2011). Thus, ascertaining the extent of the association between remodelling and inflammation in asthma will certainly be of interest in future studies.

1.2.2 Asthma – current therapy and challenges

Current asthma management is mostly focused on the prevention and the control of acute and chronic symptoms with minimum side effects, stabilisation of the lung function and improvement of the quality of life of the asthmatic patients. The adjustment of asthma therapy is based on the control of asthma symptoms and follows a step-wise approach. The step algorithm and drug groups recommended by the Global Initiative for Asthma (GINA) are shown in Table 1-1.

For the majority of mild asthmatic patients, the combination of bronchodilators, such as short-acting β2-agonists (SABA), which are available without prescription, and the low dose of anti-inflammatory drugs, such as inhaled corticosteroids (ICS) is sufficient to successfully control and relief asthma symptoms. The use of long-acting β2-agonists (LABA), as well as the increase of ICS concentration is mostly related to moderate and severe asthma phenotype. Additional
treatment options for persistent asthma include leukotriene antagonists, or tiotropium, a long-
acting muscarinic antagonist (Horak et al., 2016). Tiotropium has been particularly shown to be
effective in patients with uncontrolled severe asthma (Rodrigo and Castro-Rodriguez, 2015).

Although current asthma management is very effective at controlling symptoms, it is not
curative. Many challenges in the therapy still remain, such as overcoming the severe adverse
effects, or glucocorticoid resistance in the subset of patients with severe asthma. Thus, the future
management of asthma will involve novel therapeutic agents that modulate specific components
of the inflammatory pathways in asthma, mostly likely in combination with current established
therapies.
**Table 1-1 The Global Initiative Asthma (GINA) guidelines for asthma therapy with main drug groups.** The adjustment of asthma therapy is based on asthma control, and follows a stepwise approach. SABA=short-acting β2 agonists; ICS=inhaled corticosteroids; LABA=long-acting β2 agonists; LTRA=leukotriene antagonists; LAMA=long-acting muscarinic antagonist. Adapted from (Horak et al., 2016).

<table>
<thead>
<tr>
<th>ASTHMA SEVERITY</th>
<th>TREATMENT</th>
<th>ADVANTAGES</th>
<th>SIDE EFFECTS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Step 1</strong></td>
<td>Mild asthma</td>
<td>1&lt;sup&gt;st&lt;/sup&gt; choice: <strong>SABA</strong>; Persistent symptoms or need for SABA &gt;2×/month - consider low dose of ICS</td>
<td>SABA: rapid relief of symptoms (bronchoconstriction)</td>
</tr>
<tr>
<td><strong>Step 2</strong></td>
<td>1&lt;sup&gt;st&lt;/sup&gt; choice: <strong>SABA</strong> + <strong>low dose ICS</strong>; 2&lt;sup&gt;nd&lt;/sup&gt; choice: <strong>LTRA</strong></td>
<td>ICS: reduce airway inflammation; prevent asthma exacerbation</td>
<td>ICS: local side effects; potential systemic side effects; resistance; poor compliance</td>
</tr>
<tr>
<td><strong>Step 3</strong></td>
<td>Moderate asthma</td>
<td>1&lt;sup&gt;st&lt;/sup&gt; choice: <strong>low dose of ICS</strong> + <strong>LABA</strong>; 2&lt;sup&gt;nd&lt;/sup&gt; choice: medium/high dose of ICS or low dose of ICS + LTRA</td>
<td>LABA: better relief of symptoms</td>
</tr>
<tr>
<td><strong>Step 4</strong></td>
<td>Severe asthma</td>
<td>1&lt;sup&gt;st&lt;/sup&gt; choice: <strong>medium dose of ICS</strong> + <strong>LABA</strong>; 2&lt;sup&gt;nd&lt;/sup&gt; choice: add <strong>tiotropium</strong>/ high dose of ICS/add LTRA</td>
<td>Tiotropium (LAMA) – addition of tiotropium effective in uncontrolled severe asthmatics</td>
</tr>
<tr>
<td><strong>Step 5</strong></td>
<td>Other therapy options (i.e. Anti-IgE) or oral corticosteroids (oral CS)</td>
<td>Oral CS: minimisation of symptoms; prevention of severe asthma exacerbations</td>
<td>Oral CS: significant musculoskeletal, endocrine and metabolic, cardiovascular, gastrointestinal side effects; resistance</td>
</tr>
</tbody>
</table>

*Step 3-5 SABA or low dose ICS/formoterol as needed for symptom relief*
1.2.3 **Asthma and stress**

In the past two decades, studies have investigated the link between stress and asthma, demonstrating that stress is a trigger of asthma exacerbations and may also play a role in asthma development in childhood (Kozyrskyj et al., 2008). However, the mechanism by which stress triggers asthma remains insufficiently explored.

Psychological and physiological stresses activate the hypothalamic–pituitary–adrenal (HPA)-axis, increasing levels of the physiological glucocorticoid, cortisol (Oelkers 1996, Carl Coeck et al., 1991, Kudielka et al., 2004). Furthermore, pathogen-induced stresses, such as viral and bacterial infections activate immune and structural cells, inducing their production of pro-inflammatory cytokines, including TNFα, IL-1α and interferons. These cytokines can directly activate HPA-axis, leading to elevation of adrenocorticotrophic hormone and increased cortisol release (Rhen and Cidlowski, 2005). Stress also increases the levels of adrenaline, which is generally regarded to synergise with cortisol, providing multiple beneficial effects in inflammation and bronchoconstriction (Chen and Miller, 2007).

A recent model has illustrated the interaction of physiological stress with environmental stimuli in triggering asthma exacerbations. This model has suggested that, apart from activating HPA-axis and sympathetic pathways, stress also operates by increasing the airway inflammatory response to allergens, irritants or virus infections in asthmatics, prolonging and worsening their symptoms. Although one would expect that the increased secretion of cortisol and adrenaline results in reduced airway inflammation and bronchodilation, emerging evidence suggests that upon exposure to chronic stress, several changes occur in the receptor-signalling pathways of stress-released mediators, leading to defective regulation of inflammatory responses to asthma triggers (Chen and Miller, 2007). In addition, increased corticosteroid levels in chronic stress were shown to induce a shift in the Th1/Th2 cytokine balance toward Th2 cytokine response, the principal inflammatory agency in asthma (Griffin et al., 2014). Moreover, adrenaline has been also shown to contribute to goblet cell metaplasia though activation of β2-adrenoceptors in a murine model of asthma (Thanawala et al., 2013).
Although several studies have indeed investigated normal and stress-induced cortisol levels in asthmatics and non-asthmatics (Laube et al., 2002, Landstra et al., 2002, Kapoor et al., 2003, Vink et al., 2012, Sutherland et al., 2003), they delivered conflicting findings, leaving the role of cortisol in asthma and asthma development uncertain.

### 1.2.4 Asthma vs. COPD: similarities and differences

Chronic obstructive pulmonary disease (COPD) is a progressive respiratory condition with increasing degree of reversible airflow limitation, associated with aberrant systemic and local inflammatory response to inhaled toxic stimuli, such as particles or gases (Pauwels and Rabe, 2004).

Whilst in asthma, most patients have an atopic phenotype, around 18% of COPD patients are also atopic (Postma and Rabe, 2015). Atopy represents a genetic predisposition to produce IgE antibodies against common environmental allergens (Froidure et al., 2016). Additionally, emerging evidence suggests that atopy is a possible risk factor for respiratory symptoms and exacerbations in COPD patients (Fattahi et al., 2013). Acute exacerbations, defined as the sudden worsening of symptoms and lung function, are the key features of both asthma and COPD that significantly contribute to the progression of these diseases (Soler-Cataluña et al., 2005, Bai et al., 2007).

Similarly to asthma, chronic inflammation is the essential component of COPD pathogenesis. However, whilst asthmatic inflammation is generally located in the airways, with limited effects on the lung parenchyma (Barnes, 2008b), chronic inflammation and increased oxidative stress caused by the cigarette smoke in COPD patients contributes to emphysema and increased destruction of the alveolar walls. This leads to enlargement of the alveolar spaces and reduced surface for the gas exchange, with consequent limited respiratory function (Hogg et al., 2013). The inflammation in COPD is mostly neutrophilic and CD8+ driven (Postma and Rabe, 2015). However, elevated eosinophil levels have been also found in 15 to 40% of patients with COPD, particularly during the acute exacerbations (Barker and Brightling, 2013).
Drugs currently available for the COPD treatment include bronchodilators (selective β2-agonists, anticholinergic agents and methylxanthines), glucocorticoids and other groups such as mucolytic agents, α1-antitrypsin augmentation therapy in those with deficiency, antioxidants and immunoregulators (Vogelmeier et al., 2017). In particular, the role of glucocorticoids in the treatment of COPD remains controversial. Although inflammation is one of the main targets for glucocorticoids in COPD treatment, current evidence suggests that their anti-inflammatory effects are limited, perhaps due to complex inflammatory pathways and mediators, and the overall heterogeneity of the disease (Barnes and Adcock, 2009). Nonetheless, clinical studies have shown a better response to inhaled corticosteroids (ICS) in COPD patients with the sputum eosinophilia. Moreover, ICS treatment in these patients reduced the number of acute exacerbations and hospitalizations (Postma et al., 2014).
1.3 The role of the airway epithelium in the pathogenesis of asthma and other chronic respiratory diseases

The mammalian epithelium represents one of the four major tissues found in the human body, forming fundamental layers of the cells that line the cavity and lumen of different organs. With high proliferative rates and the ability to differentiate into cell types that perform a range of highly specialized functions, epithelial cells have an important role in both physiological and pathological conditions (Blanpain et al., 2007).

1.3.1 The structure and physiological functions of the airway epithelium

The airway epithelium is of a pseudostratified columnar type, consisting of distinct cell types - ciliated, goblet, basal and other specialized cells (i.e. club cells), that are firmly attached to the basal membrane (Walters et al., 2013). The airway epithelial cells were once thought to be primarily a physical barrier. However, given their ability to secrete different mediators and cytokines and interact with other structural and inflammatory cells in the lung, airway epithelial cells also represent one of the core participants in the protection of the lungs from the environmental distress, and preservation of pulmonary homeostasis (Lambrecht and Hammad, 2012) (Figure 1.3).

Given their high proliferative capacity, basal cells are thought to be progenitors of the ciliated and goblet cells in the airways (Rock, 2009). Their specific morphology with specialized junctional complexes allows them firm attachment to the basement membrane, which not only supports the upper layers of differentiated cells, but also confers a central position for the interaction with the airway environment: neurons, basement membrane, underlying mesenchymal cells and inflammatory cells (Rock et al., 2010). Basal cells can secrete different cytokines and growth factors (Hackett et al., 2011a), contributing to both physiological and pathological processes in the airways. It has also been reported that in the culture of primary bronchial epithelial cells, basal cells are the main cell type producing antimicrobial protein RNase 7,
significant not only for the antimicrobial activity towards different pathogens, but also in the protection from the mechanical injury (Amatngalim et al., 2015).

Figure 1.3 Simplified graphic representation of the structure of the epithelium in the airway of a human lung. Airway epithelium consists of various cell types (ciliated, goblet and basal cells) firmly attached to the basal membrane. Given their position in the lung, epithelial cells can interact with other structural and inflammatory cells, contributing to the protection of the lungs from the environmental distress, and preservation of pulmonary homeostasis.

*Ciliated cells* are the most common cell type in the airways, which characteristically have high rate of metabolism and up to 200-300 cilia/cell. Cilia represent tubular organelles typically
found in the respiratory, reproductive and nervous system, and are a vital component in the motility process and removal of exogenous substances from the lung (Singla and Reiter, 2006). Although they were considered to be terminally differentiated with a key role in muco-ciliary clearance (Inayama et al., 1989), recent findings suggest that ciliated cells can transdifferentiate into distinct epithelial cell phenotypes for the regeneration of the airway epithelium post-injury (Park et al., 2006, Tyner et al., 2006).

Initially identified in the gut lining, as cup-shaped cells with secretory granules, *goblet cells* are thought to be fully differentiated epithelial cells specialized in the production of mucus, which help coordinated cilia movements in the clearance of the inhaled pathogens and particles in the airway tracts (Ganesan et al., 2013). Different stimuli, such as proteinases, inflammation and irritant gases can induce secretion of mucus, a complex viscous mixture of lipids, glycoproteins (i.e. mucins), enzymes and oxidants, which requires appropriate viscosity and elasticity for the optimal clearance (Rogers, 2012). Although mucus production is one of the essential components in the maintenance of lung homeostasis, in some cases, goblet cell hyperplasia with chronic mucus hypersecretion and production of pro-inflammatory mediators, can significantly contribute to the pathology of various lung diseases (Barnes, 2008b).

*Club cells*, formerly known as Clara cells, are primarily located in the bronchial epithelium. Although they are known as non-ciliated and non-mucous cells, they can serve as progenitors for ciliated cells (Reynolds and Malkinson, 2010), and are able to secrete different physiologically important mediators, such as uteroglobin (CC16), contributing to the lung protection (Laucho-Contreras et al., 2015). One of the most significant roles of club cells noted in the airways is their ability to bio-transform inhaled xenobiotics, suggesting their additional importance in protecting the lungs from the detrimental effects of the external environment (Reynolds and Malkinson, 2010).

Alveolar epithelial cells, also known as *pneumocytes*, line the alveoli of the lungs. Although both type I and II pneumocytes contribute to the airway protection, type II pneumocytes are the ones that appear more functionally active and have various essential roles in the lung homeostasis. They secrete a wide variety of factors, cytokines and chemokines, necessary for the activation of
the immune cells, specifically lung macrophages, therefore contributing to the clearance of the pathogens from the airways (Thorley et al., 2006). More importantly, they produce pulmonary surfactant, which reduces the surface tension of the alveolar air-liquid interface, and therefore is essential for the normal lung function (Fehrenbach, 2001).

1.3.2 Airway epithelial differentiation: double-edge sword

The airway epithelial cell layer regulates the interface with the environment, including maintaining microbial defence, and has well-recognized immune-modulatory and barrier functions (Lambrecht and Hammad, 2012). However, changes in epithelial differentiation and functions can significantly contribute to inflammation in chronic respiratory diseases, including asthma (Erle and Sheppard, 2014).

It is well established that epithelium responds to harmful stimuli with increase in proliferation and phenotypic changes. Imbalance in the normal epithelial proliferation and differentiation can lead to either epithelial hyperplasia or hypoplasia (Yamada et al., 2016). An early expression of the airway remodelling in asthma includes epithelial shedding and metaplasia. Goblet cell hyperplasia and mucus hypersecretion, induced by coordinated action of IL-13 and EGF, are also critical contributors to the airway obstruction observed in both asthma and COPD (Tang et al., 2006). Additionally, basal cell hyperplasia, airway wall tissue damage and defects in ion transport in epithelial cells have been also reported in cystic fibrosis (Voynow et al., 2005, Mall and Galietta, 2015). Airway biopsies from the asthmatic patients show lower expression of cell junctional molecules E-cadherin and ZO-1 (de Boer et al., 2008) and disruption of tight junction complexes (TJs) in the epithelial cultures from asthmatic subjects differentiated in vitro compared to normal subjects, suggesting that epithelial barrier function may be impaired in asthmatics (Xiao et al., 2011).

An epithelial – mesenchymal transition (EMT) is a biological process, physiologically required for the normal developmental processes like embryogenesis, organ development and wound healing (Li and Li, 2015). Polarized epithelial cells undergo several phenotypic and biochemical changes which allow them to acquire mesenchymal (fibroblast-like) phenotype.
They lose their polarity and connections with the basal membrane, followed by the reorganization and expression of cytoskeletal proteins. In addition, they start to lose cell-cell epithelial adhesion markers (E-cadherin), and have increased expression of specific cell-surface markers, characteristic for mesenchymal phenotype (α smooth muscle actin, N-cadherin, Vimentin) (Lamouille et al., 2014). Their production of matrix metalloproteinases (MMPs) is also greatly increased, suggesting their ability to modify the extracellular matrix (ECM), with the higher frequency of migration and invasion of other tissues. Due to these characteristics, EMT is one of the fundamental processes in the progression of epithelial tumours. Inappropriate induction of EMT with increased number of myofibroblasts and deregulated MMP expression, may stimulate fibrosis, tumorigenesis and metastasis (Bissell and Radisky, 2001). The origins and potential signalling factors of EMT in cancers remain insufficiently explored. Research so far has shown that epigenetic modifications in cancer cells during the primary tumour development cause their higher sensitivity to EMT-inducing signals (Tam and Weinberg, 2013). In the case of different carcinomas, participants established to date include HGF, EGF, PDGF, and TGF-β, with different intracellular signalling networks, including ERK, PI3K, MAPK, Smads, Ras and c-Fos, as well as the cell surface integrins, which ease the disruption of cell-cell adherent junctions (De Craene and Berx, 2013).

Along with their structural changes, epithelial cells can secrete multiple cytokines, chemokines and peptides, such as GM-CSF, IL-1, IL-6, IL-8, IL-13, TGFβ and TNFα, promoting further activation of immune cells and contributing to the chronic airway inflammation (Erle and Sheppard, 2014). GM-CSF is a colony-stimulating factor and inflammogen which regulates the accumulation and activity of neutrophils at the inflammatory site (Fossati et al., 1998), additionally promoting their survival (Cox et al., 1992). Asthmatic airway epithelium expresses higher levels of GM-CSF protein than non-diseased airway epithelium (Sousa et al., 1993).

The airway epithelium is also considered to be a major source of the pleiotropic cytokine, interleukin (IL)-6, which was found to inhibit pro-inflammatory effects of tumour necrosis factor-α and IL-1 in macrophages (Tilg et al., 1994). Elevated circulating IL-6 levels have been also found in asthmatic patients (Rincon and Irvin, 2012). Furthermore, primary bronchial epithelial
cells from COPD patients were demonstrated to have significantly higher expression and secretion levels of IL-8 compared with smokers and control subjects (Schulz et al., 2004). IL-8 is a powerful chemokine in inducing neutrophil recruitment and activation (Kolaczkowska and Kubes, 2013).

In response to oxidative stress, type II pneumocytes can release a variety of pro-inflammatory mediators, including enzymes nitric oxide (NO)-synthase and hemoxygenase-2 (McCourtie et al., 2008). These enzymes are found to be increased in smokers and patients with severe COPD (Maestrelli et al., 2003). Moreover, constituents of the cigarette smoke, such as different oxidants can affect the normal activity of neutrophil elastase and MMPs, subsequently influencing pulmonary surfactant function (Zhao et al., 2010). Finally, different mutations of genes implicated in the epithelial synthesis of pulmonary surfactant phospholipids have been recognised as a rare cause of lung cancers and risk factor for the idiopathic pulmonary fibrosis (IPF) (Lawson et al., 2004, Wang et al., 2009).
1.4 Glucocorticoids: the mainstay of anti-inflammatory therapy

Glucocorticoids (GCs) are a class of corticosteroid (CS) hormones, physiologically both present in the human body and laboratory-manufactured. The secretion of physiological glucocorticoid, cortisol, is mostly regulated by the circadian rhythm and stress. Cortisol is essential for the variety of physiological processes, including embryonic development, metabolism, normal function of immune, respiratory, cardiovascular, nervous, and reproductive system (Figure 1.4) (Kadmiel and Cidlowski, 2013). Since the discovery of their impressive anti-inflammatory potential in 1940s (Munck et al., 1984), synthetic GCs have remained the most potent anti-inflammatory drugs widely used for the suppression of the inflammation in different acute and chronic inflammatory conditions. The clinical use of GCs includes asthma, COPD, rheumatoid arthritis, inflammatory bowel disease and different dermal and ocular inflammatory conditions (Rhen and Cidlowski, 2005). In addition to their anti-inflammatory effects, GCs are known to possess an anti-proliferative and immunosuppressive actions, which is beneficial in the treatment of some cancers (Lin and Wang, 2016).

In 2013, out of 9% of the total Australian population who received one or more respiratory medications, inhaled corticosteroids (ICS) were the most common class of drugs, dispensed on prescription to 6% of the total population, mainly for use in asthma (AIHW: Correll PK, 2015). Although these agents remain a “gold standard” in the prophylactic treatment of asthma, long-term use of oral GCs can be associated with many side effects, including osteoporosis, myopathy and different endocrine, metabolic and cardiovascular effects (Moghadem-Kia and Werth, 2010). Furthermore, GC-resistance remains a major clinical obstacle, as a fraction of patients, particularly with severe disease are less sensitive or completely unresponsive to GC therapy. Several molecular mechanisms of GC-insensitivity have been elucidated. However, this continues to be a field of active research.

Considering their broad range of actions and signalling pathways in almost all cell types in the body, clarifying which cell type is the most important for their actions, or which signalling pathways are of essence, remains a challenge. Thus, given this complexity and the need to better
understand GC actions, we have recently reviewed in depth the molecular mechanisms of GC effects in distinct cell types, with the emphasis on the currently described mechanisms of GC-insensitivity (Keenan et al., 2015b). For the purpose of this thesis, we are providing the general overview of the mechanisms of GC-actions and insensitivity, with the particular focus on their actions in the airway epithelium.

1.4.1 Physiological vs synthetic glucocorticoids

The physiological, endogenous glucocorticoids (cortisol in humans and corticosterone in rodents) are cholesterol-derived hormones, secreted from zona fasciculata of the adrenal glands. The secretion is regulated primarily by the hypothalamic-pituitary-adrenal (HPA) axis, an integral component of the adaptive responses to stress (Cain and Cidlowski, 2017). In response to stress, hypothalamus releases corticotropin-releasing factor (CRF) which binds to its receptors on the pituitary gland, subsequently inducing secretion of adrenocorticotropic hormone (ACTH) into the circulation. ACTH further acts on the adrenal cortex and stimulates the synthesis and release of GCs (Smith and Vale, 2006). The activity of HPA-axis is then inhibited by the circulating GCs, though distinct mechanisms of the negative feedback (Keller-Wood and Dallman, 1984) (Figure 1.4).

Although present in humans at low circulating levels, corticosterone, also known as 17-deoxycortisol, represents the main GC in rodents, in which the final step of cortisol synthesis is blocked due to their natural deficiency in 17α-hydroxylase, enzyme necessary for hydroxylation of the precursor molecule, pregnenolone (Raubenheimer et al., 2006, Keeney et al., 1995) (Figure 1.4). Once delivered to the cells, physiological GCs bind with high affinity to both glucocorticoid and mineralocorticoid receptors (Cain and Cidlowski, 2017), activating complex signalling pathways, that overall contribute to tissue development, metabolism and immunomodulation of both innate and adaptive immunity.
Figure 1.4 The regulation of glucocorticoid secretion by the hypothalamic-pituitary-adrenal (HPA) axis. The hypothalamus releases corticotropin-releasing factor (CRF) which binds to its receptors on the pituitary gland, subsequently inducing secretion of adrenocorticotropic hormone (ACTH) into the circulation. ACTH further acts on the adrenal cortex and stimulates the synthesis and release of glucocorticoids, which in return inhibit their further secretion through mechanisms of the negative feedback. Once delivered to the cells, physiological GCs bind with high affinity to both glucocorticoid and mineralocorticoid receptors, contributing to normal bone development and regulation of cardiovascular, immune, respiratory, nervous and metabolic system. Adapted from (Kadmiel and Cidlowski, 2013).

Exogenous GCs are synthetic derivatives which resemble and share the same mechanisms of action as cortisol (hydrocortisone). However, due to the chemical modifications in the “original” molecular structure, they have different levels of efficacy, potency, pharmacokinetic profile and
overall altered physiological effects. The production of the synthetic GCs for systemic delivery has already started back in 1950s, and as a result of specific modifications, such as 1-dehydrogenation in the structure of prednisolone, 16α-methylation in methylprednisolone and fluorination in dexamethasone and betamethasone, these synthetic derivatives acquired more potent anti-inflammatory actions, with fewer associated side-effects, such as fluid retention associated with mineralocorticoid activity (Buttgereit et al., 2005). However, these molecules still caused significant suppression of HPA-axis with considerable systemic side-effects, including osteoporosis, growth suppression and metabolic disorders. Therefore, further improvement of the therapy was evident in the 1960s with the development of topical glucocorticoids. Their targeted delivery to the specific site of inflammation minimised systemic side-effects, as in the case of inhaled glucocorticoids, budesonide and fluticasone for the treatment of asthma (Barnes, 2006) (Figure 1.5).
Figure 1.5 The evolution of the synthetic GCs. Due to the chemical modifications in the steroidal structure of the physiological GCs (cortisol), synthetic systemic GCs (i.e. prednisolone, methylprednisolone, dexamethasone and betamethasone) acquired greater anti-inflammatory actions, with less associated side effects, compared with cortisol (hydrocortisone). As their systemic delivery still caused significant suppression of HPA axis, with side effects such as osteoporosis, growth suppression and metabolic disorders, further improvement of the therapy involved optimisation of their target delivery to the specific site of inflammation, as in the case of ICS (budesonide and fluticasone propionate).
1.4.2 Glucocorticoid bioavailability

In the circulation, under normal circadian rhythm, only 5-10% of cortisol is free. Around 80-90% of cortisol is bound to the corticosteroid-binding globulin (CBG), which acts as a transporter and can release or bind free cortisol, depending on its concentration in the plasma. Therefore, plasma levels of CBG are essential in adaptive stress response, as they control the concentration of free, biologically active GCs (Perogamvros et al., 2012). Interestingly, both endogenous and exogenous GCs have been reported to inhibit CBG production and secretion, through mechanism involving the glucocorticoid receptor (Verhoog et al., 2014).

GC bioavailability is also controlled by the enzymes 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) and type 2 (11β-HSD2), which catalyse the interconversion between the active and inactive forms of GCs and are differentially expressed between different cell types (Seckl, 2004). For example, 11β-HSD1, which converts inactive GCs to active metabolites (i.e. cortisol to cortisone), is highly expressed in the epithelial and mesenchymal cells, whilst low expression is observed in the inflammatory cells (Zhang et al., 2005, Chapman et al., 2006). The expression of 11β-HSD2, which catalyses inactivation of the active GCs, is noted mostly in the epithelial and peripheral blood mononuclear cells (Garbrecht et al., 2007). Increasing evidence suggests that the expression of these enzymes is altered during acute and chronic inflammation (Chapman et al., 2013). In particular, the 11β-HSD1 activity was found to be induced early during the inflammatory response, promoting local amplification of the GC activity in the macrophages and leukocytes (Chapman et al., 2006).

1.4.3 Overview of the molecular mechanisms of GC actions

1.4.3.1 Glucocorticoid receptor – localisation, isoforms and activation by GCs

The majority of the GC effects are mediated through their binding of the functional isoform of glucocorticoid receptor (GR), initiating multiple complex signalling pathways. GR is a product of NR3C1 (Nuclear Receptor Subfamily 3 Group C Member 1) gene, which belongs to the superfamily of ligand-activated nuclear receptors. It is expressed in nearly all tissues and cell
types throughout the human body. However, alternative splicing of the primary transcript of the NR3C1 gene produces two main, functionally different receptor isoforms – GRα and GRβ (Hollenberg et al., 1985, Oakley and Cidlowski, 2011). Although Pujols et al. (2002) were able to detect GRβ mRNA in a variety of the human tissues and cell types, the abundance was approximately one four-hundredth of the GRα mRNA. The GRα was considered to be the only active form of the receptor that mediates GC effects, and GRβ a transcriptionally inactive form that does not bind GCs (Lu and Cidlowski, 2006). However, increasing evidence suggests that GRβ not only might have an autonomous transcriptional activity, which is modulated by synthetic GR antagonist RU486 (mifepristone), but also could interfere with GRα-mediated GC signalling pathways (Kino et al., 2009). Recently, a GRγ isoform was also shown to bind GCs in the human osteosarcoma cell line, though it lacked the ability to bind GREs and induce the expression of distinct GC-regulated genes (Meijsing et al., 2009).

GRα is mostly located in the cell cytoplasm, together in a multiprotein complex that contains heat-shock proteins, immunophilins and other chaperones, that enhance the affinity of the receptor for ligand (Kirschke et al., 2014). Upon binding of its ligands, either cortisol or exogenous synthetic GCs, GRα undergoes several conformational changes, dissociates from the other proteins and as a homodimer translocates to the nucleus. Once located in the nucleus, GRα dimer interacts with DNA, further activating or repressing the transcription of target genes (Cain and Cidlowski, 2017). However, GCs can also exert their actions through mechanisms independent of their classical cytosolic receptor and transcription of genes. Different genomic and non-genomic actions are summarized in Figure 1.6.
Figure 1.6 The overview of the currently described genomic and non-genomic actions of glucocorticoids (GCs). Glucocorticoids bind to the glucocorticoid receptor (GRα) that can directly interact with target DNA (protein-DNA interaction) through binding of glucocorticoid responsive elements (GRE), further activating (transactivation) or repressing transcription of target genes. Alternatively, GRα can indirectly repress transcription of genes through protein-protein interactions with different transcriptional factors (TFs) (transrepression). GC-activated GRα can additionally either enhance or sequestrate the activity of TFs, and affect the stability of mRNA. Finally, GCs can exert their effects through rapid, non-genomic actions. From (Keenan et al., 2015b).

1.4.3.2 Genomic GC effects

The genomic effects of GCs are mediated through binding of GCs to classical cytosolic GRα, receptor dimerization and translocation of the complex to the nucleus. Subsequent interaction of this complex with specific palindromic sequences in the target DNA (glucocorticoid responsive elements (pGREs)), results in the increase in the mRNA levels of the specific target. This process is known as transactivation of target genes (Keenan et al., 2015b). Several mediators involved in the resolution of inflammation or important for modulation of the cell cycle are shown to be transactivated by GCs in different cell types: glucocorticoid-inducible leucine zipper (GILZ),

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MAP kinase phosphatase 1 (MKP-1), cyclin-dependent kinase inhibitor 1C (CDKN1C) and others (Ronchetti et al., 2015, Lasa et al., 2002, Keenan et al., 2014). Although transactivation of target genes has been recognized as an important mechanism of GC actions, the true extent of the contribution of this mechanism to their beneficial effects is still not fully understood. Furthermore, transactivation has also been noted as an important mechanism behind GC side effects, especially osteoporosis (Schacke et al., 2002).

Ligand-activated GRα can indirectly repress transcription of genes through protein-protein interactions with different transcriptional factors. This process is known as trans-repression. For example, GC-stimulated GRα can directly increase the export of transcriptionally important p65 subunit of transcriptional factor NF-κB, from the nucleus (Nelson et al., 2003), subsequently inhibiting signalling pathways which lead to both gene transcription and production of pro-inflammatory cytokines, such as IL-1, IL-6, IL-8, and TNF-α (Newton, 2014). Moreover, cross-talk between GRα and transcriptional factor AP-1 occurs through binding and inhibiting its proto-oncogene unit c-jun, which not only mediates GC suppression of inflammatory mediators (such as IL-6 and metalloproteinases), but also inhibits cell proliferation in mouse fibroblasts cells (Wei et al., 1998).

Glucocorticoids can repress gene transcription through GRα-binding to negative GRE (nGRE) in the promoter of the target gene, preventing the binding of other transcriptional factors. This type of GC genomic mechanism is observed in the GC regulation of different gene expressions, including osteocalcin, important for osteoblast differentiation, and FAS-ligand, which has an essential role in the immunity and progression of cancer (Mitre-Aguilar et al., 2015). Interestingly, GRα can form a complex with other transcriptional factors, additionally both enhancing their transcriptional activity, as in the case of STAT5 (Stocklin et al., 1996), or sequestering the activity state of other transcriptional regulatory factors and co-activators (Ratman et al., 2013).

In addition, expression levels of the pro-inflammatory cytokine tumour necrosis factor alpha (TNFα) can be modified post-transcriptionally, through the effect of GCs on the stability of mRNA. This mechanism involves GRα-induced increases in expression of tristetrapolin (TTP),
which can bind to specific sequences in the mRNA and initiate the cascade reaction with different RNAses which degrade target mRNA. Similarly, post-transcriptional regulation has been also noted in the expression of GM-CSF, IL-6 and IL-8 and COX-2 (Newton, 2000).

1.4.3.3 Non-genomic GC effects

Not all glucocorticoid actions can be explained by delayed, genomic effects in the nucleus. Rapid effects of GCs on stress-related cognition, adaptive behaviour and neuroendocrine function (Groeneweg et al., 2011) or GC-effects on vasoconstriction within minutes (Brieva and Wanner, 2001) suggest additional rapid non-genomic actions. They are characterized by the rapid onsets (within seconds to minutes) and short duration (up to 1-2 hours). These GC effects have been reported in various studies. Zhou et al. (2008) have demonstrated that GC-rapid inhibition of IgE-mediated histamine release of mast cells in vitro, is not due to transcriptional effects on individual components of the secretory machinery, but it is a result of the inhibition of Ca\(^{2+}\) influx. Another study has reported rapid immunosuppressive effects of dexamethasone and prednisolone on T-cell activation, in which the membrane-bound GR inhibited many pathways downstream from T-cell receptor, subsequently blocking the cell activation (Lowenberg et al., 2006). Recently discovered localisation of GR to the adherent junctions at the plasma membrane of primary human keratinocytes (Stojadinovic et al., 2013) suggests another novel, unexplored non-genomic action of GCs in these cells.

1.4.4 Differentiated epithelium as a target for GC actions

Glucocorticoids have profound anti-inflammatory and immunosuppressive effects in various types of cells, which place them in the centre of the therapy for chronic inflammatory diseases. Although their anti-inflammatory actions have been so far linked mainly to activated immune cells, epithelial cells can be additional targets for GC actions (Newton et al., 2010). Extensive studies have established that epithelial expression and release of pro-inflammatory mediators, including IL-6, IL-8 and granulocyte-macrophage colony stimulating factor (GM-CSF) can be inhibited by GCs (Stellato, 2007). The mechanism of action in the primary human bronchial
epithelial cells and human immortalized BEAS-2B cell line was shown to require the α isoform of glucocorticoid receptor and direct GRα interaction and inhibition of signalling pathways downstream of two major pro-inflammatory transcriptional factors, AP-1 and NF-κB (LeVan et al., 1997). The in vitro study in A549 lung cancer cell line confirmed GC inhibition of cytokine-induced NF-κB binding to the promoter of iNOS, which is highly expressed in the inflammatory environment in asthma, COPD and cancers (Kleinert et al., 1996, Lechner et al., 2005). However, subsequent studies have demonstrated that this mechanism may be less important than initially anticipated (Newton and Holden, 2007).

In the airway epithelial cells, recent studies have identified two key anti-inflammatory GC-inducible genes: glucocorticoid-induced leucine zipper (GILZ) and MAP kinase phosphatase-1 (MKP-1). The induction of GILZ by GCs inhibits NF-κB transcriptional activity, suppressing signalling pathways which trigger the release of chemokines (Eddleston et al., 2007). The induction of MKP-1 was found to inhibit the production of inflammatory cytokines IL-6, IL-8 and COX-2 by airway epithelial cells (Turpeinen et al., 2010). MKP-1 activity is also recognised as an important contributor to the restoration of the airway epithelium integrity during wound healing processes (White et al., 2005). Furthermore, using filter-grown Caco-2 monolayers as an in vitro model of the intestinal epithelial barrier, Fischer et al. (2014) demonstrated that GC effects on the epithelial barrier restoration and diminishing inflammation are also dependent on MKP-1 induction.

Epithelial cells can also contribute to inflammation with the release of lipid mediators. The topical application of GCs has been shown to inhibit these pathways in psoriatic skin, through induction of annexin A1. In healthy skin, annexin A1 is mostly found in the cytoplasm of the basal keratinocytes. However, in the lesional skin, annexin A1 seems to appear only in the cell membrane, which facilitates its binding to and inhibition of the activity of phospholipase (PLA2). Therefore, GC-induction of the annexin A1 expression further blocks the synthesis and release of pro-inflammatory lipid mediators (Uva et al., 2012). The inhibition is additionally supported by
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GC-repressing COX-2, via GR-dependent transcriptional and post-transcriptional mechanisms involving mRNA destabilisation (Newton et al., 1998).

Synthetic GCs are a potent inducers of apoptosis in many cell types and tissues (Gruver-Yates and Cidlowski, 2013). Transactivation of cyclin-dependent kinase inhibitor (CDKN1C) and promyelocytic leukaemia zinc finger (PLZF) has been shown to mediate anti-proliferative and apoptotic actions of GCs in different cell types (Wasim et al., 2012, Samuelsson et al., 1999). There have been few reports of GC anti-proliferative actions in the epithelial cells. Glucocorticoids were demonstrated to inhibit both epidermal mitosis (Ahuwalia, 1998) and airway epithelial repair in vitro (Liu et al., 2013b). However, several studies suggest a pro-survival effect (Wen et al., 1997, Pelaia et al., 2003), leaving the effect of GCs on the airway epithelial cell survival and apoptosis less clear.

Rapid, non-genomic GC effects in the airway epithelium have also been reported. In the human bronchial epithelial cells, dexamethasone caused rapid stimulation of Na+/H+ exchanger, with subsequent change in pH. The proposed mechanism involved cAMP-dependent protein kinase (PKA) and mitogen-activated protein kinase (ERK1/2) pathways. Similar effects were also observed in Dex-regulation of intracellular Ca²⁺ levels, where a reduction in the levels of Ca²⁺ was sustained for about 90 minutes. Given that ion transport and Ca²⁺-signalling pathways are important for mucin production and secretion from the airway epithelial cells, these rapid GC effects might be of essence in inhibition of mucus hyperproduction, observed in asthma and COPD (Urbach et al., 2006).

Although it has been established that GCs can inhibit TGFβ - induced EMT through ROS-dependent pathways (Zhang et al., 2010), more recent study has revealed that TGFβ is able to impair EMT sensitivity to GCs, by reducing nuclear translocation of GRα, with resulting loss of GR-dependent gene regulation in adenocarcinoma-derived human alveolar basal epithelial cell line A549 (Salem et al., 2012).

Many studies of the effects of GCs in the epithelium have uncovered various mechanisms which involve both classical GR-dependent gene regulation, post-transcriptional regulation and rapid non-genomic actions. As novel pathways of GC actions in epithelium are constantly being
uncovered, the view of epithelium as a therapeutic target for GC most certainly has plenty of scope to evolve.

1.4.5 Glucocorticoid resistance

The vast majority of asthmatic patients have well-controlled asthma symptoms with inhaled GCs, usually in the combination with short-acting or long-acting bronchodilators. However, approximately 10% of asthmatic patients do not respond to GC therapy and develop severe form of the disease. This condition is generally recognized as GC-resistant (Trevor and Deshane, 2014). From the clinical point of view, GC-resistant asthma is characterized by several criteria, of which the most important one is the persistent obstruction in the airways, following two weeks treatment with a systemic GC (Hew and Chung, 2010). Pharmacologically speaking, GC-resistance refers to the rightward shift in the dose-response curve, as well as the diminution in the maximum response to GCs (Keenan et al., 2012).

The involvement of the splice variant of the “normal” GR, the GRβ, has been mentioned in various studies investigating GC-resistant pathways, as the “negative”, non-ligand binding, and inhibitory receptor form. Whilst Oakley et al. (1996) have demonstrated clear inhibition of GRα-mediated induction of gene expression in human cells transfected with GRβ, the exact mechanism of “inhibition” is still insufficiently characterised. The potential explanations involve the ability of GRβ to bind the GRα isoform, preventing GRα-signalling pathways, or direct binding of GRβ to GRE in the target gene, outcompeting the GRα isoform (Leung et al., 1997, Keenan et al., 2012). Considering that pro-inflammatory cytokines were shown to induce the expression of GRβ in both inflammatory (Webster et al., 2001) and epithelial cells of asthmatics (Vazquez-Tello et al., 2010), this pathway of GC-resistance may be of importance in chronic inflammation.

Some of the other mechanisms of resistance involving GR translocation to the nucleus, GR expression or ligand-binding activity have been demonstrated across variety of different cells types. Defective GR nuclear translocation was noted in the peripheral blood mononuclear cells from asthmatic patients who showed no response, even to the high doses of oral GCs (Matthews et al., 2004). Likewise, in the immortalized human lung carcinoma cell line A549, TGFβ1 was
able to reduce the expression and impair nuclear translocation of GRα, inhibiting GR-dependent GC-signalling pathways (Salem et al., 2012). Although this effect of TGFβ was also later confirmed in the immortalised human bronchial epithelial cell line BEAS-2B and differentiated air-liquid interface culture of primary epithelial cells, the impairment was not due to decreased receptor translocation. Moreover, the TGFβ effect was shown not to be exerted through its known classical canonical or non-canonical signalling pathways, suggesting another potential mechanism through which TGFβ impairs GC effects in the airway epithelium (Keenan et al., 2014). Interestingly, TGFβ was also found to be essential for the respiratory syncytial virus (RSV)-mediated impairment of GC anti-inflammatory actions in the airway epithelium (Xia et al., 2017).

Different factors were demonstrated to modify the structure of GR, affecting the receptor ability to bind to GC molecules (Barnes and Adcock, 2009). Increased expression of P-glycoprotein, transporter in control of the export of GCs from the cells (Crowe and Tan, 2012), has also been reported in some GC-resistant inflammatory conditions (Farrell and Kelleher, 2003). Finally, there is a compelling evidence linking decreased levels of enzyme histone deacetylase 2 (HDAC2) to GC-resistance, particularly observed in the COPD and severe asthmatic patients (Barnes, 2013).

The complexity behind the concept of GC-resistance grows every day, as new potential pathways linked to this phenomenon are being described in different inflammatory conditions. Therefore, current efforts are focused not only on improving the efficiency of GC therapy and reducing side effects, but also, more importantly, on preventing or reversing GC-resistance.

1.4.6 Does the physiological GC, cortisol possess partial agonist activity in GR-dependent gene transactivation in the bronchial epithelium?

A full agonist can bind and activate the receptor to produce the biological response in the tissue, and the amplitude of that response is typically proportional to the fraction of receptors that are occupied. In particular, with an increase in the agonist concentration, the number of the occupied receptors increases, leading to an increase in the tissue response until the maximum is
reached (Kenakin, 2004). Low efficacy agonists can bind and activate target receptors to produce lower levels of agonist response that may manifest as reduced maximal responses. These agonists are typically referred to as partial agonists. One predictable characteristic of a partial agonist is competition with a full agonist for the same receptor. In this circumstance the partial agonist is able to limit the effect of the full agonist over a range of concentrations of each ligand (Jackson, 2010) (Figure 1.7).

![Dose-response curves for a full agonist in the presence and absence of a partial agonist.](image)

**Figure 1.7 Dose-response curves for a full agonist in the presence and absence of a partial agonist.** Full agonist binds and activates the receptors to produce the maximum response. Partial agonist competes with a full agonist for the same receptor, limiting the maximum effect of the full agonist over a range of concentrations of each ligand.

Interestingly, a recent pharmacodynamic investigation of GR-mediated gene transactivation in human bronchial epithelial cell line BEAS-2B revealed that a single GC can behave as antagonist, partial agonist or even antagonist, depending on the gene being analysed. More importantly, the study has demonstrated a lower maximum response to hydrocortisone (cortisol) compared to several other synthetic GCs, suggesting a potential partial agonist activity of cortisol at GR in the bronchial epithelium (Joshi et al., 2015).
1.5 PLZF: GC-inducible transcriptional repressor with many roles

1.5.1 Acute Promyelocytic Leukaemia (APL) and first recognition of PLZF

The Promyelocytic Leukemia Zinc Finger (PLZF) or zinc finger 145 and BTB domain containing 16 (Zbtb16) protein was first identified 20 years ago in one of the retinoid–acid resistant cases of acute promyelocytic leukaemia (APL), in which chromosomal translocation (11;17) caused the fusion of retinoid-acid–receptor-α (RARα) and PLZF. This resulted in the formation of fusion proteins with a prominent role in the pathogenesis of APL (Chen et al., 1994), a condition characterised by accumulation of undifferentiated granulocytes - “promyelocytes”. Expressed in the haematopoietic progenitors, PLZF can interfere with cellular proliferation and differentiation, repressing the activity of cyclin A2, c-myc, and Box genes. Therefore, many of these important functions were reduced by its “captivity” in the fused complex with RARα. It is increasingly accepted that RA–RARα pathway not only plays a major role in neutrophil maturation, it is also essential for differentiation of pluripotent haematopoietic progenitors to granulocytic lineage. The PLZF–RARα complex could interfere with this pathway and modulate the RA–RARα–mediated transcription of genes crucial for differentiation of immature granulocytes (Mistry et al., 2003). Although APL is a highly curable disease, there have been various reports of resistance to the current forms of therapy, such as ATRA (all trans retinoic acids) and Arsenic-based induction therapy (Zhu et al., 2014). Therefore, a better understanding of pathways downstream of RARα-fusion proteins, as well as the role of PLZF alone in cell differentiation and cell cycle, are essential to guide efforts to overcome the impediments in treatment of resistant leukaemias, as well as in the other PLZF-related pathological conditions.

1.5.2 PLZF - a member of highly abundant family of DNA binding transcriptional factors

With more than 200 different regulatory proteins, the family of zinc finger proteins represents one of the largest families in eukaryotic genomes that plays an important role in DNA, RNA binding and protein interactions. Although the diversity in their structures and functions is well recognized, the structure pattern comprising multiple cysteine and histidine residues, which
requires zinc ions for its stabilisation, is a consistent feature of all family members (Laity et al., 2001). The full-length of the primary transcript of ZBTB16 gene encoding PLZF protein (Figure 1.8) shows three functionally important domains (Suliman et al., 2012).

**Figure 1.8 The primary transcript of ZBTB16 gene encoding PLZF protein, with 3 functionally important domains.** The BTB or POZ domain is critical for localisation of PLZF in the nuclear speckles, protein interactions, formation of multi-protein complexes on target DNA and transcriptional activity. The RD-2 domain is a subject to post-translational regulation which affects PLZF DNA-binding capacity. The C-terminus contains 9 zinc-finger motifs, which contribute to sequence specific binding of PLZF to its target DNA molecules. Adapted from Suliman et al. (2012).

On the N-terminus is a highly conserved BTB or poxvirus and zinc finger (POZ) domain, responsible for the localisation of PLZF in the specific nuclear compartments (Bardwell and Treisman, 1994). The BTB-POZ domain is also important for the protein dimerization, formation of a multi-protein complexes on DNA and consequential transcriptional activity (Ball et al., 1999). Although the middle RD-2 domain remains insufficiently characterized, Kam et al. (1993) were able to demonstrate that SUMO-1 (small ubiquitin related modifier-1) modifications of this domain can increase PLZF DNA-binding capacity. The C-terminus contains zinc finger patterns, which together with the POZ domain are essential for sequence-specific binding of PLZF to its target DNA molecules (Li et al., 1997).
1.5.3 Localization in nuclear speckles and modifications of transcriptional activity

Studying PLZF expression in human purified bone marrow (BM) CD 34+ cells, Reid et al. (1995) discovered that this transcriptional repressor is localized in the nuclear speckles, and taken together with the previous findings for similar zinc finger protein ZID (Bardwell and Treisman, 1994), it was shown that the presence of POZ-domain in the structure is required for this localisation. Nuclear speckles are defined as a subnuclear structures located in the interchromatin regions of mammalian cells, and contain a great number of different pre-messenger RNA splicing factors (Lamond and Spector, 2003). Interestingly, Koken et al. (1997) anticipated that co-localisation and direct interaction of PLZF and promyelocytic leukaemia protein (PML) in the nuclear speckles in different leukaemia cell lines, might be necessary for its transcriptional activity.

Different post-translational modifications can affect PLZF transcriptional activity. Phosphorylation and ubiquitination are the ones better understood. There have been a couple of milestone reports on PLZF phosphorylation by various molecules. Immunoprecipitation and kinase assay of transient transfected human embryonic kidney cell line 293T have shown that PLZF is a substrate for Cyclin-B/cdc2 kinase, with two potential sites for phosphorylation in the RD-2 domain. In the presence of exogenous phosphatase, levels of PLZF-DNA complex were greatly reduced, suggesting that phosphorylation of PLZF may have an important role in its binding to target DNA. Interestingly, the PLZF component of the RARα fusion complex could not be phosphorylated by cdc2 (Ball et al., 1999).

The proposed underlying mechanism of the stimulation of PLZF expression by interferons (IFNs) in natural killer cells is its phosphorylation within BTB domain, through the c-Jun amino-terminal kinase cascades (JNK). This phosphorylation was found to be necessary for the induction of interferon-stimulated genes (ISGs) (Xu et al., 2009). In particular, following phosphorylation triggered by IFN, PLZF binds to histone deacetylase 1 (HDAC1), allowing HDACs binding to ISG promoters, their transcription and NK-cell activation. On the contrary, the well-known gene-repressor activity is allowed by de-phosphorylation and acetylation by histone acetyl-transferase
(HAT). Thus, these post-translational modifications appear to be the switch from the classical role of transcriptional repression (established in cell proliferation and apoptosis, as well as in the renewal and stem cell maintenance) to this recently described role as a transcriptional activator (Ozato, 2009).

Furthermore, the biological function of PLZF is also modulated by sumoylation with small ubiquitin-like modifier (SUMO) and ubiquitination by ubiquitin. In the oxidative stress induced by serum deprivation, intracellular levels of reactive oxygen species (ROS) determined whether the modification of PLZF function will be with SUMO or ubiquitin. In effect, ROS were able to inhibit SUMO-conjugating enzymes, and consequently reduce the levels of sumoylation, whilst increased levels of ubiquitination were observed (Kang et al., 2008). Recent findings have established the involvement of PLZF-ubiquitination process in neurogenesis in zebrafish embryos. As PLZF represses the differentiation of neuronal progenitors, its ubiquitination by BTB6a-ubiquitination adaptor protein triggers its export from the nucleus, degradation and consequent loss of this repression activity, allowing neurogenesis to continue (Sobieszczuk et al., 2010).

1.5.4 The kaleidoscope of PLZF effects in different biological processes

The levels of PLZF expression in fully differentiated cells are exceptionally low compared to the levels in undifferentiated progenitors (Ayton and Cleary, 2002). Nevertheless, this transcriptional repressor has a variety of roles in different physiological and pathological processes (Figure 1.9).
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Figure 1.9 The kaleidoscope of PLZF functions in different biological processes. PLZF is a transcriptional repressor which modifies different signalling pathways implicated in physiological and pathophysiological processes: haematopoiesis, myeloid differentiation, immunity, spermatogenesis, cell self-renewal, control of cell cycle and cancer. Adapted from Suliman et al. (2012).

1.5.4.1 Haematopoiesis

One of the first reports on the requirement of PLZF for cell differentiation and maturation was in human bone marrow progenitors, suggesting its participation in a wide network of haematopoiesis-regulatory factors (Reid et al., 1995). Furthermore, analysis of the PLZF promoter revealed that it can repress transcription factors involved in myeloid differentiation, such as growth factor independent 1 (GFI-1), CCAAT/Enhancer-binding Protein α (C/EBPα), and lymphoid enhancer binding factor 1 (LEF-1), and induce the negative regulators, dual specificity phosphatase 6 (DUSP6) and inhibitor of DNA binding 2 (ID2). In addition, stress-triggered
cytokine-induction of ERK1/2, leads to PLZF nuclear export-dependent inactivation, subsequently inducing the production of mature cells (Doulatov et al., 2009). Finally, in unique knock-in mice models with or without ability of PLZF to interact with DNA, it was discovered that PLZF may be an important protector of genome integrity of hematopoietic cells by having an inhibitory effect on retro-transposition via DNA methylation and histone deacetylation of target sequences (Guid ez et al., 2014). Retrotransposons are DNA fragments that are known to amplify quickly and change their position, affecting the human genome via generating the insertion mutations and modifying gene expressions (Cordaux and Batzer, 2009).

1.5.4.2 Spermatogenesis and cell self-renewal

PLZF knock-out mice showed a progressive defect of spermatogonia in the tubules, followed by reduced spermatogenesis. The proposed underlying molecular mechanisms included a direct PLZF induction of cell-cycle regulators cyclin D2 and forkhead box N3 (FOXN3) expression, but not of previously reported targets myc and cyclin A2 (Costoya et al., 2004). Both in vitro and in vivo, PLZF can also act as a transcriptional repressor for Kit-tyrosine kinase-coupled receptor, which, together with its ligand, KL, is essential for spermatogonial stem cell differentiation and proliferation (Filipponi et al., 2007). Just as PLZF is crucial for the maturation of stem cells, its repressive functions are also linked to the self-renewal and maintenance of germ, hematopoietic and leukemic cell lines. Research so far has shown that PLZF can repress the activity of a member of Polycomb family product BMI1, involved in the maintenance of repression of different developmental genes (Kotaja and Sassone-Corsi, 2004). In the differentiating germ cells, another transcriptional factor, Sall4, interacts with PLZF, sequestering it to chromatin fragments, which allows the induction of Kit and stem cell differentiation (Hobbs et al., 2012).

1.5.4.3 Immunity

PLZF−/− mice have an impairment of IFN-activation of natural killer cells, due to the loss of PLZF expression, which is necessary for transcription of different ISGs (Xu et al., 2009). Furthermore, PLZF deficient invariant NKT cells express notably reduced levels of granzyme B,
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a serine protease most commonly found in granules of NK cells and cytotoxic T cells, with the importance in mediating apoptosis of target cells. Even though NKT cells could develop without PLZF expression, they lacked many other features of mature T cells, including, localisation in the lymph nodes rather than in other non-lymphoid tissues and impairment in IL-4 and IFN-γ production upon activation. It was suggested that the SAP/Fyn signalling pathway, which is important for NKT cell development in general (Pasquier et al., 2005) could be responsible for PLZF induction. However, full induction of PLZF in NK thymocytes occurs in the absence of SAP (Savage et al., 2008, Kovalovsky et al., 2008). Interestingly, Kreslavsky et al. (2009) demonstrated that PLZF expression is TCR-signalling-dependent in the γδNKT subset of immune cells, considered to be a part of both innate and adaptive immunity.

A novel, additional role of PLZF was revealed in human Th17 immune cells, known by their role in the development of autoimmune diseases (Steinman, 2007). In fact, PLZF transcriptional activity was shown to be important for the expression of CCR6 chemokine receptor and acquisition and maintenance of Th17 phenotype (Singh et al., 2014). Finally, recent findings show that PLZF is specifically expressed not only during development of innate-like lymphocytes, but also in innate lymphoid cells (ILC). The innate lymphoid cells are a relatively newly described group of immune cells without B/T receptors, that are considered to play an important role in the protective immunity and tissue remodelling (Hazenberg and Spits, 2014). Altogether these findings suggest that, by programming different effector cells, PLZF plays a significant role in immunity (Constantinides, 2014).

1.5.4.4 Control of cell cycle and cancer

PLZF alone directly supressed cyclin A2 and triggered growth arrest of NIH3T3 fibroblast cell line. The proposed pathway included binding of zinc-finger domains in the PLZF structure to sequences contained within the cyclin A2 promoter, suppression of cyclin A2 expression and growth arrest in S phase of the cell cycle. On the contrary, the ectopic expression of PLZF-RARα did not repress cell growth compared to wild-type PLZF (Yeyati et al., 1999). Moreover, immunoprecipitation promotor assays in primary murine hematopoietic progenitors showed that
RARα-PLZF fusion protein promoted cell growth by repressing DUSP6 and cyclin dependent kinase inhibitor 2D (CDKN2D), together with inducing c-myc expression. This induction of c-myc was caused predominantly by PLZF captivity in fusion complex. By binding to a region of c-myc promoter that is a target for PLZF alone, the fusion molecule antagonized PLZF-mediated c-myc repression (Rice et al., 2009). Extensive investigations in human embryonic kidney cell line HEK293 and human promyelocytic leukemia cell line HL-60, demonstrated that RARα-PLZF was also able to repress the transcription of CDKN1A gene that encodes p21, the induction of which predominantly leads to cell cycle arrest. This repression was shown to be both direct, by DNA methylation of proximal CDKN1A promoter, and indirect, by repression of p53 expression or activity. Transcriptional repressor p53 is an important factor that induces CDKN1A, by binding to distal p53 regulatory elements on CDKN1A promoter (Choi et al., 2014).

Engineered overexpression of PLZF had a growth-suppressive effect in murine myeloid 32Dcl3 cells, with consequential accumulation of cells in the G0/G1 phase of the cell cycle, and increase in apoptosis. Although only 50% of the cells expressed high PLZF levels, it appeared that PLZF-expressing cells could also secrete a growth-inhibitory factor, which caused death of majority of the cells in the population (Shaknovich et al., 1998). In a later study, PLZF overexpression alone was shown to inhibit proliferation and induce apoptosis in immortalized line of human T lymphocytes (Jurkat cells), cultured at low density. Annexin V staining and cell cycle distribution confirmed large decrease in number of cells in S phase. Further analysis of this PLZF-depended cell cycle arrest revealed direct interaction of PLZF with apoptosis-related genes: upregulation of pro-apoptotic factors DNA-binding protein inhibitor 1 (ID1) and 3 (ID3) and tumour protein p53 inducible nuclear protein (TP53INP1) and downregulation of anti-apoptotic telomerase reverse transcriptase (TERT) (Bernardo et al., 2007). In addition, investigating the role of PLZF in proliferation of human corneal endothelial cells, Shiraishi et al. (2007) found that PLZF is expressed exclusively in completely confluent cultures, compared to sub-confluent HCECs in vitro. Moreover, cultured cells infected with adenovirus vector carrying genes encoding PLZF (ad-PLZF) could not proliferate, suggesting that PLZF acts as a suppressor of proliferation.
Intriguingly, tissue inhibitors of metalloproteinases (TIMPs) can also interact with PLZF, as shown in a human ovarian cancer cell line. Besides their role in binding and inhibiting matrix metalloproteinases (MMPs), which is an important modulating influence in cancer progression (Rundhaug, 2003), TIMP-1 can also promote cell proliferation and have an anti-apoptotic function (Hornebeck, 2003). In yeast two-hybrid screening, following different binding assays, Rho et al. (2007) showed that TIMP-1 can notably reduce PLZF-induced apoptosis in HeLa cervical carcinoma cell line, suggesting that anti-apoptotic role of TIMP-1 could be mediated by the reduction of PLZF-suppression of proliferation.

There have been a few additional reports on PLZF acting as a tumour-suppressor in other cancers, including melanoma, colon, hepatocellular and prostate cancer. An in vivo study of primary malignant melanomas from 41 patients revealed that PLZF was expressed in the majority of tumours in vivo, but not in melanoma cell lines in vitro. Furthermore, low PLZF expression caused increase in Pre-B-cell leukaemia transcription factor-1 (PBX1) expression, recognized as a proto-oncogene for different tumours (Park et al., 2008). Thus, this was the first time that PLZF mRNA levels could be used as a prognostic/survival marker (Brunner et al., 2008). Similar findings were acquired in colorectal cancer cell lines, in which high PLZF expression levels correlated with low levels of CUX-1, a factor implicated in breast and pancreas tumour initiation and progression (Hulea and Nepveu, 2012, Frechette et al., 2010). However, PLZF mRNA and protein levels were found to be significantly downregulated in the hepatocellular carcinoma compared to normal tissues (Hui et al., 2015). Likewise, PLZF was also found to be down-regulated in non-small-cell lung cancer cases (NSCLC). Demethylation analysis in A549 cell line revealed that PLZF downregulation was triggered by the hyper-methylation in the promoter region. In addition, overexpression of PLZF in both A549 and LTEP lung cancer cell lines caused inhibition of proliferation and increase in apoptosis. Thus, decreased PLZF expression levels could potentially contribute to the pathogenesis of NSCLC by promoting cell survival (Wang et al., 2013) (Table 1-2).
### Table 1-2 PLZF expression and potential roles in different types of cancer

<table>
<thead>
<tr>
<th>Type of cancer</th>
<th>PLZF expression</th>
<th>PLZF role</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary malignant melanoma</strong></td>
<td>Low levels <em>in vitro</em> Higher levels <em>in vivo</em></td>
<td>Repression of proto-oncogene Pre-B-cell leukaemia factor a (PBX-1)</td>
<td>(Brunner et al., 2008)</td>
</tr>
<tr>
<td><strong>Colorectal</strong></td>
<td>Higher levels <em>in vitro</em> (Cac0-2/15, HEK293) than <em>in vivo</em></td>
<td>Target for CCAAT-displacement-protein (CUX1)-stimulator of cell proliferation and motility, but also promotes genetic instability</td>
<td>(Frechette et al., 2010) (Hulea and Nepveu, 2012)</td>
</tr>
<tr>
<td><strong>Hepatocellular</strong></td>
<td>Significantly downregulated</td>
<td>Suppression of proliferation; pro-apoptotic activity</td>
<td>(Hui et al., 2015)</td>
</tr>
<tr>
<td><strong>Non-small-cell-lung cancer (NSCLC)</strong></td>
<td>Downregulated both in A549 and LTEP cell lines</td>
<td>Suppression of proliferation; pro-apoptotic activity</td>
<td>(Wang et al., 2013)</td>
</tr>
</tbody>
</table>

Given that PLZF is induced by glucocorticoids, a down-regulation of PLZF could also be potentially of interest for interpretation of TGFß impairment of sensitivity to glucocorticoids in A549 cell line (Salem et al., 2012).

Using a human umbilical vein endothelial cell model, Rho et al. (2010) showed PLZF inhibition of endothelial cell angiogenesis, following the stimulation with vascular endothelial growth factor (VEGF). Considering angiogenesis is an essential step for tumour growth (Folkman, 1992), this inhibition could confer additional tumour-suppressive activities on PLZF in different types of cancer.

Increasing amounts of evidence suggest that PLZF may also play a role in reorganization of the actin cytoskeleton. In chicken embryo fibroblasts, overexpressed PLZF was able to bind the promoter of smooth muscle α-actin, reducing its mRNA and protein levels in a concentration-dependent manner. The repression of smooth muscle α-actin caused an impressive change in fibroblast morphology from spindle shaped to polygonal, with an increase in resistance to
oncogenic transformation by the majority of oncoproteins tested, such as myc, PI3K, AKT and FOS (Shi et al., 2010, Kolesnichenko and Vogt, 2011). This recently described role of PLZF could be particularly important in the TGFβ-induced epithelial-mesenchymal transition of the epithelial cancer cell line A549, as the cells acquired spindle-shaped fibroblast-like phenotype following TGFβ-treatment (Salem et al., 2012).

1.5.5 PLZF – a GC inducible target

Algorithm-based analysis of PLZF genomic sequence revealed six GC-rich regions, and a possible GRE promoter immediately upstream of the first GC-rich region (Zhang et al., 1999), indicating that PLZF is a target for GCs. Knock down and overexpression experiments in CCRF-CEM childhood ALL cells confirmed that PLZF is a GC response gene which modulates GC-induced apoptosis in lymphoid cells. In fact, transgenic overexpression of PLZF alone did not have an effect on the cell proliferation and apoptosis, whilst it impaired the sensitivity to dexamethasone-induced apoptosis. Moreover, knockdown of PLZF resulted in a small, but significant increase in Dex-induced apoptosis. Transcriptomic analysis revealed that PLZF induction regulated various GC response genes, particularly the BCL2L11/Bim, factors accountable for the induction of apoptosis in CCRF-CEM cells. Given that PLZF overexpression did not reduce the sensitivity to cell death induced by other factors, such as chemotherapeutics, mitomycin C, doxorubicin or taxol, nor antibodies against CD95/FAS, this protective effect of PLZF appeared to be specific for GC-induced apoptosis (Wasim et al., 2010). Interestingly, in the subsequent study, the same authors showed that PLZF enhances the GC-induced cell death in leukemic model cell line NALM6 (Wasim et al., 2012).

The induction of PLZF by GCs has been also noted in cochlear damage associated with acoustic trauma, caused by different “conditioning” stimuli, such as heat stress, hypoxia and moderate level sound. Results of the study imply that synthetic GCs such as dexamethasone or methylprednisolone, widely used in treating acute hearing loss, can induce the protection of the cochlea, by elevating PLZF levels (Peppi et al., 2011).
In Jurkat cell-line, PLZF induction by dexamethasone was shown to require sufficient amounts of functional glucocorticoid receptor (Riml et al., 2004) suggesting that the induction is mediated by the GR. These findings were confirmed in the human primary endometrial stromal cells (EMC) and myometrial smooth muscle cells (SMC), in which the GR partial agonist RU486 (mifepristone) reduced Dex-induction of PLZF. Although PLZF was upregulated by both dexamethasone and progesterone, there was a greater induction by dexamethasone compared with progesterone, after 4h incubation. In an extended time-course investigation, the peak of PLZF expression occurred within 4h of steroid treatment, remaining high for more than 72h. Additionally, transient transfections in ESC revealed prominent PLZF induction by the activator protein-1 (AP-1), independent of AP-1 binding site in the PLZF promoter. Remarkably, this AP-1 mediated upregulation also remained resistant to GR-repression (Fahnenstich et al., 2003).
1.6 General hypotheses and aims of this thesis

GC-resistance remains a major clinical obstacle to successful treatment of certain patients with severe disease, due to the insufficient understanding of the mechanisms underlying their actions and insensitivity. Emerging evidence suggests that physiological GC, cortisol might act as a partial agonist at the glucocorticoid receptor in the bronchial epithelium. However, whether cortisol can be a limiting factor to beneficial effects of synthetic GCs, remains to be established. Therefore, through a better understanding of the impact of cortisol on the effects of synthetic GCs \textit{in vitro} and \textit{in vivo}, as well as of novel individual mediators mediating GC actions in the bronchial epithelium, new strategies may arise for restoring GC responsiveness.

General hypotheses of this study were:

1. The physiological GC, cortisol limits actions of synthetic, therapeutically used GCs in the airway epithelium \textit{in vitro} and \textit{in vivo}.
2. Transcriptional repressor, promyelocytic leukaemia zinc finger (PLZF) is a mediator of GC actions in the bronchial epithelium.

The major aims of this study were to:

1. Examine the impact of physiological GC, cortisol on the actions of synthetic GCs in the bronchial epithelium, using different epithelial \textit{in vitro} cell models: immortalized human bronchial epithelial cell line BEAS-2B and air-liquid interface culture of primary human bronchial epithelial cells.
2. Further explore the effects of physiological and synthetic GCs in the airway epithelium \textit{in vivo}.
3. Investigate so far unidentified role(s) of transcriptional repressor promyelocytic leukaemia zinc finger (PLZF) in the bronchial epithelial proliferation, differentiation, and in mediating GC effects, using \textit{in vitro} epithelial cell models and gene-silencing techniques.
CHAPTER 2:
GENERAL METHODS
2.1 General cell culture

2.1.1 Culture of immortalised human bronchial epithelial cell line BEAS-2B

2.1.1.1 Propagation method

The immortalized normal human AD12-SV40-hybrid virus-transformed bronchial epithelial cell line BEAS-2B (American Type Culture Collection (ATCC), VA, USA) was cultured in complete LHC-9 medium (Life Technologies, NY, USA), supplemented with 2% (v/v) heat-inactivated foetal calf serum (HIFCS), 2mM L-glutamine, 100IU/ml penicillin and 50µg/ml streptomycin (Sigma, MO, USA) and kept at 37°C in a humidified 5% CO₂/95% air atmosphere. Cells were passaged twice a week by washing 70-80% confluent cell monolayers twice with sterile phosphate-buffered saline (PBS), followed by incubation with trypsin-EDTA (0.12% w/v) (Sigma, MO, USA) for 2-3 minutes to detach the cells from the surface. Suspension of cells was then re-seeded at 8,000 cells/cm².

2.1.1.2 Starvation method

Unless otherwise indicated, BEAS-2B cells were serum-starved 24h prior to drug treatments, using incomplete phenol red-free Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies, NY, USA), supplemented with 0.25% (w/v) BSA, 0.2% (v/v) sodium bicarbonate, 15mM HEPES, 2mM L-glutamine, 1% (v/v) non-essential amino acids, 1% (v/v) sodium pyruvate, 100IU/ml penicillin and 50µg/ml streptomycin (Sigma, MO, USA), and maintained at 37°C in a humidified atmosphere containing 5% CO₂.

2.1.2 Culture of primary human bronchial epithelial cells (HBECs)

Primary human bronchial epithelial cells (HBECs) were purchased from Lonza (Mt Waverley, Australia), and the characteristics of donors are shown in Table 2-1.
Table 2-1 Main characteristics of donors of primary human bronchial epithelial cells

<table>
<thead>
<tr>
<th>TAN</th>
<th>Diagnosis</th>
<th>Age</th>
<th>Sex</th>
<th>Alcohol</th>
<th>Smoking</th>
<th>Culture passage number</th>
</tr>
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<tbody>
<tr>
<td>26573</td>
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<td>No</td>
<td>No</td>
<td>2</td>
</tr>
<tr>
<td>25062</td>
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<td>56</td>
<td>Male</td>
<td>No</td>
<td>Yes</td>
<td>2</td>
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<tr>
<td>16023</td>
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<td>56</td>
<td>Male</td>
<td>Yes</td>
<td>Yes</td>
<td>2</td>
</tr>
<tr>
<td>25745</td>
<td>Normal</td>
<td>24</td>
<td>Female</td>
<td>Yes</td>
<td>Yes</td>
<td>2</td>
</tr>
<tr>
<td>22544</td>
<td>Asthmatic</td>
<td>25</td>
<td>Male</td>
<td>Unknown</td>
<td>Unknown</td>
<td>2</td>
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<tr>
<td>27853</td>
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<td>No</td>
<td>2</td>
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<tr>
<td>28695</td>
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<td>Yes</td>
<td>2</td>
</tr>
<tr>
<td>25786</td>
<td>Asthmatic</td>
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<td>No</td>
<td>No</td>
<td>2</td>
</tr>
<tr>
<td>26680</td>
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<td>65</td>
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<td>Yes</td>
<td>No</td>
<td>2</td>
</tr>
<tr>
<td>24634</td>
<td>COPD</td>
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<td>Yes</td>
<td>Yes</td>
<td>3</td>
</tr>
<tr>
<td>24527</td>
<td>COPD</td>
<td>52</td>
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<td>Yes</td>
<td>2</td>
</tr>
<tr>
<td>25407</td>
<td>COPD</td>
<td>59</td>
<td>Female</td>
<td>Yes</td>
<td>Yes</td>
<td>2</td>
</tr>
</tbody>
</table>

Cells were cultured using the Clonetics™ BEGM™ (bronchial epithelial growth medium or 1xBEGM) Bulletkit™ (Lonza, Mt Waverley, Australia) consisting of bronchial epithelial cell basal medium supplemented with 0.4% (v/v) bovine pituitary extract (BPE), 0.1% (v/v) hydrocortisone, 0.1% (v/v) hEGF, 0.1% (v/v) adrenaline, 0.1% (v/v) transferrin, 0.1% (v/v) insulin, 0.1% (v/v) retinoic acid, 0.1% (v/v) triiodothyronine, 0.1% (v/v) GA-1000. Cells were kept in a humidified atmosphere containing 5% CO₂ at 37°C and passaged from a single T25 flask using method recommended by the manufacturer. Briefly, 70-80% confluent cell monolayers were washed with 2mL of HEPES buffered saline solution (HBSS; Lonza #CC-5022) and then detached from the surface of a flask using 1mL of trypsin/EDTA (Lonza #CC-5012). Trypsin was then neutralised with 2mL of trypsin neutralizing solution (TNS; Lonza #CC-5002), after which cells were collected by centrifugation at 220g for 5 min at room temperature. Cells were then re-seeded at 5,000 cells/cm².
2.1.3 **Mycoplasma detection**

Mycoplasma contamination was routinely tested in all cell lines, using the Mycoalert™ Mycoplasma detection kit (Lonza, Mt Waverley, VIC, Australia) according to manufacturer’s instructions.

2.2 **Air-liquid interface culture of differentiated primary HBECs**

2.2.1 **Coating of Transwell™ inserts with rat-tail collagen**

Rat tails, obtained from the animals euthanized for other studies were used for preparation of the fibrillar, type I collagen. Briefly, tails were soaked in 70% ethanol for 15 minutes, after which the tail skin was removed, exposing tendons for dissection and subsequent digestion in 0.1% sterile acetic acid at 4°C for 72 hours. Digested collagen was centrifuged every 24h at 830g for 2 hours at room temperature, after which the supernatant was collected and fresh acid was added to further digest insoluble collagen fibres. Supernatant was then dialysed against sterile, endotoxin-free water at 4°C for 24 hours and the concentration of the protein was determined by Bradford protein assay (Section 2.4.2.2). 100μL of 0.03mg/mL stock of the rat-tail collagen was used to coat Corning® Transwell® inserts containing 0.4μm pore PET membrane (Sigma #CLS3470). Following 45 minutes incubation at 37°C, collagen solution was carefully aspirated from each well and membranes were washed once with 150µL of PBS and left to air-dry.

2.2.2 **Differentiation of primary HBECs by air-liquid interface (ALI) culture**

On day 1, primary HBECs monolayers were seeded onto collagen-coated Transwell® inserts at 125,000 cells/cm² in 100μL Clonetics™ B-ALI™ supplemented growth medium (1xBEGM) (Section 2.1.2). Additional 350μL of 1xBEGM was added to the basal chamber and cells were incubated at 37°C in a humidified 5% CO₂/95% air atmosphere. On day 2, following microscopic confirmation of the cell adherence, media was aspirated from both basal and apical surfaces and replaced with fresh pre-warmed 1xBEGM. The following day, cells were ‘air-lifted’ by aspirating medium from both basal and apical chambers and adding 350μL of B-ALI™ differentiation
medium only to the basal chambers. B-ALI™ differentiation medium was prepared out of 1:1 (v/v) mixture of 2x B-ALI™ growth medium (2xBEGM) (supplemented with BSA fraction V solution at final concentration of 3 µg/ml) and Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies, NY, USA), supplemented with 0.2% (v/v) sodium bicarbonate, 15mM HEPES, 2mM L-glutamine, 1% (v/v) non-essential amino acids, 1% (v/v) sodium pyruvate, 100IU/ml penicillin and 50µg/ml streptomycin (Sigma, MO, USA). Cells were re-fed every 48–72h over a period of 4-weeks by aspirating medium from the basal chamber, and adding 350µL of fresh medium. Differentiation of the cells was confirmed through visualisation of the beating cilia and mucus production and by measurement of trans-epithelial electrical resistance (TEER) using an EVOM2 Volt ohmmeter (WPI). Specifically, measurement of TEER values was performed as follows. Sterilisation of the electrode was carried out by submerging the electrode in 80% ethanol for 5 minutes, following its air drying and rinsing in HBSS. A 100µL of pre-warmed sterile HBSS was added to the apical surface of the Transwells. The measurement was carried out by holding the electrode vertically and as steady as possible until the reading (Ω) was stabilised, and the process was then repeated for each Transwell (Srinivasan et al., 2015).

2.3 Small interfering RNA (siRNA) – mediated gene silencing

BEAS-2B cells were seeded in a 24-well plate at a density of 40000 cells/cm². Following 24h in antibiotic-free LHC-9 complete medium (Section 2.1.1.1), cells were washed once with 1mL of pre-warmed PBS, and equilibrated for an hour in 500µL of antibiotic-free DMEM, supplemented only with 0.2% (v/v) sodium bicarbonate and 15mM HEPES. Negative control siRNA and pre-designed and validated 2 different PLZF (ZBTB16) siRNA sequences (10nM/well) (#s15199, #s15200, Ambion, Silencer® Select 5nmol, Life Technologies, USA) were pre-mixed and complexed with 1µL/well Lipofectamine® RNAiMAX (Invitrogen, Life Technologies, CA, USA) in reduced serum Opti-MEM medium (Life Technologies, NY, USA) for 15 min prior to drop-wise addition of 100µL of that transfection media to each well. After 4
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hours of incubation, the transfection was quenched by replacing medium with 1mL incomplete DMEM (Section 2.1.1.2).

2.4 General analytical techniques

2.4.1 Real time – Quantitative Polymerase chain reaction (RT-qPCR)

2.4.1.1 Total mRNA extraction

Extraction of total mRNA from BEAS-2B cells was performed using TRIZol® reagent (Invitrogen, Doncaster, VIC, Australia). Initially, cells were washed with PBS twice and then lysed in 500μL TRIZol® reagent. Cell lysate was relocated to 1.7mL RNase-free tube and incubated at room temperature for 5 minutes to ensure complete detachment of the nucleoprotein complex. Further, 150μL of chloroform was added to each tube which were then vortexed with following centrifugation at 12000g for 15 minutes at 4°C. After having aqueous and organic phase successfully separated, the upper aqueous phase was then gently aspirated and transferred to a new RNase-free tubes. 500μL of isopropanol was added together with the carrier - glycogen (4μL/tube) (Invitrogen #AM9510) to precipitate RNA at room temperature for 10 minutes. After the incubation, samples were centrifuged at 12000g for 10 minutes at 4°C. Finally, the supernatant was discarded and 1mL of 75% (v/v) ethanol in diethyl-pyrocarbonate (DEPC)-treated water was used to wash the RNA pellet. Following the centrifugation at 7,500g for 5 minutes at 4°C, the supernatant was aspirated, observable RNA pellet was then air-dried and resuspended in 40μL DEPC-treated water. Quantification of RNA concentration was performed by the NanoDrop 1000. Samples were stored at -80°C until further analysis.

Total RNA from ALI-cultures and animal tissues was extracted using Illustra™ RnaSpin Mini RNA Isolation Kit (#25-0500-72, GE Healthcare, UK) and the extraction process was performed at room temperature. Briefly, cells or animal tissue (following homogenisation) were firstly lysed in 350μL of RnaSpin lysis buffer with 1% β-mercaptoethanol (Sigma, MO, USA) and each sample was transferred into corresponding RnaSpin Mini Filter Units (violet ring). Clear lysates were collected following centrifugation at 11000g for 1min. Adjustment of RNA
binding conditions was carried out through addition of the same volume of 70% Ethanol in DEPC-
water as of lysate, mixing and centrifuging the solution in RNASpin Mini Columns (blue ring) at
8000g for 30s. After desalting the silica membrane with 350μL of MDB reagent and centrifuging
for 1min at 11000g, DNA was digested by incubation of each sample with reconstructed DNase
I, diluted 10x in DNase reaction buffer for 15 minutes. 200μL of RNASpin wash buffer 2 was
added then to inactivate DNase I and columns were centrifuged for 1min at 11000g. Next 2
washes were performed using 600μL and 250μL RNASpin wash buffer 3 for centrifuging at
11000g for 1 and 2 minutes respectively. Finally, 40μL of Nuclease-free water was added
carefully in the middle of the membrane and highly-purified RNA was eluted by centrifugation
for 1min at 11000g. Quantification of RNA concentration was performed by the NanoDrop 1000.
Samples were stored at -80°C until further analysis.

2.4.1.2 Reverse transcription

cDNA was formed from total mRNA using the High Capacity cDNA reverse transcription
kit (Invitrogen, #4387406), with total reaction volume of 5μL, (2μL of total RNA (100ng RNA),
2.5μL 2xRT buffer, and 0.5μL 20x RT enzyme mix), with one additional control sample (without
RT enzyme mix). The reaction was performed on 37°C for 60 minutes, then on 95°C for 5
minutes in Eppendorf Mastercycler® Pro. cDNA was diluted with 145μL DEPC-treated H2O.
Samples were stored at -20°C until further analysis.

2.4.1.3 RT-qPCR – protocol and primers

For the gene of interest, RT-qPCR was performed in triplicate in a 384-well plate using
QuantStudio™ 6 Flex sequence detection system (Applied Biosystems). The PCR reaction
consisted of 1.5μL diluted cDNA, 2.5μL Platinum SYBR Green qPCR Supermix-UDG
(Invitrogen, Mulgrave, VIC, Australia) and 1μL of mix of 100nM forward and reverse human
(Table 2-2) or mouse primers (Table 2-3), obtained from either previously published references,
or from pre-validated KiCqStart™ primers (Sigma-Aldrich). PCR amplification was done with
the following thermal protocol: 50°C for 2 minutes, 95°C for 10 minutes followed by 40 cycles
of 95°C for 15 seconds, and 60°C for 1 minute. The melt curve analysis was performed to confirm the specificity of the amplified products.

2.4.1.4 Analysis of the PCR data

Threshold cycle (Ct) values were automatically generated using QuantStudio™ 6 software (indicated by the red line on Figure 2.1) for each sample and analysed using ΔCt method relative to the internal standard (18S ribosomal RNA) as described by the manufacturer. Unless otherwise specified, data were expressed as relative expression for each target gene, normalised to a control/vehicle group.

![Amplification Plot](image)

Figure 2.1 Representative amplification plots for r18s housekeeping gene
### Table 2-2 Human primer sequences for RT-qPCR

<table>
<thead>
<tr>
<th>Gene product</th>
<th>Reverse primer</th>
<th>Forward primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>r18s</td>
<td>TCT TGG CAA ATG CTT TCG CTC</td>
<td>CGC CGC TAG AGG TGA AAT</td>
</tr>
<tr>
<td>CDKN1A</td>
<td>GTG GTA GAA ATC TGT CAT GCT GTT C</td>
<td>GAC TCT CAG GGT CGA AAA CGG</td>
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<tr>
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<td>CTC TTT GGG CTC TAA ATT GG</td>
<td>TCT GAT CTC CGA TTT CTT CG</td>
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<tr>
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<td>ATG CTG TGG TGC TTT GAG GTA G</td>
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</tr>
<tr>
<td>c-myc</td>
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<td>CCC TCA ACG TTA GCT TCA CCA</td>
</tr>
<tr>
<td>ENaCα</td>
<td>TGA GGT TGA TGT TGA GGC TG</td>
<td>AGC ACA ACC GCA TGA AGA C</td>
</tr>
<tr>
<td>FKBP5</td>
<td>TTG CCC ATT GCT TTA TTG G</td>
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</tr>
<tr>
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<td>GRα</td>
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</tr>
<tr>
<td>KCNB1</td>
<td>GAA CCT CAG CAG GTA CTC</td>
<td>AAG ATC CTT GCC ATA ATT TCC</td>
</tr>
<tr>
<td>IL-6</td>
<td>CGC TTG TGG AGA AGG AGT TCA</td>
<td>AGC TCT ATC TCG CCT CCA GGA</td>
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<tr>
<td>IL-8</td>
<td>CCT TGG CAA AAC TGC ACC TT</td>
<td>CTG GCC GTG CCT CTC TTG</td>
</tr>
<tr>
<td>MKP-1</td>
<td>TCT ATG AAG TCA ATG GCC TCG TT</td>
<td>CCA CAA GGC AGA CAT CAG CTC</td>
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<tr>
<td>PCNA</td>
<td>ATA CTG GTG AGG TTC ACG CC</td>
<td>ACA CCT ACC GCT GCG ACC</td>
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<tr>
<td>PLZF</td>
<td>CAT GTC AGT GCC AGT ATG</td>
<td>GTT CCT GGA TAG TTT GCG</td>
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Table 2-3 Mouse primer sequences for RT-qPCR

<table>
<thead>
<tr>
<th>Gene product</th>
<th>Reverse primer</th>
<th>Forward primer</th>
</tr>
</thead>
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<tr>
<td>r18s</td>
<td>CCT GCT GCC TTC CTT GGA T</td>
<td>TCC GGC GAG GGA GCC TG</td>
</tr>
<tr>
<td>GILZ</td>
<td>TCT TCA CGA GGT CCA TGG C</td>
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<tr>
<td>KC</td>
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<td>GCA TTC AAA GGG GAT ATC AG</td>
<td>CTA CTA CCA GAC ATA CTG CC</td>
</tr>
</tbody>
</table>

2.4.2 Western Blotting

2.4.2.1 Collection of total protein lysates

Cells were washed twice with ice-cold PBS, and then lysed with lysis buffer (1% v/v TritonX-100, 1 mM EDTA, 100 mM NaCl; 10 mM Tris–HCL (pH 7.4)), which additionally contained phosphatase inhibitor cocktail (#P5726 Sigma-Aldrich, Castle Hill, Australia) and protease inhibitor cocktail (#P1860 Sigma-Aldrich) for 20 min on ice. Lysates were then centrifuged at 8000g 10 minutes at 4°C and supernatants were transferred to a fresh new tubes and stored at -20°C until further analysis.

2.4.2.2 Bradford protein assay

To determine the protein concentration in samples, 100µL of PBS-diluted duplicate samples or BSA standards (final concentrations of 0; 2.5; 5.0; 7.5; 10; 15; 20µg/mL) were vortexed and incubated with 100µL of 0.2M NaOH for 15 minutes at room temperature. After addition of 600µL of water and 200µL of Protein Assay Dye Reagent Concentrate in (Bio-Rad #500-0006), tubes were again vortexed thoroughly and 200µL of solution was finally used to measure the absorbance at 595nm on a Multiskan Ascent plate reader (Thermo Scientific). The standard curve,
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fitted from the absorbance of BSA standards, was used to calculate the concentration of the proteins.

2.4.2.3 SDS-PAGE/Western Blot analysis

To 20-30µg of protein samples, sample buffer containing 1% (v/v) β-mercaptoethanol, 0.125M Tris-HCL (pH 6.8), 20% (v/v) glycerol, 4% (w/v) SDS, 0.1% (w/v) bromophenol blue, was added and the mix was heated on 95°C for about 3 minutes. Samples were then resolved using SDS-PAGE on precast 4–20% Mini-PROTEAN® TGX™ gels (BioRad #4561094) for 40 minutes at 200V, and transferred onto nitrocellulose membranes using wet transferring method with transfer buffer containing 30% (v/v) methanol, 25mM Tris, 0.2M Glycine, 0.1% (w/v) SDS, at 100V for 1h on ice. After the blocking of unspecific binding sites on the membranes with 5% skim milk in TBS/0.1% Tween, membranes were incubated overnight at 4˚C with primary antibodies as follows. PLZF mouse monoclonal antibody (#sc-28319, Santa Cruz Biotechnology, CA, USA) was diluted 1:100, and both total GRα rabbit polyclonal antibody (#sc-1003 Santa Cruz Biotechnology, CA, USA) and ENaCα goat polyclonal antibody (#sc-22239 Santa Cruz Biotechnology, CA, USA), were diluted 1:200 in 5%BSA in TBS/0.1% Tween. Following the imaging of proteins of interest and stripping the membranes using stripping buffer (50mM glycine, 0.1% (w/v) SDS, 1% Tween-20, pH 2.2), membranes were re-probed with either β-actin mouse monoclonal (1:2000 in 5%BSA in TBS/0.1% Tween) (#8226 Abcam, Cambridge, UK) or β-tubulin mouse monoclonal (1:2000 in 5%BSA in TBS/0.1% Tween) (#2146, Cell Signalling, Danvers, MA, USA), which were used as housekeeping controls for the protein loading. All signals were detected using horseradish peroxidase-conjugated secondary antibody (Polyclonal goat anti-mouse Immunoglobulins/HRP, Dako, Denmark) and Thermo Scientific™ Pierce™ ECL Western Blotting Substrate (GE Healthcare, NSW, Australia). Images of Western blots were captured using Bio-Rad ChemiDoc™ MP imaging system (#731BR01006, NSW, Australia) and densitometry was carried out using ImageJ (v1.44, National Institute of Health, Bethesda, MD, USA).
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#### 2.4.3 Trypan blue assay

Total cell number (viable and non-viable cells) was assessed using Trypan blue exclusion method, in which the non-viable cells take up trypan blue. Cell count was performed using hemocytometer. Briefly, supernatant from each well was collected and after centrifugation for 5 minutes at 1100g at room temperature, supernatant was discarded and 20µL of Trypan blue (0.2% (w/v)) (Sigma, USA) was added to the cell pallet and left for 5 minutes on ice. Adherent monolayer of cells was washed once with 1mL PBS, and incubated with Trypsin-EDTA (Sigma, USA) until the cells were detached from the bottom of the well. Trypan blue solution in 2%FCS in PBS (0.2% (w/v)) was added to the suspension of the cells, and the total content of the well was transferred to an open test tube and incubated for 5 minutes on ice for the counting of stained and unstained adherent and supernatant cells using Olympus IX51 (Japan).

#### 2.4.4 Resazurin-based cell proliferation assay

BEAS-2B cells were seeded at different densities in a 96-well plates, and following the treatments, supernatants were collected, cells were washed once with 150µL warm PBS, and 100µL of resazurin working solution (1:10 diluted resazurin stock solution (Invitrogen) in incomplete DMEM (Section 2.1.1.2)) was added to each well. Plate was kept in the incubator, and fluorescence readings were carried out using FlexStation 3 microplate reader (Molecular Devices, California, US) at 30min, 1, 2, 3, 4 and 5h following resazurin addition. The fluorescence filters used were 530nm for excitation and 580nm for emission.

#### 2.4.5 Enzyme-linked immunosorbent assay (ELISA)

##### 2.4.5.1 Supernatant collection

Supernatants were collected in duplicates (250µL) from confluent monolayers of cells into a new 96-well plates. Plates were sealed with adsorbent tape and stored at -20°C until further analysis.
2.4.5.2 Detection of cytokines

Supernatants obtained from BEAS-2B cells were assayed for IL-8, IL-6 and GM-CSF cytokine levels, using commercial sandwich ELISA kits (BD Biosciences, NSW, Australia) according to the manufacturers’ instructions. In summary, medium affinity flat bottom 96-well plates (Greiner bio-one #655001) were incubated with specific capture antibodies (diluted to the recommended concentration in coating buffer (0.1M sodium carbonate, pH 9.8)) overnight, washed several times in 0.1% (v/v) Tween20/PBS and blocked with 10% FCS in PBS for an hour to block non-specific binding sites. Following additional washing, suitable dilutions of standards and supernatants were incubated with detecting antibodies for 2-3h at room temperature, after which HRP-conjugated streptavidin complex was added in the recommended concentration for 30 minutes incubation. Plates were then finally washed 3 times with wash buffer, and visualization was carried out using 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (1:1 parts A and B, BD Biosciences). Sulphuric acid (2 M) was used to stop the reaction and the absorbance was measured at \( \lambda = 450 \) nm on Multiskan Ascent\textsuperscript{®} plate reader. Concentration of the cytokines in the samples were determined using the absorbance of the cytokine standards and 4 parameter logistic curve fitting.

2.4.6 Immunofluorescence

BEAS-2B cells were seeded in 8-chamber Permanox\textsuperscript{®} cell culture slides (Nunc, Roskilde, NL). Following drug treatments, cells were first washed in 500\,\mu L PBS/chamber and then fixed for 15 minutes in 10% (v/v) neutral buffered formalin (NBF). Cells were then permeabilised in 0.1% Triton X-100 in PBS for 3 minutes, washed in PBS and blocked in 2% normal goat and normal horse serum for an hour at room temperature. Appropriate dilutions of primary antibodies were made as follows. Detection of rabbit polyclonal anti-PLZF (#sc-22839, Santa Cruz Biotechnology, CA, USA) was first optimised for IF using 4 different concentrations of the this antibody (stock 200\,\mu g/mL) in 0.25%BSA in PBS (4\,\mu g/mL, 1\,\mu g/mL, 0.4\,\mu g/mL and 0.2\,\mu g/mL). The best signal for PLZF detection was observed using 1:500 dilution (0.4\,\mu g/mL) which was
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Further used for all IF staining procedures. Mouse monoclonal anti-SC-35 antibody against nuclear speckles (#11826, Abcam, UK) was used at 1µg/mL. Appropriate matching concentrations of both rabbit (#3900, Cell Signalling, USA) and mouse (#0931, DAKO, VIC, Australia) normal IgG, as negative controls were also prepared in 0.25%BSA in PBS and cells were incubated with primary antibodies overnight at 4°C. Slides were washed twice in PBS and incubated with secondary antibodies: 1:100 FITC-conjugated horse anti-mouse IgG (Santa Cruz Biotechnology) and 1:200 Alexa-Fluor® 594-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) for 1 hour at room temperature. Cell nuclei were then stained incubating the samples for 10 minutes with 4′-6-diamidino-2-phenylindole (DAPI: Santa Cruz Biotechnology) and slides were cover-slipped using fluorescence anti-fade mounting medium (DAKO, VIC, Australia). Immunoreactivity was analysed using confocal fluorescence microscopy (Zeiss LSM880), 63X oil lens and centred Z-stack with optimal interval between each slice (approximately 13 slices). Image analysis was performed using Image J and Volocity software.

2.4.7 Immunohistochemistry

The three layer immunoperoxidase staining method was used. 3µm paraffin embedded sections of human airway were dewaxed by immersing in histolene (2x5 minutes), rehydrated through graded ethanol (2x100%, 1x70% each 5 minutes) and washed in tap water (2x5 minutes) at room temperature. Antigen retrieval was performed using citric acid and microwaving sections for 3 minutes. Sections were then dehydrated through grade ethanol (70%, 2x100%) and endogenous peroxidase activity was blocked with 0.3% H₂O₂ in methanol for 10 minutes, followed by rehydration in ethanol and blocking of non-specific sites for 20 minutes using 1.5% normal goat serum. Sections were incubated overnight with rabbit polyclonal 2µg/mL anti-PLZF (#sc-22839, Santa Cruz Biotechnology, CA, USA). Appropriate matched concentration of normal rabbit IgG (#3900, Cell signalling, USA) was used as the negative control. Recommended 1:100 dilution was used for rabbit monoclonal pan-actin antibody (#8456, Cell signalling, USA) as a positive control. On the next day, after washing, sections were incubated for 30 minutes with Vectastain® biotinylated goat anti-rabbit secondary antibody (1:200 of secondary in 1.5% normal
General Methods

goat serum in PBS) (Vector laboratories, USA). Sections were further incubated for 30 minutes with ABC reagent (Vectastain®, Vector laboratories, USA) by mixing equal volumes of both A (avidin) and B (biotinylated enzyme) in PBS and subsequent specific staining was visualized using stable peroxidase substrate buffer DAB (DAKO, CA, USA). Sections were the counterstained with Mayer’s haematoxylin, differentiated in 1% acid alcohol and coverslipped with DePeX® (Sigma, USA). Images were taken using Zeiss Axioscop (Germany).

2.5 Statistical analyses

All data were statistically analysed using GraphPad Prism 5.0 (Graphpad, San Diego, CA) and presented as the mean ± standard error of mean (SEM) for n individual experiments in BEAS-2B cell line, n individual donors for ALI-cultures of primary HBECs and n number of animals per group. Statistical test mostly used for data analysis was two-way analyses of variance (ANOVA) with Bonferroni post-hoc tests. Otherwise, one-way analysis of variance (ANOVA) with Dunnett’s post-hoc tests or two tailed t-test were used when appropriate. P <0.05 was considered to be statistically significant.
CHAPTER 3:
CORTISOL LIMITS ACTIONS OF SYNTHETIC GLUCOCORTICOIDS IN THE HUMAN BRONCHIAL EPITHELIUM
3.1 Introduction

The physiological glucocorticoid (GC), cortisol is essential for normal growth and differentiation of the airway epithelium (Fulcher et al., 2005). The airway epithelial cell layer regulates the interface with the environment, including maintaining microbial defence, and has well-recognised immune-modulatory and barrier functions (Lambrecht and Hammad, 2012). However, changes in epithelial differentiation and functions can significantly contribute to inflammation in chronic respiratory diseases, including asthma (Erle and Sheppard, 2014).

Psychological and physiological stresses activate the hypothalamic–pituitary–adrenal (HPA)-axis, increasing levels of cortisol (Oelkers 1996, Carl Coeck et al., 1991, Kudielka et al., 2004). Furthermore, pathogen-induced stresses, such as viral and bacterial infections elevate cortisol levels. These effects occur through activation of the immune and structural cells. Subsequent release of pro-inflammatory cytokines can directly stimulate the HPA-axis (Rhen and Cidlowski, 2005). Stress also increases the levels of adrenaline, which is generally regarded to synergise with cortisol providing multiple beneficial effects in inflammation and bronchoconstriction (Chen and Miller, 2007). However, stress is an established trigger of asthma exacerbations, and may also play a role in asthma development in childhood (Kozyrskyj et al., 2008). Although several studies have investigated normal and stress-induced cortisol levels in asthmatics and non-asthmatics (Laube et al., 2002, Landstra et al., 2002, Kapoor et al., 2003, Vink et al., 2012, Sutherland et al., 2003), they delivered conflicting findings, leaving the role of cortisol in asthma and asthma development uncertain.

Synthetic GCs, including inhaled corticosteroids (ICS) exert profound influences on the airway epithelium (Prosperini et al., 2002, Heino et al., 1988, Lundgren et al., 1988). Glucocorticoid anti-inflammatory effects are mediated by transactivation of selected genes, such as glucocorticoid inducible leucine zipper (GILZ) and MAP kinase phosphatase 1 (MKP-1) (Keenan et al., 2015b), and by inhibition of synthesis and release of pro-inflammatory cytokines, including granulocyte macrophage colony-stimulating factor (GM-CSF), IL-6 and IL-8 (Stellato, 2007). Glucocorticoid transactivation of GILZ inhibits NF-kB transcriptional activity,
Cortisol Limits Actions of Synthetic GCs in the Human Airway Epithelium

suppressing signalling pathways which trigger the release of chemokines (Eddleston et al., 2007). The induction of MKP-1 was found to inhibit the production of inflammatory cytokines IL-6, IL-8 and COX-2 by airway epithelial cells (Turpeinen et al., 2010). MKP-1 activity is also recognised as an important contributor to the restoration of the airway epithelium integrity during wound healing processes (White et al., 2005).

Synthetic GCs are potent inducers of apoptosis in many cell types and tissues (Gruver-Yates and Cidlowski, 2013). Transactivation of cyclin-dependent kinase inhibitor (CDKN1C) and promyelocytic leukaemia zinc finger (PLZF) has been shown to mediate anti-proliferative and apoptotic actions of GCs in different cell types (Wasim et al., 2012, Samuelsson et al., 1999). However, whilst some studies have reported GC-induced apoptosis in cultured human airway epithelial cells (Dorscheid et al., 2006), others suggest a pro-survival effect (Wen et al., 1997, Pelaia et al., 2003), leaving the effect of GCs on epithelial cell survival and apoptosis less clear.

Despite their proven efficacy in the control of chronic inflammatory diseases, GC insensitivity in some patients with severe disease remains a challenging clinical problem. Several molecular mechanisms of steroid-insensitivity have been elucidated in airway structural and immune cells (Keenan et al., 2015b), although mechanisms may vary depending on the context and cell phenotype under consideration. There are no specific GC-sensitising strategies yet available, even though combination therapy, using ICS and long-acting β2-adrenoceptor agonists in asthma and COPD could be considered to meet some of the criteria for such an approach, given the evidence for their synergy (Pauwels et al., 1997).

The majority of the GC effects are mediated through their binding of glucocorticoid receptor (GR), which is expressed in the airway epithelial cells (LeVan et al., 1997). A recent pharmacodynamic investigation of GR-mediated gene transactivation in the human bronchial epithelial cell line BEAS-2B, revealed a lower maximum response to hydrocortisone (cortisol) compared to several other synthetic GCs, suggesting a potential partial agonist activity of cortisol at GR in the bronchial epithelium (Joshi et al., 2015). Considering the well-established potential of partial agonists to behave as antagonists in the presence of a full agonist (See section 1.3.6),
Cortisol Limits Actions of Synthetic GCs in the Human Airway Epithelium

we postulated that endogenous corticosteroids may limit the actions of synthetic GCs therapeutically used in chronic inflammatory diseases, including asthma.

3.1.1 Outline and aims of the chapter

In this chapter, we will investigate the effect of cortisol (hydrocortisone) on GR-mediated transactivation of genes and inhibition of the release of pro-inflammatory cytokines by inhaled corticosteroid fluticasone propionate in the airway epithelium, using an immortalised bronchial epithelial cell line BEAS-2B. Furthermore, the effect of cortisol on transactivation of genes by different synthetic glucocorticoids will be examined in the air-liquid interface (ALI) organotypic culture of primary human bronchial epithelial cells, which is widely considered to recapitulate the characteristics of the differentiated human airway epithelium.
3.2 Methods

3.2.1 Cell culture and treatments

3.2.1.1 BEAS-2B cells

BEAS-2B were seeded at 25,000 cells/cm² in complete LHC-9 medium, supplemented as described in Section 2.1.1.1. Cells were growth-arrested in FCS- and steroid-free DMEM medium (Life Technologies, NY, USA), supplemented as described in Section 2.1.1.2, 24h prior to drug treatments. Cells were then exposed for 4h to glucocorticoid (GR) or mineralocorticoid receptor (MR) agonists (hydrocortisone (HC) (1nM-10µM), fluticasone propionate (FP) (1pM-100nM) or aldosterone (ALD) (10-100nM) (Sigma-Aldrich, Castle Hill, Australia)) alone, or 30 min after addition of vehicle or one of the antagonists, mifepristone (RU486) (1µM) or spironolactone (SPL) (1µM) (Sigma-Aldrich, Castle Hill, Australia). In subsequent experiments, BEAS-2B cells were treated with HC, 30 min prior to the incubation with FP for 4h and 24h for gene and protein analysis, respectively.

3.2.1.2 Air-liquid interface culture of primary human bronchial epithelial cells (pHBECs)

Primary human bronchial epithelial cells (HBECs) from healthy, asthmatic and COPD donors (Section 2.1.2, Table 2-1) were cultured using BEGM Bulletkit™ (Lonza) and differentiated into air-liquid interface cultures for 4 weeks on collagen coated Corning® Transwell® inserts (Sigma-aldrich, MO, USA) as described in Section 2.2.2. Following visualisation of beating cilia and mucus production, and measurement of transepithelial electrical resistance (TEER), cells were kept in normal ALI-growth medium (containing approximately 1.4µM HC), or exposed to low-HC medium (0.1µM HC) 24h prior to the treatment with fluticasone propionate (10nM) or dexamethasone (100nM) for 4h, at which time total RNA was extracted and expression of GC-inducible gene was measured by RT-qPCR. Data for the gene expression changes in the low hydrocortisone medium and normal ALI-growth medium.
were pooled from differentiated ALI cultures from individual healthy, asthmatic and COPD donors.

### 3.2.2 Determination of gene expression in BEAS-2B cells and ALI culture of primary HBECs

Following drug treatments, total RNA from BEAS-2B cells was extracted using TRIzol® reagent (Invitrogen, Doncaster, VIC, Australia) and extraction protocol as described in Section 2.4.1.1. Extraction of total RNA from ALI cultures of primary HBECs was performed using Illustra™ RNAspin Mini RNA Isolation Kit (GE Healthcare, UK) according to manufacturer’s instructions (Section 2.4.1.1). cDNA was formed from total RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, VIC, Australia) with a total reaction volume of 5μL (Section 2.4.1.2). The expression of glucocorticoid-inducible genes (GILZ, MKP-1, CDKN1C, PLZF, ENaCα, FKBP5, KCNB1) was measured by RT-qPCR (Section 2.4.1.3).

### 3.2.3 Determination of protein expression in BEAS-2B cells by Western blot

Following drug treatments, BEAS-2B cells were lysed in lysis buffer (1% TritonX-100, 1 mM EDTA, 100 mM NaCl; 10 mM Tris–HCL (pH 7.4)), containing phosphatase and protease inhibitor cocktail (Sigma-Aldrich, Castle Hill, Australia) (Section 2.4.2.1). Protein concentration was determined by Bradford assay (Section 2.4.2.2), and the expression of PLZF and ENaCα was determined by Western blotting using a PLZF and ENaCα specific antibodies (Section 2.4.2.3). Densitometry was performed using ImageJ (v1.44, National Institute of Health, Bethesda, MD, USA).

### 3.2.4 Detection of IL-6, IL-8 and GM-CSF in supernatants by ELISA

Following steroid treatment, BEAS-2B cells were stimulated with pro-inflammatory cytokine, tumor necrosis factor (TNFα) (10ng/mL) (R&D systems, Victoria, Australia) for 24h, at which time at which time supernatants were collected (Section 2.4.4.1) and assayed for IL-8, IL-6 and GM-CSF cytokine levels, using commercial sandwich ELISA kits, according to
manufacturers’ instructions (Section 2.4.4.2). The selected concentrations of TNFα and durations of exposure have been validated in a number of previous studies from our laboratory (Keenan et al., 2014, Keenan et al., 2015a).
3.3 Results

3.3.1 Comparison of the effects of hydrocortisone and fluticasone propionate in GR-mediated gene induction in BEAS-2B cells

In BEAS-2B cells, both HC and FP induced promyelocytic leukaemia zinc finger (PLZF), MAP-kinase phosphatase (MKP-1), cyclin-dependent kinase inhibitor 1 (CDKN1C) and epithelial sodium channel subunit α (ENaCα) mRNA levels in a concentration-dependent manner, at 4h (Figure 3.1). However, there was a lower maximum response and lower potency for HC compared to FP across all gene expression measurements. The effects of HC (1µM) and FP (10nM) were both strongly inhibited by the GR antagonist RU486 (mifepristone) (1µM), indicating that gene-induction is mediated by GR (Figure 3.2).

In order to ascertain whether the HC maximum response may be confounded by the ability of HC to bind both GR and mineralocorticoid receptors (MR) in the bronchial epithelial cells, we treated BEAS-2B cells with MR antagonist, spironolactone (1µM), 30 min prior to HC or the MR agonist aldosterone (10 or 100nM), to examine the expression of GC-inducible genes. Consistent with the previous experiment, HC induced PLZF, MKP-1, CDKN1C and ENaCα mRNA levels. However, the induction of these genes was neither activated by aldosterone nor modulated by spironolactone (Figure 3.3 A, B, C, D). In this set of experiments, the expression of glucocorticoid-induced leucine zipper (GILZ) and FK506 binding protein 5 (FKBP5) was additionally analysed. Whilst HC highly induced GILZ mRNA, the expression of this gene was neither induced by aldosterone nor modulated by spironolactone (Figure 3.3 E). On the other hand, a 4-fold increase was observed in the expression of FKBP5 levels following the treatment with aldosterone (100nM), and this induction was markedly inhibited by spironolactone (Figure 3.3 F).
Figure 3.1 The effect of hydrocortisone (HC) and fluticasone propionate (FP) on gene expression in BEAS-2B cells. The effects of the physiological GC, HC (cortisol) and the inhaled corticosteroid, FP on PLZF (A), MKP-1 (B), CDKN1C (C) and ENaCα (D) mRNA expression levels. Cells were serum-starved in the steroid-free medium 24h prior to the treatment with either vehicle control (0.1% DMSO) or with the range of concentrations of HC or FP for 4h, after which total RNA was extracted and analysed by RT-qPCR. Results are expressed as a % of gene expression levels induced by 10nM or 100nM FP. Data are presented as means and SEM for n=4-5 independent experiments.
Figure 3.2 The effect of the glucocorticoid receptor (GR) antagonist RU486 (mifepristone) on the induction of gene expression by hydrocortisone (HC) and fluticasone propionate (FP) in BEAS-2B cells. Cells were incubated with RU486 (1µM) for 30 min prior to addition of HC (1µM) or FP (10nM) for 4h, after which total RNA was extracted and gene expression of PLZF (A), MKP-1 (B), CDKN1C (C), and ENaCα (D) was measured by RT-qPCR. Results are expressed as fold change from control. Data are presented as means and SEM for n=4 independent experiments; two-way ANOVA with repeated measures, Bonferroni post-hoc test, **P<0.01, ***P<0.001, ns=non-significant.
Figure 3.3 The effect of hydrocortisone (HC) and aldosterone (ALD) on gene expression in the presence and absence of MR antagonist spironolactone (SPL) in BEAS-2B at 4h. Cells were starved in the steroid-free medium for 24h and incubated with SPL (1µM) for 30 minutes prior to addition of HC (1µM) or ALD (10/100nM) for 4h, at which time total RNA was extracted and gene expression of PLZF (A), MKP-1 (B), CDKN1C (C), ENaCα (D), GILZ (E), FKBP5 (F) was measured.
by RT-qPCR. Results are expressed as fold change from control and displayed as a log scale for ease of comparison of baseline and HC/ALD-induced changes in the presence and absence of SPL. Data are presented as means and SEM for n=4 independent experiments; two-way ANOVA with repeated measures, Bonferroni post-hoc test, *P<0.05, ns=non-significant.

3.3.2 The effect of hydrocortisone on the gene transactivation by fluticasone propionate in BEAS-2B cells

Pre-treatment of BEAS-2B cells with HC (1µM) impaired the maximum induction of certain genes by FP. This effect was most pronounced in the PLZF gene expression levels, although similar patterns of results were observed in GILZ and MKP-1 gene expression, as the addition of HC constrained the maximum response to FP. Furthermore, there was a lack of concentration-response relationship to FP in the presence of HC (Figure 3.4 A, B, E). This effect of HC was not observed in the expression of some other GC-inducible genes, including CDKN1C, ENaCα and K+ voltage-gated channel subfamily B M1 (KCNB1) (Figure 3.4 C, D, F), suggesting a differential effect of HC on FP-induction of different genes in the bronchial epithelium.

To further ascertained whether the limitation of maximum FP-induction of PLZF gene expression was simply related to a common effect of concurrent exposure to a high concentration of GC, we incubated BEAS-2B cells with the potent GR agonist Dex (0.01-1µM) 30 minutes prior to the addition of FP. Whilst FP alone induced PLZF expression, as in the previous experiment, none of the three Dex concentrations had reduced the maximum FP-induction of PLZF gene expression. Thus, the inhibitory effect of cortisol does not appear to be an effect common to high concentrations of other GCs (Figure 3.5).

We performed western blot analysis to ascertain whether PLZF protein induction by FP was similarly restricted by HC pre-treatment, since the gene encoding PLZF was the most affected of the genes analysed. Indeed, HC (10µM) inhibited maximum PLZF protein induction by FP (Figure 3.6 A, B). Membranes were additionally probed for the expression of ENaCα, and consistent with the gene expression, hydrocortisone did not affect the induction of ENaCα protein levels by fluticasone propionate (Figure 3.6C).
Figure 3.4 The effect of fluticasone propionate (FP) on the expression of glucocorticoid-inducible genes in the presence and absence of hydrocortisone (HC) (1µM) in BEAS-2B cells at 4h. Following 24h starvation in the steroid-free medium, BEAS-2B cells were treated with either vehicle control (0.01% DMSO) or HC (1µM) 30 minutes prior to treatment with FP (0.01-10nM) for 4h incubation, after which total RNA was extracted and expression of glucocorticoid-inducible genes
PLZF (A), MKP-1 (B), CDKN1C (C), ENaCα (D), GILZ (E), KCNB1 (F) was measured by RT-qPCR. Results are expressed as a % of gene levels induced by 1nM FP. Data are presented as means and SEM for n=4 independent experiments; Given that the specific aim of this experiment was to determine whether the maximum induction of gene expression with FP (achieved with 1nM) will be compromised in the presence of HC, student’s paired t-test was performed to compare the mean difference between these two data sets (the response to 1nM FP in the presence and absence of 1µM HC); *P<0.05.
Figure 3.5 The effect of fluticasone propionate (FP) on the PLZF gene expression in the presence and absence of dexamethasone (Dex) ((A) 0.01µM; (B) 0.1µM; (C) 1µM) in BEAS-2B cells at 4h. Following 24h starvation in the steroid-free medium, BEAS-2B cells were treated with either vehicle control (0.01% DMSO) or Dex (0.01-1µM) 30 min prior to the treatment with FP (0.01-10nM) for 4h, after which total RNA was extracted and expression of promyelocytic leukaemia zinc finger (PLZF)
was measured by RT-qPCR. Results are expressed as a % of gene levels induced by 1nM FP. Data are presented as means and SEM for n=3 independent experiments. Student paired t-test was performed to compare the response to 1nM FP in the presence and absence of Dex.

Figure 3.6 The effect of fluticasone propionate (FP) on PLZF and ENaCα protein expression in the presence and absence of hydrocortisone (HC) (10µM) in BEAS-2B cells at 24h. (A) Representative Western Blot and (B) densitometry analysis for PLZF expression at 24h; (C) densitometry analysis for ENaCα protein expression. BEAS-2B cells were serum-starved in the steroid-free medium for 24h followed by treatment with either vehicle control (0.1% DMSO) or HC (10µM) 30 min before the treatment with FP (0.1-10nM) for 24h, after which protein lysates were collected and protein expression was analysed by Western Blot. β-tubulin was used as a loading control. Results are expressed as a % of the protein levels induced by 1nM FP. Data are presented as means and SEM for n=4 independent experiments; Student’s paired t-test was performed to compare the response to 1nM FP in the presence and absence of 10µM HC; **P<0.01.

3.3.3 The effect of hydrocortisone on FP inhibition of TNFα-induced cytokine release in BEAS-2B cells

In order to ascertain whether HC also limited FP inhibition of the production of pro-inflammatory cytokines in BEAS-2B cells, 30 min after HC (50nM or 1µM) addition, cells were
treated with FP (1 or 10nM), followed by the simulation of cytokine production with pro-inflammatory cytokine TNFα (10ng/mL) for 24h. As anticipated, TNFα significantly induced levels of IL-6, IL-8 and GM-CSF from BEAS-2B cells (Table 3-1). Although the elevated cytokine levels were significantly reduced by either HC or FP alone, HC (1µM) prior to FP treatment markedly reduced FP-inhibition of GM-CSF release. FP-inhibition of IL-6 and IL-8 release was not significantly affected by HC (Figure 3.7).

Table 3-1 Vehicle- and TNFα-induced levels of pro-inflammatory cytokines GM-CSF, IL-8 and IL-6 in BEAS-2B cells determined by ELISA. In 4 independent experiment, BEAS-2B cells were incubated with pro-inflammatory cytokine TNFα (10ng/mL) for 24h, at which time levels of GM-CSF, IL-8 and IL-6 were determined in the supernatants by commercial sandwich ELISA kits.

<table>
<thead>
<tr>
<th>Experiment replicate</th>
<th>Condition</th>
<th>GM-CSF (pg/mL)</th>
<th>IL-8 (pg/mL)</th>
<th>IL-6 (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Vehicle control</td>
<td>12.9</td>
<td>22.3</td>
<td>9.6</td>
</tr>
<tr>
<td>A</td>
<td>10ng/mL TNFα</td>
<td>30.5</td>
<td>1530</td>
<td>387</td>
</tr>
<tr>
<td>B</td>
<td>Vehicle control</td>
<td>undetectable</td>
<td>35.9</td>
<td>3.3</td>
</tr>
<tr>
<td>B</td>
<td>10ng/mL TNFα</td>
<td>55.8</td>
<td>1610</td>
<td>572</td>
</tr>
<tr>
<td>C</td>
<td>Vehicle control</td>
<td>15.5</td>
<td>36.2</td>
<td>5.1</td>
</tr>
<tr>
<td>C</td>
<td>10ng/mL TNFα</td>
<td>226</td>
<td>2830</td>
<td>302</td>
</tr>
<tr>
<td>D</td>
<td>Vehicle control</td>
<td>undetectable</td>
<td>23.2</td>
<td>undetectable</td>
</tr>
<tr>
<td>D</td>
<td>10ng/mL TNFα</td>
<td>293</td>
<td>1980</td>
<td>362</td>
</tr>
</tbody>
</table>
Table 3-2 TNFα-induced levels of pro-inflammatory cytokine GM-CSF (pg/mL) in the presence of HC (1µM) and FP (10nM) in BEAS-2B cells, determined by ELISA. BEAS-2B cells were incubated with HC for 30 minutes prior to FP treatment, following the stimulation with pro-inflammatory cytokine TNFα (10ng/mL) for 24h, at which time levels of GM-CSF were determined in the supernatants by commercial sandwich ELISA kits. Data are presented as means and SEM from n=4 independent experiments.

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>1µM HC</th>
<th>10nM FP</th>
<th>HC+FP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Levels</td>
<td>151±64</td>
<td>34±17</td>
<td>24±14</td>
<td>80±30</td>
</tr>
</tbody>
</table>
Figure 3.7 The effect of fluticasone propionate (FP) on TNFα-induced production of pro-inflammatory cytokines in the presence and absence of hydrocortisone (HC) (50nM or 1µM) in BEAS-2B cells at 24h. Cells were serum starved in the steroid-free medium for 24h prior to the steroid
treatment. At time 0h, cells were treated with either vehicle (0.01% DMSO) or HC (50nM or 1µM), followed by treatment with FP (1 or 10nM) for 30 min prior to the addition of pro-inflammatory cytokine TNFα (10ng/mL) for 24h, at which time supernatants were collected and analysed for pro-inflammatory cytokines GM-CSF (A), IL-8 (B) and IL-6 (C) levels by ELISA. Results are expressed as a % of the level of the cytokine induced by TNFα in the presence of vehicle. Data are presented as means and SEM for n=4 independent experiments; two-way ANOVA with repeated measures, Bonferroni post-hoc test, ***P<0.001, **P<0.01, *P<0.05.

3.3.4 The effect of hydrocortisone ALI-growth medium concentrations on GC-transactivation mechanisms in ALI-cultures of primary HBECs

As cortisol is essential for growth and differentiation of bronchial epithelial cells into ALI organotypic culture, we sought to ascertain whether standard concentrations of HC in ALI growth medium would inhibit gene transactivation by the synthetic GC-agonists, fluticasone propionate and dexamethasone.

Primary human bronchial epithelial cells from healthy, asthmatic and COPD donors were differentiated for 4 weeks by culture at an air-liquid interface (ALI) and cell-differentiation was confirmed by microscopic visualisation of beating cilia, mucus production and by measurement of TEER values (Figure 3.8). TEER values were measured between day 6 and day 27 of differentiation and substantial increase in TEERs in all ALI cultures was observed on day 13, with no further significant change up to day 27 (Figure 3.8 A). Although TEER values appear to have increased more in the ALI cultures of healthy donors (NHBE) than in the ALI cultures of asthmatic (ASTH) and COPD donors, this difference was not statistically significant (Figure 3.8 B).

Differentiated cells were maintained in the standard ALI-growth medium with 1.4µM HC, or exposed to low-HC medium (containing 0.1µM HC) for 24h, and subsequently treated with dexamethasone (Dex) (100nM) or FP (10nM) for 4h. In ALI medium containing the lower HC level, Dex or FP significantly induced mRNA expression of all measured genes. However, this gene induction was prevented in the standard growth medium containing the higher concentration of HC (Figure 3.9). Moreover, the absence of gene induction by FP or Dex in the presence of the higher HC concentration is not explained by HC (1.4µM) having induced maximum gene
expression levels, because gene expression in HC (1.4µM)/FP(10nM) or Dex (100nM) was significantly less than that in HC (0.1µM)/FP(10nM) or Dex (100nM) (Figure 3.9).

This result was observed across all ALI cultures, regardless of the disease-state, as shown in the comparable analysis of the effect of different concentration of HC in the medium on Dex-induction of PLZF and GILZ between individual healthy, asthmatic and COPD donors (Figure 3.10).

Figure 3.8 Transepithelial electrical resistance (TEER) values of primary HBECs cultured at air-liquid interface (ALI). Primary human bronchial epithelial cells from healthy (NHBE), asthmatic and COPD donors were seeded onto 0.4µm pore transwell membrane coated with type-I rat-tail collagen and cultured at air-liquid interface for up to 27 days. Transepithelial electrical resistance (TEER) values, which are a strong indicator of the integrity of the epithelial barrier function were obtained using an EVOM2 volt ohmometer. (A) TEERs for 4 different donors of NHBE between day 6 and day 27 of differentiation; (B) Comparison of TEERs between primary HBECs in ALI-cultures from healthy, asthmatic and COPD donors measured on day 6 and day 27 of differentiation; Data are presented as means and SEM for n=3-4 individual donors. One-Way ANOVA, Dunnett’s post hoc tests, ***p<0.001.
Cortisol Limits Actions of Synthetic GCs in the Human Airway Epithelium

**A**

**PLZF mRNA** ($2^{\Delta Ct} \times 10^5$)

- Vehicle
- 100nM Dex

**GILZ mRNA** ($2^{\Delta Ct} \times 10^5$)

- Vehicle
- 100nM Dex

**CDKN1C mRNA** ($2^{\Delta Ct} \times 10^5$)

- Vehicle
- 100nM Dex

**MKP-1 mRNA** ($2^{\Delta Ct} \times 10^5$)

- Vehicle
- 100nM Dex

---

**B**

**PLZF mRNA** ($2^{\Delta Ct} \times 10^5$)

- Vehicle
- 10nM FP

**GILZ mRNA** ($2^{\Delta Ct} \times 10^5$)

- Vehicle
- 10nM FP

**CDKN1C mRNA** ($2^{\Delta Ct} \times 10^5$)

- Vehicle
- 10nM FP

**M KP-1 mRNA** ($2^{\Delta Ct} \times 10^5$)

- Vehicle
- 10nM FP

Legend:
- Low-HC medium (0.1µM HC)
- ALI-growth medium (1.4µM HC)
Figure 3.9 The effect of different concentrations of HC in the growth media in ALI- cultures of primary HBECs on induction of genes by (A) dexamethasone (Dex) and (B) fluticasone propionate (FP). Cells were treated with either vehicle or Dex (100nM) (A) or FP (10nM) (B) for 4h in either low-HC medium (containing 0.1µM HC), or normal ALI- growth medium (1.4µM HC). Results are expressed as $2^{-\Delta\Delta Ct}\times10^5$ for PLZF, GILZ and MKP-1 mRNA and $2^{-\Delta\Delta Ct}\times10^7$ for CDKN1C mRNA and are displayed as a log scale for ease of comparison of baseline and glucocorticoid induced changes under different growth conditions. Data are presented as means and SEM for n=4-9 individual donors; two-way ANOVA with repeated measures, Bonferroni post-hoc test, ***P<0.001, **P<0.01, *P<0.05.
Figure 3.10 The effect of different concentrations of HC in the growth media in the ALI- cultures of primary HBECs from healthy, asthmatic and COPD donors on the induction of PLZF and GILZ by dexamethasone (Dex). Primary human bronchial epithelial cells from healthy (NHBE), asthmatic and COPD donors were seeded onto 0.4µm pore transwell membrane coated with type-I rat-tail collagen and cultured at air-liquid interface for up to 27 days. Cells were treated with either vehicle control or Dex (100nM) for 4h in either low-HC medium (containing 0.1µM HC), or ALI- growth medium (1.4µM HC). Result is expressed as $2^{-\Delta Ct}$x10^5. Open symbols represent vehicle and filled symbols represent Dex-treated cells from individual healthy (blue circle), asthmatic (red square) and COPD donors (green triangle).
3.4 Discussion

Alterations in the airway epithelial differentiation and function can significantly contribute
to inflammation in chronic respiratory diseases, including asthma. Synthetic glucocorticoids,
including the inhaled corticosteroids (ICS), exert anti-inflammatory effects in the airway
epithelium by GR-mediated transactivation of genes and by inhibition of release of pro-
inflammatory cytokines (Keenan et al., 2015b). Despite their proven efficacy in the control of
chronic inflammatory diseases, glucocorticoid insensitivity in some patients with severe disease
remains a challenging clinical problem. Here we present the evidence that cortisol, the
physiological glucocorticoid in humans, acts like a partial agonist at GR in the airway epithelium,
consequently limiting selected beneficial actions of synthetic glucocorticoids.

3.4.1 Cortisol has a lower maximum response than fluticasone propionate in activating
GR-dependent gene transcription in the bronchial epithelium

Conventional molecular GC actions occur through binding of the glucocorticoid receptor,
GR, which is expressed in airway epithelial cells (LeVan et al., 1997). Although GR-dependent
transcriptional activity is complex and incompletely characterized, transactivation of target genes
by glucocorticoids is one of the most extensively investigated and better understood mechanisms.
Upon ligand binding, GR as a homodimer translocates to the nucleus and binds to glucocorticoid
response elements (GREs) on DNA, further promoting activation of gene transcription (Smoak
and Cidlowski, 2004). Structural modifications of different synthetic glucocorticoid molecules
significantly modulate their affinity for GR (Daley-Yates, 2015), which influences their potency
for transcription of target genes. A recent pharmacodynamic investigation of GR-mediated gene
transactivation in the human bronchial epithelial cell line BEAS-2B revealed a lower maximum
response of a transfected reporter construct to cortisol (hydrocortisone) compared to several other
synthetic glucocorticoids (Joshi et al., 2015), raising the question of whether endogenous cortisol
could antagonise synthetic anti-inflammatory GCs.
We compared the potencies and the maximal transactivational effects of the clinically used ICS fluticasone propionate (FP) with cortisol (HC) in the BEAS-2B cell line. Both HC and FP robustly induced PLZF, MKP-1 and CDKN1C mRNA in a concentration-dependent manner. Transactivation of these genes is considered important in facilitating glucocorticoid anti-inflammatory and anti-proliferative actions in various types of cells (Rider et al., 2015, Fahnenstich et al., 2003, Stellato, 2007). We observed distinct lower maxima to HC compared to FP in inducing these targets. The lower HC maxima could have been due to HC additionally binding mineralocorticoid receptors (MR) (Menshanov et al., 2013), a property not shared by FP (Harding, 1990), or other ICS. The mineralocorticoid aldosterone and the glucocorticoid, cortisol bind MR with high affinity, regulating salt and fluid transport in the epithelium of the kidney and the colon (Funder, 2005). MR are expressed in BEAS-2B cells (Viengchareun et al., 2007), establishing the potential of HC agonist actions at MR to confound the GR effect. However, as the MR agonist aldosterone did not induce HC-responsive genes and the MR antagonist spironolactone did not modulate HC actions, we excluded any potential HC actions at MR as an explanation for the reduced HC maxima.

The regulation of the fluid transport by glucocorticoids and aldosterone was shown to require direct stimulation of the expression of specific ion transporters, including the epithelial sodium channel (ENaC) (Rossier et al., 2002, Sayegh et al., 1999). We investigated the mRNA expression of α subunit of the epithelial sodium channel (ENaCα) in BEAS-2B cells. Although aldosterone stimulates the expression of ENaCα in renal epithelial cells (Masilamani et al., 1999), no such regulation by aldosterone was evident in BEAS-2B cells. Moreover, ENaCα induction by HC was completely inhibited by GR antagonist, RU486, but unaffected by MR antagonist spironolactone, confirming that this effect required GR binding in the airway epithelial cells.

The induction of FK506 binding protein 5 (FKBP5) gene expression by aldosterone was significantly inhibited by MR antagonist spironolactone. This result confirmed previous findings that FKBP5 is not only induced by glucocorticoid-GR pathways, but also by mineralocorticoids through their binding of MR (Murck et al., 2014, Hubler and Scammell, 2004). FKBP5 is a
member of the immunophilin protein family that acts as a co-chaperone to maintain GR activity (Zannas et al., 2016).

3.4.2 Cortisol impairs transactivation of selected genes by fluticasone propionate in the bronchial epithelium

Low efficacy agonists can bind and activate target receptors to produce lower levels of agonist response that may manifest as reduced maximal responses. These agonists are typically referred to as partial agonists. One predictable characteristic of a partial agonist is competition with a full agonist for the same receptor. In this circumstance the partial agonist is able to limit the effect of the full agonist over a range of concentrations of each ligand (Jackson, 2010) (Section 1.3.6, Figure 1.6).

In our experiments, HC impaired the maximal induction of PLZF, GILZ and MKP-1 mRNA by FP, but not of other genes, such as CDKN1C, ENaCα and KCNB1, implying perhaps, that there are different receptor reserves for these genes (Joshi et al., 2015). Furthermore, for a given GRE-regulated gene, the lower the efficacy of HC, the greater was the extent of antagonism of the full agonist, FP. The implication of this finding is that HC-mediated reductions in the maximum effect of ICS may interfere with some of the beneficial ICS actions in the airway epithelium. Through limitation of ICS-induction of GILZ and MKP-1, cortisol may interfere with the ICS-inhibition of the signalling pathways leading to the release of pro-inflammatory cytokines and chemokines in the airway epithelium (Eddleston et al., 2007, Turpeinen et al., 2010). Glucocorticoid transactivation of MKP-1 was also shown to inhibit mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK)-signalling pathways (Kassel et al., 2001), which may block the airway epithelial cell migration (White et al., 2005). Thus, cortisol may also modulate glucocorticoid effects on the epithelial restitution following epithelial injury in inflammation and infection.

Cortisol appeared to have the greatest inhibitory effect on FP-induction of PLZF. Whilst this transcriptional repressor is predominantly involved in cell cycle and differentiation processes in different types of cells (Suliman et al., 2012), the potential role(s) of PLZF in the airway
epithelium are not well understood. PLZF has been reported to mediate GC anti-proliferative and apoptotic actions in lymphocytes (Wasim et al., 2012) and to modulate inflammatory responses to pathogens through repression of NF-κB pathways in bone marrow-derived macrophages (Sadler et al., 2015). Limiting induction of PLZF by ICS may therefore impair their anti-inflammatory effects, particularly those mediated through repression of NF-κB-signalling pathways in the airway epithelium.

3.4.3 Cortisol limits fluticasone propionate inhibition of GM-CSF release in the bronchial epithelium

In response to various signals from inflammatory cells, such as lung macrophages, and to activation by pathogens and their associated molecular pattern products (Xia et al., 2017), the airway epithelium can release GM-CSF, IL-6, IL-8 and other cytokines that initiate and/or amplify inflammation through recruitment of eosinophils and neutrophils (Lambrecht and Hammad, 2012, Stellato, 2007, Trevor and Deshane, 2014). ICS can notably repress transcription of genes encoding these pro-inflammatory cytokines, inhibiting their synthesis and release by the airway epithelium (Korn et al., 2001, Levine et al., 1993). Glucocorticoid-activated GRα can directly interact with and increase the export of p65 subunit of the transcription factor NF-κB from the nucleus (Nelson et al., 2003), subsequently inhibiting signalling pathways which lead to both transcription and production of IL-6 and IL-8 (Lawrence, 2009). Similarly, glucocorticoids have been shown to inhibit the production of GM-CSF in the airway epithelium through pathways involving CREB binding protein (CBP)-mediated DNA-methylation and inhibition of NF-kB transcriptional activity (Kagoshima et al., 2001).

GM-CSF is a colony-stimulating factor and inflammogen that regulates the accumulation and activity of neutrophils at the inflammatory site (Fossati et al., 1998), additionally promoting their survival (Cox et al., 1992). Asthmatic airway epithelium expresses higher levels of GM-CSF protein than non-diseased airway epithelium (Sousa et al., 1993). Furthermore, IL-17 and TNFα-induced GM-CSF release from human bronchial epithelial cells in vitro promotes neutrophil survival (Laan et al., 2003). The Th17-associated asthma phenotype is considered to be
neutrophil-predominant and steroid-resistant (Chambers et al., 2015). In our study, inhibition by FP of the TNFα-induced GM-CSF release was compromised by HC. Moreover, in the presence of HC, GM-CSF levels were maintained in the concentration range known to promote neutrophil numbers and activity (Table 3-2) (Laan et al., 2003). Thus, cortisol may contribute to steroid insensitivity and disease severity in asthmatic patients.

3.4.4 Cortisol limits transactivation of glucocorticoid-inducible genes in the air-liquid interface (ALI)-culture of primary HBECs

3.4.4.1 Air-liquid interface (ALI)-culture of primary HBECs: a better physiological in vitro model of the bronchial epithelium

Although the BEAS-2B cell line originated from a normal human bronchial epithelium and is responsive to inflammatory stimuli, it is adenovirus-transformed and immortalized and fails to replicate many of the characteristics of cell differentiation, such as mucus production and development of beating cilia in vitro (Stewart et al., 2012). More importantly, perhaps, as it is derived from one human donor, it does not give insight into the natural biological variation observed between individuals. It has also been found that BEAS-2B cells have significantly lower levels of 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2), enzyme that inactivates active forms of glucocorticoids, suggesting that their metabolism may be less influenced than in primary HBECs (Feinstein and Schleimer, 1999). Therefore, in order to assess a better physiological model exhibiting a differentiated phenotype with goblet and ciliated epithelial cells, we cultured primary HBECs at air-liquid interface (ALI) for 4 weeks to form an organotypic culture.

Typical criteria that fully differentiated ALI-cultures have to meet are: 1) microscopic visualisation of beating cilia; 2) mucus production; and 3) TEER values within the range of 700-1200 Ωcm² (Pezzulo et al., 2011). Transepithelial electrical resistance is a quantitative measurement of epithelial barrier integrity and it is most commonly used parameter to evaluate the functionality of the air-liquid interface cultures (Srinivasan et al., 2015). In our study, TEER values were measured between day 6 and day 27 of differentiation and substantial increase in TEERs across all cultures was observed on day 13, with no further significant change up to day
27. Interestingly, TEER values appeared to have increased more in the cultures of healthy donors (NHBE) compared with cultures of asthmatic and COPD donors. Although some studies have found decreased TEERs and defective tight junctions in the cells from asthmatic donors (Xiao et al., 2011), this difference in our experiments was not statistically significant.

3.4.4.2 *Cortisol and glucocorticoid-responsiveness in ALI-cultures of primary HBECs*

As cortisol is essential for growth and differentiation of the primary human bronchial epithelial cells (Fulcher et al., 2005), we used medium containing 1.4µM HC to generate and maintain the ALI organotypic cultures. Differentiated cells were maintained in the standard ALI-growth medium, or exposed to low-HC medium (containing 0.14µM HC) for 24h, and subsequently treated with synthetic glucocorticoids, Dex and FP. Induction of genes by both Dex and FP was remarkably constrained in ALI cultures maintained in the growth medium with 1.4µM HC compared to those exposed to low-HC (0.14 µM) medium for 24h, strongly supporting the relevance of the partial agonist activity of cortisol to human bronchial epithelium *in situ.*

3.4.4.3 *Combining the data from ALI-cultures of healthy, asthmatic and COPD donors*

Primary human bronchial epithelial cells (HBECs) from different donors generally exhibit high variability in their response to exogenous stimuli and phenotypic characteristics, particularly if the cultures originate from healthy donors and patients diagnosed with pulmonary disease, such as asthma and COPD (Hackett et al., 2011b, Leclercq et al., 2016). Since several donors used in this study were diagnosed with either asthma or COPD, it may be argued that it is invalid to group results from these donors. Primary HBECs from all donors, however, consistently showed glucocorticoid-responsiveness across all gene expressions measured in the low-HC medium, as shown in the comparable analysis of the effect of HC on Dex-induction of PLZF and GILZ between individual healthy, asthmatic and COPD donors. More importantly, there was no significant difference in the magnitude of induction between different disease states, suggesting cortisol effect was not disease-specific.
3.4.5 Clinical relevance of cortisol concentrations used in this study

Physiological total plasma cortisol levels show circadian rhythm ranging from a trough of ~50nM to peaks of ~0.5µM in healthy and asthmatic individuals (Landstra et al., 2002), and reach 1-2µM in acute stress (Jung et al., 2014). We selected the concentrations of hydrocortisone (0.1µM - 10µM) that are within the ranges encountered physiologically. The highest hydrocortisone concentration appeared to produce maximum transactivation of the genes encoding PLZF, MKP-1 and CDKN1C. Bioavailable cortisol levels however, may differ from the circulating cortisol levels in plasma due to influences such as the activity of the enzymes, 11β-HSD type 1 and 2, which control the conversion of bioactive cortisol to and from its inactive metabolites (Seckl, 2004). The expression of type 2 11β-HSD, which is principally accountable for inactivation of cortisol and synthetic glucocorticoids, has been demonstrated in the airway epithelium of both asthmatics and non-asthmatics (Orsida et al., 2002). Cortisol levels in plasma are also controlled by the corticosteroid-binding globulin (CBG). CBG binds 90% of total cortisol in plasma and limits its delivery to tissues and cells (Perogamvros et al., 2012). The CBG concentration in the extracellular fluid is lower than in plasma (Holliday, 1999). Therefore, as the precise range of cortisol concentrations at the receptors in tissues is not known, we consider it is justified to examine a range that includes those reported in health and under stressed conditions. Acute stress activates the HPA-axis, doubling total plasma cortisol concentrations (Kudielka et al., 2004). Furthermore, pathogen-induced stresses, such as viral and bacterial infections induce the production of pro-inflammatory cytokines, including TNFα, IL-1 and interferons, that directly activate HPA-axis, leading to elevation of adrenocorticotropic hormone and increased cortisol release (Rhen and Cidlowski, 2005). Cortisol availability may be further increased during inflammation by the activity of neutrophil elastase and serine proteases, which cleave the CBG (Henley et al., 2016). The hydrocortisone concentration range used in this study therefore spans the physiological range. Acute stress also leads to substantial increases in circulating adrenaline levels (McEwen, 1998). Adrenaline has been shown to contribute to goblet cell metaplasia though activation of β2-adrenoceptors in a murine model of asthma (Thanawala et al., 2013). Since
adrenaline and cortisol are elevated simultaneously in vivo, ascertaining their combined influences on ICS-sensitivity will be of interest in future studies.

3.4.6 The hypothesis of the “heterodimer” formation

The limiting effect of cortisol on the gene transactivation by synthetic glucocorticoids may not be completely explained by the classical partial agonist activity of cortisol and the glucocorticoid receptor occupancy. Upon ligand binding, GR monomers form GR-GR homodimers which translocate to the nucleus and bind to target DNA, activating transcription of genes (Smoak and Cidlowski, 2004). The ability of GR to form both homo- and heterodimers prior to their binding to the glucocorticoid responsive elements on target DNA may also influence glucocorticoid signalling through GR. For example, heterodimers formed from different GR isoforms, GRα-GRβ are known to have limited ability to transactivate genes compared to GRα homodimers (de Castro et al., 1996). However, the protein expression of GRβ isoform is not detectable in human bronchial epithelial cells (Pujols et al., 2002). Similarly, the GR-MR heterodimers are also shown to have different properties in activating GR-signalling pathways compared with their respective homodimers (Trapp et al., 1994). Our findings prompt the question of whether binding of cortisol and synthetic GC to cytosolic GR monomers generates a “heterodimer” GR (GR\textsuperscript{(Synthetic GC)}-GR\textsuperscript{(Cortisol)}) that has a distinct signalling effect to GR homodimers bound uniquely to either synthetic GC (GR\textsuperscript{(Synthetic GC)}-GR\textsuperscript{(Synthetic GC)}) or endogenous cortisol (GR\textsuperscript{(Cortisol)}-GR\textsuperscript{(Cortisol)}) (Figure 3.11). “Hetero” here denotes the different shape and electronic properties of the GRα when bound to cortisol compared to the synthetic GC. Such “heterodimers” may have lower transactivation activity than the homodimers, with limited activation of gene transcription, even in the presence of higher concentrations of synthetic GCs.
Figure 3.11 The hypothesis of the unique GR\textsuperscript{(Synthetic GC)}-GR\textsuperscript{(Cortisol)} “heterodimer” formation. The limiting effect of cortisol on the gene transactivation by synthetic GCs may not be completely explained by the classical partial agonist activity of cortisol and the glucocorticoid receptor occupancy. The binding of cortisol and synthetic GC to cytosolic GR monomers may generate a “heterodimer” GR (GR\textsuperscript{(Synthetic GC)}-GR\textsuperscript{(Cortisol)}) that has a distinct signalling effect to GR homodimers bound uniquely to either synthetic GC (GR\textsuperscript{(Synthetic GC)}-GR\textsuperscript{(Synthetic GC)}) or endogenous cortisol (GR\textsuperscript{(Cortisol)}-GR\textsuperscript{(Cortisol)}). Such “heterodimers” may have lower transactivation activity than the homodimers, with limited activation of gene transcription, even in the presence of higher concentrations of synthetic GCs.

3.4.7 Conclusions from Chapter 3

The airway epithelium is a target for profound anti-inflammatory actions of glucocorticoids, which are mostly mediated through both GR-dependent gene transactivation and inhibition of the synthesis and/or release of pro-inflammatory cytokines. As glucocorticoid insensitivity remains a challenging clinical problem, a better understanding of the mechanisms and mediators leading to the limitation of their actions is needed. In this chapter, we investigated the effect of physiological glucocorticoid, cortisol on the actions of synthetic glucocorticoids using different
human epithelial in vitro models – immortalised bronchial cell line and the air-liquid interface culture of primary human bronchial epithelial cells. Our observations suggest that cortisol acts like a partial agonist at the glucocorticoid receptor in the airway epithelium, limiting synthetic glucocorticoid-induced changes in gene expressions. As some of the beneficial anti-inflammatory effects of ICS/oral glucocorticoids on the epithelium are mediated through these gene expression pathways, cortisol may compromise the efficacy of therapeutically useful glucocorticoids, particularly when its levels are elevated by stress.
CHAPTER 4:
THE EFFECTS OF
PHYSIOLOGICAL AND
SYNTHETIC GLUCOCORTICOIDS
IN THE AIRWAY EPITHELium IN
VIVO
4.1 Introduction

In the preceding chapter, using various in vitro models of the human airway epithelium, we presented the evidence demonstrating that cortisol acts like a partial agonist at the glucocorticoid receptor, limiting selected actions of synthetic, therapeutically used glucocorticoids in the human airway epithelium.

In general, in vitro cell models using primary cells or immortalized cell lines are invaluable tool for basic, discovery-driven research, as they provide important insights into drug actions and interactions on the cellular levels. However, the prediction of the glucocorticoid effects in the patients, based only on the in vitro studies may not be reliable, as there are many factors in situ known to control glucocorticoid bioavailability at their receptors in tissues (Oakley and Cidlowski, 2013). In order to establish whether epithelial responses in vivo are limited by the presence of physiological corticosteroids, further studies should be carried out using animal models.

4.1.1 The physiological glucocorticoids vs. clinically used synthetic glucocorticoids

The main physiological glucocorticoids (cortisol in humans, corticosterone in rodents) are secreted from zona fasciculata of the adrenal glands. Their secretion is primarily regulated by the hypothalamic-pituitary-adrenal (HPA) axis (Biddie et al., 2012). The basal levels of the physiological glucocorticoids, maintained by the negative feedback regulation, are known to modulate a wide range of physiological processes, contributing to the tissue development, metabolism and the immunomodulation of both innate and adaptive immunity (Rhen and Cidlowski, 2005).

Exogenous glucocorticoids are synthetic derivatives of cortisol, clinically used to treat the broad spectrum of inflammatory diseases, including asthma, rheumatoid arthritis and different skin disorders. The clinical efficacy of the synthetic glucocorticoids depends on their pharmacokinetic and their pharmacodynamic parameters. In particular, the biological half-life reflects the duration of the glucocorticoid influence in the target tissues (Czock et al., 2005).
Structural modifications in the molecules of systemically used glucocorticoids, including 1-dehydrogenation in the structure of prednisolone, 6α-methylation in methylprednisolone and fluorination in dexamethasone result in more potent anti-inflammatory actions with the lower mineralocorticoid activity. These modifications also confer different pharmacokinetic profiles (Buttgereit et al., 2005). Whilst cortisol (hydrocortisone) has a biological half-life of 8-12h, methylprednisolone and dexamethasone acquired longer biological half-lives of 18-36h and 36-54h, respectively (Becker, 2013). However, these systemically used glucocorticoids still caused considerable side-effects, including osteoporosis, growth suppression and metabolic disorders. Therefore, further improvement of the therapy was evident in the 1960s with the development of topical glucocorticoids. Their targeted delivery to the specific site of inflammation minimised systemic side-effects, as in the case of inhaled glucocorticoids, budesonide and fluticasone for the treatment of asthma (Barnes, 2006).

4.1.2 The hypothalamic-pituitary-adrenal axis (HPA) axis and its suppression by glucocorticoids

The HPA-axis is the primary regulator of the secretion of the physiological glucocorticoids and an integral component of the adaptive responses to stress. In particular, the hypothalamus releases corticotropin-releasing factor (CRF) which binds to its receptors in the pituitary gland, subsequently inducing the release of adrenocorticotropic hormone (ACTH) into the circulation. ACTH acts on the adrenal cortex to stimulate the release of glucocorticoids from zona fasciculata (Smith and Vale, 2006). The activity of the HPA-axis and further release of cortisol are inhibited by the circulating glucocorticoids, which act at the hypothalamus, the pituitary gland and extra-hypothalamic centres. This inhibitory feedback acts to terminate the stress response, whilst protecting tissues from the potential harmful effects that may occur following glucocorticoid overexposure (Uchoa et al., 2014).

On the other hand, the treatment with systemic glucocorticoids is an established cause of HPA-axis suppression in patients. The major factors linked to the suppression include the biological half-life and potency of the given glucocorticoid, routes of the administration, dosage
regimen and the duration of the treatment (Alten and Wiebe, 2015). Synthetic glucocorticoids with longer biological half-life, such as methylprednisolone and dexamethasone, have been shown to cause a more pronounced and longer suppression of HPA-axis compared with cortisol (hydrocortisone) (Nebesio et al., 2016, Andrews et al., 2012). Systemic glucocorticoid therapy is more likely to suppress the HPA-axis than other routes of administration, including inhalation and topical therapy (Mortimer et al., 2006, Hengge et al., 2006). Administering glucocorticoids in several different doses during the day can also result in a greater adrenal suppression (Broersen et al., 2015). Finally, patients who receive high doses of systemic glucocorticoids for a longer period of time (more than 2-3 weeks) are also in the high risk of developing the HPA-axis suppression (Gordijn et al., 2015).

4.1.3 Animal models for the manipulation of corticosterone levels in vivo

4.1.3.1 Models to reduce corticosterone levels in mice

The most common technique for reducing levels of corticosterone in mice is adrenalectomy (ADX). ADX is a surgical procedure used for a total removal of adrenal function, which can produce more than 90% reduction in corticosterone levels in the plasma (Hoekstra et al., 2013). Whilst generally successful, this procedure comes with certain limitations, including complications during and after the surgery, which in some cases can be fatal (Castro, 1974).

On the other hand, the targeted inhibition of the activity of individual components of the HPA-axis may also result in the reduction of corticosterone release. Various preclinical studies in rodents are currently focused on the development of specific antagonists for the corticosterone releasing factor (CRF1) and adrenocorticotropin (ACTH) receptors for the treatment of Cushing’s syndrome, congenital adrenal hyperplasia and other disorders accompanied by the glucocorticoid excess (Million et al., 2013, Clark et al., 2016).

The suppression of the HPA-axis by systemic synthetic glucocorticoids, including dexamethasone, has been also demonstrated as an effective tool for reducing corticosterone levels in mice (Khemissi et al., 2014, Snyder et al., 2011).
4.1.3.2 Models to elevate corticosterone levels in mice

As corticosterone is involved in the regulation of stress responses in mice, different stress models can be used to increase its levels through activation of the HPA-axis. The forced swimming test (FST) is commonly used to increase the levels of corticosterone in rodents, including mice (Keers et al., 2012). Other non-invasive procedures involve the exposure of mice to predator odour, trimethylthiazoline (TMT), which can induce intense stress and innate anxiety responses (Yang et al., 2016), or restraint stress, which produces over 5-fold increase in the corticosterone concentration (Gong et al., 2015).

In some cases, subcutaneous administration of corticosterone has also proven to be a good alternative for a short-term increase in corticosterone levels in mice (Flint and Tinkle, 2001).

4.1.4 Outline and aims of this chapter

In this chapter, in order to further ascertain whether epithelial responses in vivo are limited by the presence of the physiological glucocorticoids, we initially obtained tracheal epithelial cells and lung tissue (containing alveolar epithelium) from BALB/c mice treated for 5 days with Dex to contrast gene regulation in these tissues.

The aim of this study was to also create an iatrogenic corticosterone insufficiency in mice by administration of clinically used synthetic glucocorticoid, 6α-methylprednisolone, in order to investigate whether suppression of corticosterone levels can restore the epithelial responses to the other synthetic glucocorticoids, such as the inhaled corticosteroid, budesonide.
4.2 Methods

4.2.1 Animals and ethics

All experimental protocols were performed in agreement with the Animal Ethics Committee of the University of Melbourne (ethics #1212356; #1613955) and in compliance with the guidelines of the National Health and Medical Research Council of Australia (NHMRC) for animal experimentation. Specific pathogen-free female 9-week-old BALB/c mice were obtained from Animal Resource Centre (Western Australia). Mice were housed under normal light-dark cycle and were given food and water ad libitum in the Biomedical Sciences Animal Facility (the University of Melbourne). All mice were acclimatized for 5-7 days prior to drug treatments and were daily weighed and monitored during the experimental period.

4.2.2 Experimental design: 5-day dexamethasone treatment

Mice received dexamethasone (1mg/kg/day) (Sigma-Aldrich, Castle Hill, Australia) or vehicle (90% peanut oil and 10% DMSO) by intraperitoneal (i.p.) administration for 5 days. On day 6, mice were euthanized, after which tracheal and whole lung tissue were dissected, epithelial cells were obtained by tracheal scraping and expression of genes in the tracheal epithelial cells and lungs was measured by RT-qPCR.

4.2.3 Experimental design: pilot study 1

Mice were weight-matched and divided into 5 groups (3 mice per group). Each group received 6α-methylprednisolone (MP) (3mg/kg/day) (Sigma-Aldrich, Castle Hill, Australia) or vehicle (90% peanut oil and 10% DMSO) by intraperitoneal (i.p.) administration for 7 days as described in the experimental design in Figure 4.1. On day 10, mice were euthanized, after which blood was collected from posterior vena cava (Section 4.1.5) for the measurement of the corticosterone concentration in the serum by ELISA (Section 4.1.8).
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**Figure 4.1 Experimental design for the pilot study 1.** Female 9-week-old BALB/c mice were divided into 5 different groups (3 mice/group) and received vehicle (90% peanut oil and 10% DMSO) or 3mg/kg (as a single i.p. injection in the morning) of 6α-methyl-prednisolone (MP) for 7 days as follows: **vehicle group** (treated with vehicle day 0-9), **96h post MP group** (received MP day 0-6; last MP dose was 96h prior to cull), **72h post MP group** (received MP day 1-7; last MP dose was 72h prior to cull), **48h post MP group** (received MP day 2-8; last MP dose was 48h prior to cull), **24h post MP group** (received MP day 3-9; last MP dose was 24h prior to cull). On day 10, all mice were culled and the blood was collected for further analysis of the serum corticosterone concentrations.

4.2.4 **Experimental design: pilot study 2**

Mice were weight-matched and divided into 10 groups (3 mice per group). Experimental design and treatments are described in Figure 4.2. On day 0 all groups received either vehicle (90% peanut oil and 10% DMSO) in the morning and afternoon, the loading dose of 6α-methylprednisolone (MP) (30mg/kg) divided into 2 equal doses in the morning and afternoon or dexamethasone (Dex) (1mg/kg) in the morning (Sigma-Aldrich, Castle Hill, Australia) by intraperitoneal (i.p.) administration. From day 1-5, mice received either vehicle (twice a day), 22.5mg/kg/day MP (7.5mg/kg in the morning and 15mg/kg in the afternoon) or Dex (1mg/kg/day in the morning) as specified in the Figure 4.2. On day 6, treatments were as follows. At t=0, mice received either vehicle (PEG-400) or corticosterone (6mg/kg) using 10μL Hamilton syringe. Fifteen minutes after corticosterone treatment, mice were treated with either vehicle (saline) or a total of 1.5mg at 0.5mg/mL of budesonide inhalation suspension - Pulmicort® respules using ultrasonic nebuliser (AstraZeneca, NSW, Australia). Additional corticosterone treatment (6mg/kg) was performed at 2h. At t=4h, mice were euthanized, after which lung tissue and tracheal epithelial cells were collected for gene expression analysis, and blood was collected from posterior vena cava (Section 4.1.5) for the measurement of the corticosterone concentration in the serum by ELISA (Section 4.1.8).
### Figure 4.2 Experimental design for pilot study 2.

Female 9-week-old BALB/c mice were divided into 10 different groups (3 mice/group). Groups 1 and 6 (green), and 5 and 10 (yellow) received vehicle from day 0-5 (two injections – morning and afternoon). Groups 2 and 7 (blue), 3 and 8 (grey) all received MP loading dose of 30mg/kg (15mg/kg morning+15mg/kg afternoon) on day 0. Groups 2, 3, and 8 received MP and Dex on days 0-4. Groups 4, 5, and 9 received CORT on days 0-4. The protocol included daily injections of vehicle (PEG-400) s.c., vehicle (saline) inh., or budesonide inh. at different time points (0h, 15min, 2h, 4h). The study duration was 6 days.
and 7 (blue) received 7.5mg/kg MP in the morning and 15mg/kg in the afternoon from day 1-4, and groups 3 and 8 (grey) received the same treatment, but from day 1-5. Groups 4 and 9 (purple) received 1mg/kg dexamethasone (Dex) i.p. from day 0-4 (Dex in the morning, vehicle in the afternoon). On day 6, at t=0h and t=2h groups received either vehicle (PEG-400) or 6mg/kg corticosterone s.c. At t=15min groups received either vehicle (saline) or a total of 1.5mg at 0.5mg/mL of budesonide inhalation suspension (Pulmicort® respules) using ultrasonic nebuliser. At t=4h, all mice were culled and tissue was collected for further analysis.

4.2.5 Experimental end-point: basic procedures

On the day of the cull, mice were euthanized with mixture of 150mg/kg ketamine and 15mg/kg xylazine delivered i.p., after which blood was collected from posterior vena cava using 1ml syringe with 21 gauge needle into Microvette® 500 Z-Gel (#20.1344, Sarstedt, Germany). Following centrifugation of the blood at 4°C for 10 minutes at 8000g, serum was separated as the upper phase and approximately 100µL of clear serum was collected into fresh set of tubes and stored at -80°C until further analysis.

Organs (lungs, thymus, heart, liver, spleen, kidney and uterus) were dissected, weighed, and lungs were snap-frozen in liquid nitrogen and stored at -80°C until further analysis.

Prior to RNA extraction, lungs were crushed in liquid nitrogen, using sterile mortar and pestle and collected lung tissue was homogenised in the appropriate volume of RA1 buffer (Illustra™ RNASpin Mini RNA Isolation Kit (GE Healthcare, UK))/ 1% β-mercaptoethanol, by passing 5 times through 23 gauge needle.

4.2.6 Tracheal epithelial scraping

Mouse trachea was dissected and tracheal epithelial cells were obtained by scraping of the tissue though single pass with sharp blade into 1.7mL RNAse free Eppendorf tubes loaded with 350µL of RA1 buffer (Illustra™ RNASpin Mini RNA Isolation Kit (GE Healthcare, UK)) and 1% β-mercaptoethanol for total RNA extraction. Samples were immediately snap-frozen in liquid nitrogen and stored at -80°C until further analysis.
4.2.7 Determination of gene expression in lungs and tracheal epithelium

Extraction of total RNA from mouse lungs and tracheal epithelial cells was performed using Illustra™ RNAspin Mini RNA Isolation Kit (GE Healthcare, UK) according to manufacturer’s instructions (Section 2.4.1.1). cDNA was formed from total RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, VIC, Australia) with total reaction volume of 5μL (Section 2.4.1.2). Expression of genes was measured by RT-qPCR using mouse primers as described in Section 2.4.1.3.

4.2.8 Detection of corticosterone in mouse serum

Determination of corticosterone concentration in mouse serum was performed using Corticosterone ELISA Kit (#ADI-900-097, Enzo Life Sciences, US) according to the manufacturer’s instructions. Briefly, corticosterone standards (#80-0916) (32, 160, 800, 4000, 20,000pg/mL) were prepared in the ELISA Assay buffer 15 (#80-0921) and thoroughly vortexed. Samples were prepared using the proposed protocol for small volume of serum, in which 10μL of each serum sample was firstly mixed with equal volume of Steroid Displacement Reagent (SDR) (#80-0925) to inhibit steroid binding to proteins in the samples, and then vortexed and incubated 10 minutes at room temperature before further diluting with 380μL of ELISA Assay buffer 15 (for final dilution of 1:40). 100μL of samples and standards were pipetted into a microtiter plate with wells coated with donkey antibody specific to sheep IgG (#80-0045) and incubated with 100μL of blue solution of alkaline phosphatase conjugated with corticosterone (#80-0917) and 50μL of yellow solution of sheep polyclonal antibody to corticosterone (#80-0918). The plate was incubated for 2 hours at room temperature on a plate shaker (~500rpm). Following the incubation period, wells were washed three times in 400μL of Wash Buffer (#80-1286), incubated at room temperature with 200μL of the pNpp Substrate (#80-0075) for 1 hour, and finally reaction was stopped using 50μL of Stop Solution (#80-0247). The absorbance was measured at λ=405 nm on a Multiskan Ascent® plate reader and the absorbance of blank wells was subtracted from all the other absorbance values. The concentration of the corticosterone in
the samples was determined using the absorbance of the corticosterone standards and 4 parameter logistic curve fitting.

4.3 Results

4.3.1 The effect of 5-day dexamethasone treatment on body, spleen and thymus weight in female 9-week-old BALB/c mice

![Graph A: Body weight (%Day 1)](image)

![Graph B: Organ weight (% body weight)](image)

Figure 4.3 The effect of 5-day dexamethasone (Dex) treatment on (A) body weight and (B) spleen and thymus weight in female 9-week-old BALB/c mice. Mice received either vehicle (90% peanut oil and 10% DMSO) or 1mg/kg/day Dex by intraperitoneal (i.p.) administration for 5 days. (A) Result is expressed as a % of the body weight on day 1, obtained prior to the treatment with Dex. Data are presented as means and SEM for n=4 mice/treatment group; two-way ANOVA, Tukey’s multiple comparisons test, **P<0.01. (B) On day 5, mice were euthanized, after which spleen and thymus were
dissected and weighed. Organ weights are expressed as a % of the body weight. Data are presented as means and SEM for n=4 mice/treatment group. Student unpaired t-test; ***P<0.001.

On day 4 and 5 we observed significantly lower body weight in Dex-treated group compared with the vehicle-treated group (Figure 4.3A). On day 5, mice were euthanized, after which spleen and thymus were dissected and weighed. Mice treated with 1mg/kg/day dexamethasone also had notably lower weight of the spleen than mice treated with vehicle. Similarly, Dex-treatment produced a substantial reduction in the thymus weight (Figure 4.3B).

4.3.2 Mouse tracheal epithelium is resistant to selected dexamethasone effects

We obtained tracheal epithelial cells (by scraping) and the whole lung tissue, to assess the expression of gene targets, either transactivated or transrepressed by dexamethasone. The basal PLZF, GILZ and MKP-1 expression levels were lower in the tracheal epithelium compared to whole lung. Nonetheless, it appears that Dex treatment had at most only a minor effect on inducing PLZF gene expression in the tracheal epithelium compared with the significant induction in lung tissue. On the contrary, whilst GILZ was significantly induced by Dex in the tracheal epithelium, mRNA expression in the lung was not affected by Dex treatment. Interestingly, Dex failed to induce MKP-1 expression in both tracheal epithelium and lung tissue.

The basal gene expression levels of pro-inflammatory cytokines IL-6, KC and CSF-2 were similar in both tracheal epithelium and lung tissue. Remarkably, although IL-6 mRNA levels in the lung were substantially reduced by Dex treatment, no such regulation was evident in the tracheal epithelium. Whilst KC expression was significantly reduced by Dex in both tracheal epithelium and the lung, CSF-2 gene expression remained unaffected by the Dex treatment (Figure 4.4).
Figure 4.4 Comparison of the effect of dexamethasone on gene expression in mouse tracheal epithelium and lung tissue. Mice were treated with either vehicle control (10% DMSO/90% peanut oil) or 1mg/kg/day dexamethasone (Dex) i.p. for 5 days, after which total mRNA was extracted and gene expression was measured by RT-qPCR in tracheal epithelium and lung. Results are expressed as $2^{-\Delta C_t} \times 10^5$ for PLZF (A), GILZ (B), MKP (C) and CSF-2 (E) mRNA and $2^{-\Delta C_t} \times 10^7$ for IL-6 (D) and
KC (F) mRNA and are displayed as a log scale for ease of comparison of baseline and Dex induced changes between tracheal epithelium and lung tissue. Data are presented as means and SEM for n=4 mice/treatment group; Student unpaired t-test; *P<0.05, ns=non-significant.

4.3.3  The effect of 7-day methylprednisolone treatment on the body and organ weight and the serum corticosterone concentrations in 9-week-old BALB/c mice

A pharmacokinetic pilot study was performed to investigate whether 7-day methylprednisolone treatment delivered as a single dose of 3mg/kg/day can suppress the hypothalamic–pituitary–adrenal (HPA) axis and lead to decrease in the secretion of the physiological glucocorticoid, corticosterone. Furthermore, to study the duration of the effect of methylprednisolone in suppressing HPA-axis, mice were divided into several groups, in which the clearance of methylprednisolone was allowed for different periods of time: 24h, 48h, 72h and 96h prior to the analysis of the serum corticosterone concentrations.

The weight of the animals was not significantly altered in the vehicle group and in most of MP treated groups during the experimental period. Interestingly, mice which received MP from day 1-7 showed a substantial decrease in their body weight on day 4. We further observed a small increase in the weight from day 9 (48h from the last MP dose). Nonetheless, the weight remained significantly low in this group up to day 10 (Figure 4.5A).

The MP treatment significantly reduced the weight of thymus, but not of the other organs, including spleen (Figure 4.5B-D). The decrease in the thymus weight was greater in the groups with the shorter period of MP clearance (up to 48h) than in the groups in which the MP elimination was allowed for 72h or 96h (Figure 4.5C). Regardless of the clearance period, serum corticosterone levels were not different in the mice treated for 7 days with MP to the vehicle treated ones (Figure 4.6).
The Effects of Physiological & Synthetic GCs in the Airway Epithelium In Vivo

A

Body weight (% Day 0)

Day

B

Organ weight (% body weight)

Lung

Liver

C

Organ weight (% body weight)

Spleen

Thymus

D

Organ weight (% body weight)

Kidney

Uterus
Figure 4.5 Pharmacokinetic pilot study: the effect of 7-day 6α-methylprednisolone (MP) treatment on body weight (A) and organ weight (B-D) in female 9-week-old BALB/c mice. Mice received vehicle (90% peanut oil and 10% DMSO) or 3mg/kg (as one i.p. injection in the morning) of MP for 7 days as follows: 96h post MP group (received last dose 96h prior to cull), 72h post MP group (received last dose 72h prior to cull), 48h post MP group (received last dose 48h prior to cull), 24h post MP group (received last dose 24h prior to cull). (A) Result is expressed as a % of the body weight on day 0 for each group, obtained prior to the treatments. Data are presented as means and SEM for n=3 mice/treatment group; two-way ANOVA, Tukey’s multiple comparisons post-test, ****P<0.0001, ***P<0.001, **P<0.01 (stars on the graph relate to statistical difference in the % body weight compared to day 0 in 72h post MP group); (B-D) Organ weights are expressed as a % of the body weight. Data are presented as means and SEM for n=3 mice/treatment group. Student unpaired t-test; **P<0.01, *P<0.05, ns=non-significant.

Figure 4.6 Pharmacokinetic pilot study: the effect of 7-day 6α-methylprednisolone (MP) treatment on the serum corticosterone levels in female 9-week-old BALB/c mice. Mice were divided into 5 groups and received vehicle (90% peanut oil and 10% DMSO) or 3mg/kg (as one i.p. injection in the morning) of MP for 7 days as follows: vehicle group, 96h post MP group (received last dose 96h prior to cull), 72h post MP group (received last dose 72h prior to cull), 48h post MP group (received last dose 48h prior to cull), 24h post MP group (received last dose 24h prior to cull). Determination of corticosterone concentration (µmol/L) in mouse serum was performed using Corticosterone ELISA Kit. Data are presented as means and SEM for n=3 mice/treatment group; one-way ANOVA, Dunnett’s post-hoc test.
4.3.4 The effect of different synthetic glucocorticoids on the body and organ weight and the serum corticosterone levels in female 9-week-old BALB/c mice

The result from the previous pilot study suggested that the methylprednisolone dose of 3mg/kg/day may not be sufficiently high to produce the suppression of HPA-axis and subsequent decrease in the serum corticosterone levels. Therefore, in the second pilot study, mice were initially treated on day 0 with the loading dose of 30mg/kg methylprednisolone, equally divided into two doses in the morning and afternoon. Other groups were treated with either vehicle or 1mg/kg dexamethasone for comparative analysis of the effects on the serum corticosterone levels and gene expression in the tracheal epithelium and lung tissue.

Whilst there was no significant change in the body weight of the mice treated with vehicle, we observed significant weight loss in the groups treated with both MP and Dex between day 0 and 1. From day 1-5, all glucocorticoid-treated groups continued to lose weight. However, 24-48h following the glucocorticoid withdrawal, a significant increase in weight was observed in both MP and Dex-treated groups (Figure 4.7).

Mice treated with MP and Dex had significantly lower weight of the spleen and thymus than mice treated with vehicle. In both MP groups, regardless of the clearance period, MP-treatment produced substantial reduction in thymus weight. However, the decrease in spleen weight was greater in the group with the shorter period of MP clearance (24h). Furthermore, neither corticosterone treatment nor additional 4h budesonide treatment had any effect on the organ weights (Figure 4.8).

In the saline groups, we observed a slight decrease in the serum corticosterone levels following the treatment with Dex for 5 days and MP for 6 days. Moreover, 4h budesonide treatment alone produced similar reduction in the corticosterone concentrations. However, considering the sample size of each group, the difference in corticosterone levels between these groups and the vehicle group didn’t reach statistical significance. The treatment of the saline groups with corticosterone (total of 12mg/kg) within 4h prior to the cull did not produce the increase in serum corticosterone levels compared to the s.c. treatment with vehicle (PEG-400) (Figure 4.9).
Figure 4.7 Pilot study 2: Progressive weights of female 9-week-old BALB/c mice treated with different synthetic glucocorticoids - 6α-methylprednisolone (MP) and dexamethasone (Dex). Mice received either vehicle, MP or Dex from day 0-4 or day 0-5 as described in the experimental design (Figure 4.2). On day 6, all groups were weighted prior to corticosterone and budesonide treatment. Results is expressed as a % of the body weight on day 0 for each group. Data are presented as means and SEM for n=6-12 mice/treatment group; two-way ANOVA, Tukey’s multiple comparisons post-test, ****P<0.0001 (stars on the graph (blue for MP 0-4, black for MP 0-5, pink for Dex 0-4) relate to statistical difference in the % body weight between day 0 and 1 and day 5 and 6).
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Figure 4.8 Pilot study 2: The effect of different synthetic glucocorticoids (6α-methylprednisolone (MP), dexamethasone (Dex), budesonide and corticosterone on organ weights in female 9-week-old BALB/c mice. Mice received either vehicle, MP, Dex or corticosterone treatments as described in the experimental design (Figure 4.2). 4h prior to cull, mice were treated with either saline or budesonide using ultrasonic nebulizer. Organ weights are expressed as a % of the body weight. Data are presented as means and SEM for n=3 mice/treatment group. Student unpaired t-test; ***P<0.001, **P<0.01, *P<0.05, ns=non-significant.
The Effects of Physiological & Synthetic GCs in the Airway Epithelium In Vivo

Figure 4.9 The effect of different synthetic glucocorticoids (6α-methylprednisolone (MP), dexamethasone (Dex), budesonide and corticosterone on the serum corticosterone levels in female 9-week-old BALB/c mice. Mice received either vehicle, MP, Dex or corticosterone treatments as described in the experimental design (Figure 4.2). Mice were treated with either saline or budesonide using ultrasonic nebulizer, 4h prior to cull. Determination of corticosterone concentration (µmol/L) in mouse serum was performed using Corticosterone ELISA Kit. Data are presented as means and SEM for n=3 mice/treatment group; two-way ANOVA, Tukey’s multiple comparisons post-test, ns= non-significant.

4.3.5 The effect of systemic and inhaled glucocorticoids on the gene expression in mouse tracheal epithelium and lung tissue

Although the present pilot study didn’t achieve the intended substantial reduction in corticosterone levels, we also aimed to investigate the effect of different synthetic glucocorticoids used in this study on gene expression in both tracheal epithelium and the lung.

The basal PLZF, MKP-1 and GILZ mRNA expression levels were approximately 10x higher in the lungs than in the tracheal epithelium. Nonetheless, there was a lack of induction of these genes by MP, Dex and corticosterone in either tracheal epithelial cells or the lung. More importantly, 4h treatment with budesonide also failed to induce the expression of these gene targets (Figure 4.10).

Additionally, gene expression of pro-inflammatory cytokines IL-6, KC and CSF-2 was measured. Whilst there was no difference in the basal expression of IL-6 and KC between tracheal epithelium and the lung, CSF-2 mRNA levels were lower in the lung tissue. Furthermore, in both
epithelium and the lung, neither KC, nor CSF-2 expression was affected by the glucocorticoid treatment. While there was also no inhibition of IL-6 by glucocorticoids in the tracheal epithelium, strong reduction of IL-6 levels in the lungs was observed in the groups treated with MP alone for 6 days or with budesonide alone for 4h. Remarkably, IL-6 inhibition by budesonide appeared to be compromised in mice pre-treated with MP or Dex. This result was most pronounced in the group which received the last MP dose 12h prior to the budesonide exposure (Figure 4.11).
The Effects of Physiological & Synthetic GCs in the Airway Epithelium In Vivo

Figure 4.10 Comparison of the gene transactivation by 6α-methylprednisolone (MP), dexamethasone (Dex) and corticosterone in the presence and absence of inhaled glucocorticoid, budesonide in the tracheal epithelium (A) and lung (B). Mice received either vehicle, MP, Dex or corticosterone treatment as described in the experimental design (Figure 4.2). 4h prior to cull, mice were treated with either saline or budesonide using ultrasonic nebulizer, after which total mRNA was extracted and gene expression of PLZF, GILZ and MKP-1 was measured by RT-qPCR in tracheal epithelium and lung. Results are expressed as $2^{-\Delta Ct}\times 10^5$ and are displayed as a log scale for ease of comparison of all treatments in saline and budesonide treated groups in tracheal epithelium and lung.
Data are presented as means and SEM for n=3 mice/treatment group; two-way ANOVA, Tukey’s multiple comparisons post-test; ns= non-significant.

Figure 4.11 Comparison of the gene transrepression by 6α-methylprednisolone (MP), dexamethasone (Dex) and corticosterone in the presence and absence of inhaled glucocorticoid, budesonide in the tracheal epithelium (A) and lung (B). Mice received either vehicle, MP, Dex or corticosterone treatment as described in the experimental design (Figure 4.2). 4h prior to cull, mice were treated with either saline or budesonide using ultrasonic nebulizer, after which total mRNA was
extracted and gene expression of IL-6, KC and CSF-2 was measured by RT-qPCR in the tracheal epithelium and lung. Results are expressed as $2^{-\Delta\Delta CT} \times 10^7$ and are displayed as a log scale for ease of comparison of all treatments in saline and budesonide treated groups in tracheal epithelium and lung. Data are presented as means and SEM for n=3 mice/treatment group; two-way ANOVA, Tukey’s multiple comparisons post-test, **P<0.01, *P<0.05, ns=non-significant.

We sought to compare the effects of budesonide on the lung gene expression between the mice exposed and not exposed to subcutaneous treatment concurrently with the budesonide treatment. We obtained samples of extracted lung mRNA from one of the previous studies in our laboratory, in which the mice were not exposed to subcutaneous injections concurrently with budesonide treatment, to contrast gene expression with the present study.

Intriguingly, s.c. treatment with vehicle (PEG-400) highly induced the expression of PLZF mRNA to levels similar to those following budesonide in the absence of s.c. treatment. On the other hand, s.c. treatment appeared to significantly inhibit GILZ mRNA expression. Subcutaneous injections significantly reduced basal expression of IL-6 mRNA to the levels comparable to the ones following budesonide treatment in the absence of s.c. treatment. However, the inhibitory effect of budesonide on the gene expression of this cytokine was even greater in the presence of s.c. treatment.

Figure 4.12 Comparison of the gene expression in the lungs of BALB/c mice exposed or not exposed to subcutaneous injections concurrently with budesonide treatment. Comparison of the PLZF, GILZ and IL-6 mRNA levels in the mice exposed to subcutaneous injection 15 min prior and
2h post inhaled budesonide or saline treatment. Gene expression was measured by RT-qPCR. Results are expressed as $2^{-\Delta \Delta Ct} \times 10^5$ for PLZF, $2^{-\Delta \Delta Ct} \times 10^4$ for GILZ and $2^{-\Delta \Delta Ct} \times 10^7$ for IL-6 mRNA and are displayed as a log scale for ease of comparison of saline and budesonide induced changes between the mice exposed and not exposed to s.c. treatment. Data are presented as means and SEM for n=3-6 mice/group; two-way ANOVA, Tukey’s multiple comparisons post-test; *P<0.05, ns=non-significant.

# 4.4 Discussion

In this chapter we investigated the effects of physiological and clinically used synthetic glucocorticoids in the airway epithelium in vivo. Data from the initial animal study demonstrated that mouse tracheal epithelium is resistant to selected dexamethasone effects. We hypothesized that this result could be the consequence of the inhibitory effect of the physiological glucocorticoid in mice, corticosterone. We performed pilot studies to create a model of iatrogenic corticosterone insufficiency by administering systemic synthetic glucocorticoids, in order to investigate whether suppression of corticosterone levels can restore the epithelial responses to synthetic glucocorticoids in vivo.

## 4.4.1 Initial study: mouse tracheal epithelium is resistant to selected dexamethasone effects

In the airways of humans, the pseudostratified epithelium containing basal and fully differentiated ciliated and goblet cells is distributed from the trachea through the terminal bronchiole. However, in rodents, the presence of basal cells in the airway epithelium is mostly limited to the trachea (Rock et al., 2010). Therefore, in order to initially examine the epithelial responses to glucocorticoids in vivo, we obtained tracheal airway epithelial cells and lung tissue containing alveolar epithelium from mice treated for 5 days with Dex to contrast gene regulation in these tissues. In contrast to the induction of PLZF and the repression of IL-6 in lung tissue, there was no PLZF mRNA induction, nor repression of IL-6 mRNA by Dex in the tracheal epithelium. IL-6 release by both inflammatory and epithelial cells contributes to the pathology of chronic respiratory diseases (Morjaria et al., 2010, Neveu et al., 2010, Rincon and Irvin, 2012). IL-6 is generally considered to be strongly regulated by glucocorticoids (Strandberg et al., 2008, De Bosscher et al., 1997), but several studies show circumstances in which glucocorticoid
regulation of IL-6 is impaired (Goleva et al., 2008, Wine et al., 2013). IL-6 mRNA regulation by Dex was observed in lung but not in the tracheal epithelium, even though the cell types comprising these tissues are expected to be exposed to similar, if not, identical concentrations of Dex. This difference in tissue response was predicted from airway epithelial cell pharmacology and may be attributable to an inhibitory effect of circulating levels of corticosterone, the physiological GC in the murine epithelia. In order to investigate this further, subsequent pilot studies were focused on developing a model to modify corticosterone levels in mice.

4.4.2 Pilot studies: the outcome and future directions

Various animal models can be used for reducing levels of corticosterone, including surgical procedure, adrenalectomy (Hoekstra et al., 2013), or targeted inhibition of the activity of the individual components of the HPA-axis using their receptor antagonists (Million et al., 2013, Clark et al., 2016). However, the suppression of the HPA-axis by the treatment with systemic glucocorticoids has been demonstrated to reduce corticosterone levels in mice (Khemissi et al., 2014). More importantly, the model of iatrogenic corticosterone insufficiency is clinically more relevant, as patients on the chronic systemic glucocorticoid treatment, including severe asthmatics, are at high risk of developing the HPA-axis suppression (Liu et al., 2013a). Therefore, this type of model was chosen for the pilot studies.

Dexamethasone is established as a potent suppressor of the HPA-axis and corticosterone levels in mice (Snyder et al., 2011). However, given its high potency and long-duration of action, as well as the high risk of significant systemic side effects, the clinical use of dexamethasone is mostly reserved for short-term use in severe, acute conditions (Liu et al., 2013a). In pilot studies we used methylprednisolone, another systemic glucocorticoid commonly used in the treatment of asthma (Alangari, 2014, Shefrin and Goldman, 2009). Methylprednisolone has a shorter biological half-life and approximately 1/5 the potency of dexamethasone (Becker, 2013). Whilst studies in rats have used methylprednisolone to suppress the activity of HPA-axis and corticosterone levels (Yao et al., 2008), this particular use of methylprednisolone remains insufficiently explored in mice. The acute treatment with 3mg/kg of methylprednisolone was
shown to have an impressive anti-inflammatory and immunosuppressive effects in a mouse model of septic shock (Iwamura et al., 2004). Thus, the first pilot study was a descriptive, pharmacokinetic study to explore whether 7-day treatment with 3mg/kg/day of methylprednisolone is sufficient to supress the HPA-axis and corticosterone release.

4.4.2.1 Pilot study 1: 7-day treatment with the single dose of 3mg/kg/day methylprednisolone is not sufficient to cause the adrenal suppression in mice

Significant weight loss was observed 4-5 days following the initial dose of methylprednisolone, although this effect was not detected in all methylprednisolone treated mice. As all groups were exposed daily to the same single dose of methylprednisolone for 7 days, the variability in this result could be the consequence of the insufficient sample size in these groups. Nonetheless, the decrease in the body weight caused by the treatment with glucocorticoids is generally a common side effect, considered to be the result of the potent catabolic effects of glucocorticoids, particularly on the muscle tissue (Schakman et al., 2013). Similarly to dexamethasone treatment in the initial study, methylprednisolone caused a substantial decrease in the thymus weight. As glucocorticoids are known inducers of T lymphocyte apoptosis (Keenan et al., 2015b), the involution of the main lymphoid organs, including thymus and spleen, is an indicator of their immunosuppressive activity. The reduction in thymus weight in methylprednisolone groups appeared to be dose-dependent, as there was a noticeable weight recovery of these organs in the groups which were given a longer clearance period. However, by contrast to 1mg/kg dexamethasone, 3mg/kg methylprednisolone was less potent in reducing the weight of the spleen.

Regardless of the clearance period, serum corticosterone levels were not different in the mice treated for 7 days with methylprednisolone to the vehicle treated mice. This result suggested that either dose of 3mg/kg/day was insufficient, or the duration of the treatment was not sufficiently long to supress the HPA-axis and cause the reduction in the corticosterone levels.
4.4.2.2 Pilot study 2: Treatment with high, split dose of methylprednisolone does not cause substantial reduction in corticosterone levels in mice

In the second pilot study we increased the methylprednisolone dose and changed the dosage regimen. To select the new dose, we followed the guidelines for the recommended dosage range of methylprednisolone for the treatment of asthma and further extrapolated and scaled the dosage for the corresponding treatment in mice (Australian_Medicines_Handbook, 2017, Nair and Jacob, 2016). Accordingly, mice were initially treated with the loading dose of 30mg/kg methylprednisolone. As administering glucocorticoids in several different doses during the day has been shown to produce a greater adrenal suppression compared to the single dose (Broersen et al., 2015), mice received 15mg/kg both in the morning and afternoon. Another group of mice received 1mg/kg dexamethasone for comparative analysis of the effects on the adrenal suppression.

Groups treated with methylprednisolone and dexamethasone had a significant weight loss overnight. As some of the mice, particularly those treated with methylprednisolone, lost more than 5% of their total body weight, we modified the methylprednisolone dose. Mice are nocturnal animals and less active during the day. Therefore, we changed the morning MP dose to half of the initial dose, whilst afternoon dose, aimed for the longer treatment overnight, remained as high as the initial dose. Single daily dose of dexamethasone also remained unchanged. Whilst all glucocorticoid-treated groups continued to lose weight in the following days, there was no significant difference between methylprednisolone and dexamethasone treated groups consistent with the difference in potencies of these two synthetic glucocorticoids. The higher potency of dexamethasone in inducing catabolic effects, particularly on the muscle tissue, is thought to be the result of the presence of the fluorinated group at C-9 (Owczarek et al., 2005).

The treatment with dexamethasone and methylprednisolone at given doses produced only minor decrease in the levels of corticosterone. Moreover, the reduction appeared to be similar to the one caused by the short exposure to the inhaled budesonide. The systemic glucocorticoid therapy is more likely to suppress the HPA-axis than other routes of administration, including
inhaled (Oelkers 1996). This result suggested therefore that mice may have also been exposed systemically to the portion of the drug, given that nebulisation is a “whole body” exposure model, and mice are likely to subsequently lick their fur and chew the drug deposited in the skin or mouth (Wong, 2007). Nonetheless, the corticosterone serum concentrations suggested that the treatment may require longer, chronic exposure to achieve concentrations of systemic glucocorticoids that elicit adrenal suppression and corticosterone insufficiency.

Subcutaneous administration of corticosterone was previously shown to cause a short-term increase in the serum corticosterone concentration in mice, with the peak reached between 1-2h following the injection (Flint and Tinkle, 2001). We treated the mice with 2 equal doses of corticosterone, administered subcutaneously at the beginning and 2h after budesonide treatment, to investigate whether increased corticosterone interfered with the budesonide effects in the tracheal epithelium. However, at the time of the cull, serum corticosterone levels were not significantly higher in the mice treated with corticosterone compared to the vehicle treated mice. This may indicate that either corticosterone dose or the time point was not appropriate to detect the increase in serum corticosterone levels in these mice.

4.4.2.3 Gene expression analysis in the pilot study 2

The additional aim of the second pilot study was to ascertain whether the reduction in corticosterone levels can restore epithelial responses to inhaled budesonide in vivo. Although the present study didn’t achieve the intended reduction in the corticosterone levels, we analysed the expression of the genes regulated by glucocorticoids in the tracheal epithelium and the lungs.

Budesonide and methylprednisolone significantly attenuated the expression of IL-6 in the lung, but not in the tracheal epithelium. This result further supported the initial findings in mice treated for 5-days with dexamethasone. However, IL-6 inhibition by budesonide appeared to be compromised in the mice receiving systemic glucocorticoid treatment. The lack of IL-6 inhibition by budesonide was most pronounced in the group which received the last dose of methylprednisolone 12h prior to the budesonide exposure. This result raises the possibility of a
clinically relevant interaction, as patients often concurrently use both systemic and inhaled glucocorticoids for their asthma management (Alangari, 2014).

The lack of gene transactivation by budesonide in the lung was a surprising outcome, as previous animal studies in our laboratory have shown that 4h budesonide treatment can strongly induce PLZF expression in the lung. In the present study, all mice, including vehicle treated ones, received one subcutaneous injection 15min prior and 2h after budesonide exposure. Therefore, we obtained lung tissue from one of the previous studies to ascertain whether the subcutaneous treatment may have compromised the budesonide effect in the lung. Surprisingly, subcutaneous treatment induced PLZF expression to levels similar to those of budesonide in the mice not subjected to the stress of subcutaneous injections. On the contrary, subcutaneous treatment inhibited GILZ and IL-6 expression. These results suggest that the intervention during budesonide treatment in these mice may have caused additional stress and increased corticosterone levels, subsequently affecting the basal expression levels of target genes.

4.4.3 Limitations and future directions for the in vivo experiments

In these experiments, gene expression was compared between tracheal epithelial cells and whole lung tissue. However, the method used for obtaining cells for gene expression analysis had certain limitations. Tracheal epithelium was simply scrapped, without a comprehensive characterisation of the pool of cells that were scrapped. Thus, the purity of the obtained tracheal epithelium was not clear. In addition, following the scraping, we dissected the lung without lavage or perfusion, so the whole lung tissue would not only have epithelial cells, but also other cell types, including inflammatory cells. Future experiments should properly acknowledge these limitations.

Current findings from the pilot studies suggest that further efforts are needed to optimise the model of iatrogenic corticosterone insufficiency in vivo. The particular parameter that should initially be investigated is the duration of the treatment with systemic glucocorticoids, sufficient to produce sustained adrenal suppression in mice. Patients who receive high doses of systemic glucocorticoids for a long period of time (more than 2-3 weeks) are at higher risk of developing
HPA-axis suppression (Gordijn et al., 2015). Given that mice have metabolism rate which is many times greater than in humans (Demetrius, 2005), it is difficult to predict the exact duration of the treatment necessary to produce suppression of the HPA-axis. This may require daily sacrifice of the animals and measurement of the corticosterone concentrations in the serum. Although the model of iatrogenic corticosterone insufficiency is clinically more relevant, other models should also be considered, including adrenalectomy, which may represent a faster and more robust method in achieving complete corticosterone insufficiency (Hoekstra et al., 2013).

To further investigate the effect of stress-elevated corticosterone levels on the actions of synthetic glucocorticoids in the airway epithelium in vivo, separate studies should be carried out using stress models, such as forced swimming test or the model of the restraint stress in mice (Keers et al., 2012, Gong et al., 2015).

Lastly, all future studies should be carried out using both female and male mice in order to draw conclusions relevant to all patient populations (Clayton and Collins, 2014).

**4.4.4 Conclusions from Chapter 4**

This chapter has demonstrated that mouse tracheal epithelium is resistant to selected effects of exogenous glucocorticoids, including transrepression of pro-inflammatory cytokine IL-6. To attempt to restore the epithelial responses in vivo, pilot studies were performed creating a clinically relevant model of iatrogenic corticosterone insufficiency. Whilst the studies didn’t achieve the required reduction in the serum corticosterone levels, they provided useful guidelines for the future in vivo work.

Furthermore, these studies measured corticosterone in mice maintained under standard conditions, including daily handling and injections, to be in the range of 0.5-1µmol/L. These concentrations are similar to those of cortisol used in the human cell experiments described in the previous chapter. Since corticosterone and cortisol have similar affinity for and activity on GR, the observations in the mouse airway epithelium in vivo further support the relevance of our findings in the human in vitro epithelial cells models.
CHAPTER 5:
THE ROLE OF GC-INDUCIBLE TRANSCRIPTIONAL REPRESSOR PLZF IN THE AIRWAY EPITHELIUM
5.1 Introduction

In the preceding chapters, evidence was presented indicating that transcriptional repressor promyelocytic leukaemia zinc finger (PLZF) is highly induced by cortisol and synthetic glucocorticoid agonists in the airway epithelium. This transcriptional repressor (also known as the zinc finger 145 and BTB domain containing 16 (Zbtb16)) was first recognised 20 years ago for its role in the pathogenesis of a rare retinoid-acid resistant form of acute promyelocytic leukaemia (APL). In APL, PLZF was shown to form a fusion protein with retinoid acid receptor α (RARα), blocking the differentiation of immature granulocytes (Chen et al., 1994). Although in the past two decades many studies have reported the involvement of PLZF in different physiological and pathological processes in various cell types, the exact role of PLZF in the airway epithelium remains unknown.

5.1.1 PLZF effect on cell cycle, cell proliferation and inflammation

The role of PLZF as tumour suppressor and its involvement in the control of cell-cycle, proliferation and differentiation was initially described in haematopoietic progenitors, and subsequently demonstrated in other cell types, such as lymphoid progenitors (Reid et al., 1995, Doulatov et al., 2009) and spermatogonial stem cells (Filipponi et al., 2007). The effect of PLZF on cell growth is thought to be carried out mostly through direct or indirect interaction with key cell-cycle regulators, such as cyclin D2, forkhead box N3 (FOXN3) (Costoya et al., 2004), as well as direct transcriptional repression of cyclin A2 (Yeyati et al., 1999), c-Myc (Rice et al., 2009), cyclin-dependent kinase inhibitor 1A (CDKN1A) and many others (Choi et al., 2014). The additional, novel role of PLZF in the inflammation has been recently described in bone marrow-derived macrophages, in which PLZF was shown to repress the activity of transcriptional factor NF-kB, inhibiting the production of certain cytokines in response to pathogens (Sadler et al., 2015).
5.1.2 **PLZF cellular expression and subcellular localisation**

Whilst PLZF is predominantly expressed in the undifferentiated progenitor cells, its expression levels in fully differentiated cells are generally extremely low (Suliman et al., 2012). Analysing PLZF expression in human purified bone marrow (BM) CD 34+ cells, Reid et al. (1995) discovered specific localisation of this transcriptional repressor in the nuclear speckles. Nuclear speckles are defined as organelle-like subnuclear structures located in the interchromatin regions of mammalian cells, functioning as a storage or an assembly site for a great number of different pre-messenger RNA splicing factors (Spector and Lamond, 2011). The expression of PLZF in the nuclear speckles has been also reported in other cell types, including spermatogonial progenitor cells (Hobbs et al., 2012). Nuclear localisation of PLZF was reported as necessary for its activity as a transcriptional repressor. In particular, inactivation of PLZF by its export from the nucleus allows myeloid cell maturation and differentiation process (Doulatov et al., 2009).

5.1.3 **PLZF and glucocorticoids**

Knock down and overexpression experiments in different lymphoid cell lines have demonstrated that PLZF is a glucocorticoid-responsive gene, mediating glucocorticoid-induced apoptosis in lymphoid cells (Wasim et al., 2010). The induction of PLZF with glucocorticoid agonists has been also noted in the study of the cochlea damage associated with acoustic trauma, caused by different “conditioning” stimuli, such as heat stress, hypoxia and moderate level sound. In this study, dexamethasone and methylprednisolone, glucocorticoids widely used in treating acute hearing loss, induced protection of the cochlea, by elevating PLZF levels (Peppi et al., 2011).

The induction of PLZF by dexamethasone in the Jurkat cell line was shown to require the functional form of glucocorticoid receptor and transactivation mechanisms (Riml et al., 2004). These findings were also established in human primary endometrial stromal cells (EMC) and myometrial smooth muscle cells (SMC), in which the glucocorticoid receptor antagonist RU486
(mifepristone) significantly inhibited dexamethasone-induction of PLZF (Fahnenstich et al., 2003).

5.1.4 **The mechanism of the small interfering RNA-mediated gene silencing using lipid-based carriers**

Small-interfering RNAs (siRNAs) are synthetic, double-stranded RNAs consisting of approximately 21 base pairs. siRNAs are the most commonly used tool to silence target genes in a sequence-specific manner via RNA interference (RNAi) pathway in mammalian cells (Castel and Martienssen, 2013). To deliver hydrophilic siRNAs into the cells, different viral and non-viral carriers can be used. Whilst viral carriers can be associated with a higher risk of triggering immune responses in the cells, non-viral lipid-based carriers can easily interact with the cell membrane and often are the first choice for the siRNA delivery (Xue et al., 2015).

In the transfection process, hydrophilic siRNA, consisting of the guide (antisense) and the passenger strand, is encapsulated in a lipophilic carrier that can then fuse with cell membrane and deliver siRNA into the cell. Whilst the passenger strand is being degraded, the guide strand becomes the part of RNA-induced silencing complex (RISC). Furthermore, complementary pairing of the guide strand with target mRNA activates the RISC, causing the cleavage of target mRNA at the specific site. This cleaved message is targeted for degradation, which ultimately results in the loss of protein expression, and changes in the cell phenotype and functions (Yin et al., 2014) (Figure 5.1).
Figure 5.1 The mechanism of the small interfering RNA (siRNA) gene silencing using lipid-based carriers for siRNA delivery into the cells.
5.1.5 Outline and aims of the chapter

Apart from a few isolated reports of investigations of PLZF involvement in the epithelial cancer cell lines, there has not been any thorough research on its subcellular localisation and its potential roles in the differentiation and proliferation of the bronchial epithelium. These processes are fundamental for many physiological and pathological conditions in the lungs. Considering that PLZF is a highly inducible glucocorticoid target and that cortisol-supplemented medium is required for growth and differentiation of the bronchial epithelium, we hypothesize that PLZF plays a role in the proliferation and differentiation of the human bronchial epithelium. We further hypothesize that this role can be modulated by glucocorticoids.

We initially investigate the specific localisation of PLZF in the normal and asthmatic human airway and normal bronchial epithelial cells using different staining techniques. Furthermore, we investigate the role of PLZF in the proliferation of bronchial epithelium and production of pro-inflammatory cytokines, using in vitro cell models of the human bronchial epithelium and small interfering RNA gene silencing method. Finally, using similar model systems, we investigate whether PLZF is a potential mediator of glucocorticoid effects in the bronchial epithelium.
5.2 Methods

5.2.1 Determination of PLZF expression in the epithelium of the large human airway biopsies from the MESCA study

5.2.1.1 MESCA study (Melbourne Epidemiological Study of Childhood Asthma)

The MESCA study began in 1964 with the specific aims to define clinical syndromes involved in the range of wheezing in children, as well as to estimate the prevalence of asthma and examine the differences in outcomes later in life. Approximately 400 7-year olds were randomly chosen out of 30,000 living in Melbourne (Victoria) and initially this cohort was divided into 4 groups as follows: controls, mild wheezy bronchitis, wheezy bronchitis and asthma. The cohort was reviewed again at ages 10, 14, 21, 28, 35 and at the age of 42. At time of each review, subjects were given the questionnaire and examined physically. At the age 42 review (May 1999-September 2000), 380 subjects were studied, with 94 consented to the bronchoscopy for airway biopsies (approved by the Royal Melbourne Hospital Ethics Committee). For the purpose of our study and IHC staining of PLZF, we used biopsies from the 7 healthy controls (non-asthmatics), 4 mild, 4 moderate and 8 severe asthmatic patients. All severe asthmatics were steroid-requiring patients. These subjects used inhaled corticosteroids (ICS) and were on a daily maintenance dose of oral prednisolone to control their symptoms and maintain their lung function. Characteristics of all MESCA subjects that were used in our study are shown in Table 5.1.
Table 5-1 Characteristics of the MESCA subjects from whom airway biopsies were obtained and then used for PLZF staining using IHC; All subjects were 42 years old at the time of biopsies. A=asthmatic.

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5.2.1.2 Bronchoscopy and processing of mucosal biopsies

Bronchoscopy was performed transnasally using a fiberoptic Pentax FB 15P bronchoscope. All patients were anesthetised with 2% lignocaine applied topically to the upper airway and nares. Two mucosal biopsies were taken from the sub-carina of the right lower and right-middle lobes, using Pentax, fenestrated, non-toothed biopsy forceps. Biopsies were fixed in 10% neutral buffered formalin (NBF) for 2h and then placed in the 70% ethanol for overnight maintenance, prior to the paraffin embedding.

5.2.1.3 Immunohistochemical staining for PLZF

The expression of PLZF in the epithelium of MESCA airway biopsies was measured by the three layer immunoperoxidase staining of paraffin embedded tissue sections, as described in Section 2.4.7. Briefly, following antigen retrieval and blocking of non-specific sites using 1.5% normal goat serum, sections were incubated overnight with a specific anti-PLZF antibody. An appropriate matched concentration of normal rabbit IgG was used as a negative control, and specific pan-actin antibody was used as a positive control. After incubation with Vectastain® biotinylated goat anti-rabbit secondary antibody, sections were further incubated with ABC reagent (Vectastain®, Vector laboratories, USA) and subsequent specific staining was visualized using stable peroxidase substrate buffer DAB (DAKO, CA, USA). Images were taken using Olympus BX51 (Japan). The PLZF expression across tissue sections was determined by semi-quantitative IHC scoring method.

5.2.2 Immunohistochemical staining for PLZF in the epithelium of non-asthmatic peripheral lung

Lung tissue was obtained by post-mortem biopsy in the Alfred hospital (Melbourne, Victoria), and fixed in 10%NBF. Tissue sections were then paraffin-embedded and the expression of PLZF in the epithelium was determined by three layer immunoperoxidase staining as described
in Section 2.4.7. Images were taken using Olympus BX51 (Japan) and PLZF expression across tissue sections was analysed by semi-quantitative IHC scoring method.

5.2.3 Determination of PLZF gene expression in BEAS-2B cells by RT-qPCR

BEAS-2B were seeded at 25,000 cells/cm² in the complete LHC-9 medium, supplemented and described as in Section 2.1.1.1. Prior to all drug treatments, cells were growth-arrested for 24h in incomplete, serum- and steroid-free DMEM medium supplemented as in Section 2.1.1.2. Cells were then exposed for 4h to glucocorticoid receptor (GR) agonists: hydrocortisone (HC) (0.001-10µM), methyl-prednisolone (MP) (0.001-10µM), dexamethasone (Dex) (0.0001-1µM), fluticasone propionate (FP) (0.000001-0.1µM) or budesonide (Bud) (0.000001-0.1µM) (Sigma-Aldrich, Castle Hill, Australia) alone, or 30 min following the addition of the GR-antagonist mifepristone (RU486) (1µM) (Sigma-Aldrich, Castle Hill, Australia). Samples were then collected and mRNA was extracted using TRIzol® reagent (Invitrogen, Doncaster, VIC, Australia) and extraction protocol, as described in Section 2.4.1.1. cDNA was formed from total RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, VIC, Australia) with the total reaction volume of 5µL (Section 2.4.1.2). The PLZF mRNA expression was then measured by RT-qPCR (Section 2.4.1.3).

5.2.4 Determination of PLZF protein expression in BEAS-2B cells by Western Blot

BEAS-2B were seeded at 25,000 cells/cm² in the complete LHC-9 medium, supplemented and described as in Section 2.1.1.1. Prior to all drug treatments, cells were growth-arrested for 24h in incomplete, serum- and steroid-free DMEM medium supplemented as in Section 2.1.1.2. Cells were then treated with Dex (1-100nM) alone or 30 min following the addition of the GR-antagonist mifepristone (RU486) (1µM) for 24h or 48h, at which time cells were lysed in lysis buffer, phosphatase and protease inhibitor cocktail (Sigma-Aldrich, Castle Hill, Australia) and total protein lysates were obtained as described (Section 2.4.2.1). Protein concentration was determined by Bradford protein assay (Section 2.4.2.2), and expression of PLZF was determined
by Western blotting using specific anti-PLZF antibody (Section 2.4.2.3). Densitometry was performed using ImageJ (v1.44, National Institute of Health, Bethesda, MD, USA).

5.2.5 Determination of PLZF subcellular localisation in BEAS-2B cells by immunofluorescence (IF)

BEAS-2B cells were seeded at 25,000 cells/cm² in the 8-chamber Permanox® cell culture slides (Nunc, Roskilde, NL) in the complete LHC-9 medium, supplemented and described as in Section 2.1.1.1. Following starvation in serum- and steroid-free medium for 24h, cells were treated with either vehicle control or 100nM Dex for 24h and staining procedure was performed as described (Section 2.4.6). Cells were fixed for 15 minutes in 10% (v/v) neutral buffered formalin (NBF), permeabilised in 0.1% Triton X-100 for 3 minutes and blocked in 2% normal goat and horse serum prior to addition of anti-PLZF and anti-SC 35 antibodies. Following incubation overnight with primary antibodies, cells were incubated with secondary antibodies and cell nuclei were then stained for 10 minutes with 4′,6-diamidino-2-phenylindole (DAPI: Santa Cruz Biotechnology). Immunoreactivity was analysed using confocal fluorescence microscopy (Zeiss LSM880). The intensity of the fluorescence was analysed with Image J (v1.44, National Institute of Health, Bethesda, MD, USA). Co-localisation of PLZF and SC-35 was analysed using Volocity 6.3. software (PerkinElmer Inc, VIC) and Pearson’s correlation analysis.

5.2.6 Small interfering RNA (siRNA)-mediated PLZF silencing in BEAS-2B cells

5.2.6.1 Optimised protocol for PLZF knock down in BEAS-2B cells

Preparation of BEAS-2B cells for transfection process was done as described (Section 2.3). Negative control siRNA and pre-designed and validated 2 different PLZF (ZBTB16) siRNA sequences (10nM) (#s15199, #s15200, Ambion, Silencer® Select 5nmol, Life Technologies, USA) were pre-mixed and complexed with cationic lipid carrier Lipofectamine® RNAiMAX (1µM) (Invitrogen, Life Technologies, CA, USA) in reduced serum Opti-MEM medium (Life Technologies, NY, USA) for 15 min prior to drop-wise addition of 100µL of the mixture to each well. After 4 hours of incubation, the transfection was quenched by replacing medium with serum-
and steroid-free incomplete DMEM (Section 2.1.1.2). On the following day, BEAS-2B cells were treated with dexamethasone (10nM) in order to increase the protein expression, and detect the knock down. Determination of PLZF protein was done by Western blotting, as described in section 5.2.4.

5.2.6.2 **Timeline, treatments and end point assays following PLZF-knock down**

The general experimental workflow used for investigating the effects of siRNA-mediated PLZF knock-down on the cell proliferation and metabolic activity, cytokine production and glucocorticoid effects in BEAS-2B cells is shown in Figure 5.2. The starting point for all the experiments was transfection of the BEAS-2B cells with either PLZF siRNA sequences or the negative control siRNA (Section 5.2.6.1).
Figure 5.2 The general experimental workflow for investigation of the effects of siRNA-mediated PLZF knock-down in BEAS-2B cells. For the assessment of BEAS-2B cell proliferation, 30min following dexamethasone (Dex) treatment, cells were treated with fetal calf serum (FCS) for 24h, and gene expression of cell proliferation markers was determined by RT-qPCR and cell count was performed using trypan blue assay. The mRNA and protein expression of glucocorticoid receptor (GRα) was determined by RT-qPCR and Western blot, respectively, 4h or 24h after Dex treatment. The mRNA expression of glucocorticoid-inducible genes was also determined by RT-qPCR at these time points. For determination of cytokine production, 30min after fluticasone propionate (FP) treatment, cells were challenged with 10ng/mL TNFα for 24h, at which time the levels of cytokines were assessed by ELISA. Metabolic activity of BEAS-2B cells was determined 48h following Dex treatment, using a resazurin-based assay.

5.2.6.3 Determination of GRα protein expression by western blot

BEAS-2B cells were treated with Dex (10nM) for either 4h or 24h, at which time cells were lysed in lysis buffer, containing phosphatase and protease inhibitor cocktail (Sigma-Aldrich, Castle Hill, Australia) and total protein lysates were obtained, as described (Section 2.4.2.1). Protein concentration was determined by Bradford protein assay (Section 2.4.2.2), and expression of GRα was determined by western blotting using a total GRα rabbit polyclonal antibody (#sc-1003 Santa Cruz Biotechnology, CA, USA) (Section 2.4.2.3). Densitometry was performed using ImageJ (v1.44, National Institute of Health, Bethesda, MD, USA).

5.2.6.4 Determination of gene expression by RT-qPCR

Following Dex treatment (10nM), BEAS-2B cells were treated with either 2% or 5% fetal calf serum (FCS) or vehicle for 24h, after which total RNA was extracted using TRIzol® reagent (Invitrogen, Doncaster, VIC, Australia) and extraction protocol, as described in Section 2.4.1.1. cDNA was formed from total RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, VIC, Australia) with the total reaction volume of 5μL (Section 2.4.1.2). The mRNA expression of cell proliferation markers (cyclin A2, c-myc, p21) was measured by RT-qPCR (Section 2.4.1.3).

In the case of mRNA determination of GRα and the glucocorticoid-inducible genes (GILZ, MKP-1, ENaCα and CDKN1C), BEAS-2B cells were treated with either vehicle control or Dex
The Role of GC-Inducible Transcriptional Repressor PLZF in the Airway Epithelium

(10nM) for 4h or 24h, at which time total RNA was extracted using TRIzol® reagent (Section 2.4.1.1), cDNA was formed from total RNA as described (Section 2.4.1.2) and gene expression was measured by RT-qPCR (Section 2.4.1.3).

5.2.6.5 Detection of IL-6, IL-8 and GM-CSF in supernatants by ELISA

Following fluticasone propionate (FP) (10nM) treatment, BEAS-2B cells were stimulated with pro-inflammatory cytokine TNFα (10ng/mL) (R&D systems, Victoria, Australia) for 24h, at which time supernatants were collected (Section 2.4.4.1) and assayed for IL-8, IL-6 and GM-CSF cytokine levels, using commercial sandwich ELISA kits, according to the manufacturers’ instructions (Section 2.4.4.2).

5.2.6.6 Determination of BEAS-2B cell number using trypan blue assay

Following Dex treatment (10nM), BEAS-2B cells were treated with either 2% or 5% fetal calf serum (FCS) or vehicle for 24h, at which time the total number of cells was determined using trypan blue assay as described (Section 2.4.3).

5.2.6.7 Determination of BEAS-2B metabolic activity and proliferation by resazurin assay

Resazurin (blue fluorescent indicator dye) undergoes enzymatic reduction in the mitochondria due to the activity of enzymes such as: flavin mononucleotide dehydrogenase, flavin adenine dinucleotide dehydrogenase, nicotinamide adenine dehydrogenase, and cytochromes. The highly fluorescent pink resorufin is excreted into the medium and the quantity of the produced resorufin is proportional to the number of viable cells. Fluorescence is measured at 530-560nm excitation wavelength and 590nm emission wavelength. An adequate fluorescent signal is generally reached between 1 and 4 hours, depending on the metabolic activity of the particular cell type and the cell density per well (Riss et al., 2016). BEAS-2B cells were seeded at 18,000, 37,000 or 50,000 cells/cm² and following 48h incubation with Dex (10nM) or vehicle control, resazurin working solution was added. Fluorescence readings were carried out using FlexStation 3 microplate reader as described (Section 2.4.4).
5.3 Results

5.3.1 PLZF expression in the non-asthmatic and asthmatic human airway

To ascertain the PLZF expression in the human airway epithelium, we used biopsies of large non-asthmatic and asthmatic human airway from Melbourne Epidemiological Study of Childhood Asthma (MESCA) (Figure 5.3C-F) and biopsies of the non-asthmatic peripheral lung (Figure 5.5C) stained with specific PLZF antibody. Additionally, normal rabbit IgG was used as a negative control (Figure 5.3A; Figure 5.5A) and antibody against pan-actin as a positive control (Figure 5.3B, Figure 5.5B). Initially, we compared PLZF expression in the airway epithelium of the healthy controls, mild, moderate and severe asthmatic patients from MESCA study using semi-quantitative IHC scoring method (Figure 5.4A). In the non-asthmatics, PLZF appeared to be expressed at low levels and localised predominantly in the nuclei of the basal and ciliated cells of the airway epithelium (Figure 5.3C). Whilst the expression of PLZF seemed to be higher in the airway epithelium of moderate asthmatics (Figure 5.3E), in the mild (Figure 5.3D) and severe asthmatics (Figure 5.3F) the expression was not altered compared with controls. Interestingly, a higher expression of PLZF was evident in the airway epithelium of asthmatic smokers compared with non-smoker asthmatics (Figure 5.4B).

In the epithelium of non-asthmatic peripheral lung, PLZF was expressed mostly in the basal and ciliated epithelial cells. PLZF didn’t appear to be expressed in the goblet cells (Figure 5.5C).
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Figure 5.3 The expression of PLZF in the large human airway biopsies from Melbourne Epidemiological Study of Childhood Asthma (MESCA) study. Paraffin-embedded sections of human airways from non-asthmatics (C), mild (D), moderate (E) and severe (F) asthmatic patients were stained using three layer immunoperoxidase method. The expression of PLZF was detected with specific PLZF antibody (2µg/mL). An appropriate matched concentrations of normal rabbit IgG were used as a negative control (A), and specific pan-actin antibody was used as a positive control (B). Images were taken using Olympus (Japan) with objective 20x or 60x. Representative images from 4-8 individual donors. Red arrows on the images refer to positive (brown) PLZF staining in the nuclei of the cells.
Figure 5.4 Determination of the PLZF expression in the epithelium of the large human airways from Melbourne Epidemiological Study of Childhood Asthma (MESCA) study. (A) Comparison of the PLZF expression in the non-asthmatics, mild, moderate and severe asthmatic patients using semi-quantitative IHC scoring method, in which the negative controls (n=3 donors) were scored with 0, and the positive controls (n=3 donors) with 5. Data are presented as means and SEM for n=4-8 individual donors. One-way ANOVA, Kruskal-Wallis post-test; (B) Comparison of the PLZF expression in the epithelium of the non-smoker and smoker asthmatic patients using semi-quantitative IHC scoring method. Data are presented as means and SEM for n=5 individual donors. Unpaired t-test, *P<0.05.
Figure 5.5 The expression of PLZF in the epithelium of non-asthmatic peripheral lung. Paraffin-embedded sections of the non-asthmatic peripheral lung were stained using three layer
immunoperoxidase method. The expression of PLZF was detected with specific PLZF antibody (2µg/mL) (C). An appropriate matched concentration of normal rabbit IgG was used as the negative control (A), and specific pan-actin antibody was used as a positive control (B). Images were taken using Olympus (Japan) with objective 20x or 60x. Representative images from 4 individual donors. Red arrows on the images refer to positive (brown) PLZF staining in the nuclei of the cells.

5.3.2 The effect of glucocorticoids on PLZF subcellular localisation and its expression in BEAS-2B cells

The investigation of the subcellular localisation of PLZF in the bronchial epithelial cells and its modification by Dex (100nM) was initially carried out using confocal immunofluorescence microscopy. Following the treatment with Dex for 24h, BEAS-2B cells were co-stained with anti-PLZF (red) and anti-SC-35 (nuclear speckles marker) (green). Very low non-specific binding of the these two antibodies was confirmed by the absence of red or green fluorescence signal in both vehicle and Dex-treated groups co-stained with normal rabbit and normal mouse IgG (Figure 5.6A/B). In the vehicle treated cells, both PLZF and SC-35 were predominantly localised in the nuclei of the cells. Nuclear speckles were detected on the images as the various compact green fluorescent foci in each cell (Figure 5.6; Figure 5.7). We observed a significant increase in the PLZF expression following Dex treatment at 24h, but not in the expression of the nuclear speckles (Figure 5.7), as established by quantitative analysis of the fluorescence intensity using Image J (Figure 5.8).
Figure 5.6 The expression of PLZF (red) and nuclear speckles marker SC-35 (green) in BEAS-2B cells in the absence (A) and presence of dexamethasone (Dex) (100nM) (B). BEAS-2B cells were starved in the serum- and steroid-free medium 24h prior to Dex (100nM) treatment for 24h. Cells were then co-stained with either rabbit anti-PLZF and mouse anti-SC-35 antibodies, or with normal rabbit and mouse IgG as the negative controls. Following the incubation with corresponding secondary antibodies, cell nuclei were detected with DAPI (all images - blue signal). Immunoreactivity was detected by confocal fluorescence microscopy. Top images show signal for the negative controls, and bottom images merged signals for anti-PLZF (red) and anti-SC-35 (green). Representative images from 4 independent experiments.
Figure 5.7 Co-localisation of PLZF (red) and nuclear speckles marker SC-35 (green) in BEAS-2B cells in the absence (A) and presence of dexamethasone (Dex) (100nM) (B). BEAS-2B cells were starved in the serum- and steroid-free medium 24h prior to Dex (100nM) treatment for 24h. Cells were then co-stained with either rabbit anti-PLZF and mouse anti-SC-35 antibodies. Following the
incubation with corresponding secondary antibodies, immunoreactivity was detected by confocal fluorescence microscopy. Representative images from 4 independent experiments.

Figure 5.8 Comparison of the expression of PLZF (A) and nuclear speckles marker SC-35 (B) in the absence (vehicle) and presence of dexamethasone (Dex) (100nM). BEAS-2B cells were starved in the serum- and steroid-free medium 24h prior to Dex (100nM) treatment for 24h. Immunoreactivity was detected by confocal fluorescence microscopy. Quantification of fluorescence intensity was performed using Image J software. Data are presented as means and SEM for n=4 independent experiments; Paired Student t-test, *p<0.05, ns=non-significant.

Quantitative co-localisation analysis of PLZF and nuclear speckles in the presence and absence of Dex was performed using Pearson’s correlation (Figure 5.9). Briefly, Pearson’s correlation coefficient (PCC) is a measure of the strength of a linear association between two variables. In our study, those two variables were fluorescence signal of PLZF and nuclear speckles. Whilst Pearson’s correlation attempts to create a line of best fit through the data of these variables, the PCC value indicates how far away are all these data points to this line. PCC can take a range of values from -1 to +1. A value of 0 indicates that there is no correlation between the variables, values greater than 0 indicate positive correlation, whilst values less than 0 indicate negative correlation (Mukaka, 2012). In both untreated and Dex-treated cells, PCC coefficient value was approximately 0.5, suggesting positive linear correlation between the fluorescence signal of PLZF and nuclear speckles. In addition to the Pearson’s correlation analysis, we used two additional Mander’s co-localisation coefficients M1 and M2. The coefficient M1 indicates
the amount of PLZF co-localised with SC-35 of the total PLZF. The coefficient M2 indicates the amount of SC-35 co-localised with PLZF out of total SC-35 (Zinchuk et al., 2007). In untreated and Dex-treated BEAS-2B cells, under basal conditions approximately 60% of PLZF expression was co-localised with SC-35 and almost all of the SC-35 was co-localised with PLZF.

Figure 5.9 Pearson’s correlation analysis of co-localisation of PLZF and nuclear speckles in the absence (vehicle) or presence of dexamethasone (Dex) (100nM) in BEAS-2B cells at 24h. BEAS-2B cells were starved in the serum- and steroid-free medium 24h prior to Dex (100nM) treatment for 24h. Immunoreactivity was detected by confocal fluorescence microscopy. Co-localisation analysis was performed using Volocity software (PerkinElmer). Intensity threshold was determined from region of interest (ROI). Calculation of each coefficient was automatically done by the software: PCC (Pearson's Correlation Coefficient) as the measure of the linear correlation between PLZF and SC-35; M1/M2 (Mander’s) coefficients – M1 showing the amount of PLZF co-localised with SC-35; M2 showing the amount of SC-35 co-localised with PLZF. Data are presented as means and SEM for n=4 independent experiments; Paired Student t-test.

To further investigate the effect of different synthetic GC-agonists on the PLZF expression in the bronchial epithelium, here we established the concentration-response curves (CRCs) for PLZF gene expression at 4h, using systemic GC agonists (dexamethasone (Dex), hydrocortisone (HC), methylprednisolone (MP)) and inhaled GC agonists (budesonide (Bud) and fluticasone-
propionate (FP)). All synthetic GCs robustly increased PLZF mRNA at 4h. Although the induction was followed by the increase in the variability in the expression levels, there was a lower maximum response to HC compared with other GC agonists (Figure 5.10A). Induction of PLZF mRNA with high concentrations of GC agonists was markedly inhibited with the addition of GR antagonist RU486 (mifepristone) (1µM) 30 min prior to the GC treatment (Figure 5.10B).

PLZF protein expression was also investigated using Dex (1-100nM), in the presence and absence of GR antagonist RU486 (1µM). Consistent with gene expression and protein analysis by immunofluorescence, western blot followed by densitometry showed substantial induction of PLZF protein by Dex in BEAS-2B cells at 4h and 24h. Addition of RU486 30 min prior to Dex treatment completely abolished Dex effect (Figure 5.10C).
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Figure 5.10 The effect of various synthetic glucocorticoid agonists on the PLZF mRNA and protein expression in BEAS-2B cells. (A) Concentration–response curves with EC50s: Assessment of the effects of systemic GC agonists (dexamethasone (Dex), hydrocortisone (HC), methylprednisolone (MP)) and inhaled GC-agonists (budesonide (BUD), fluticasone propionate (FP)) on PLZF mRNA levels (expressed as fold vehicle) in BEAS-2B cells at 4h. EC50 were calculated using GraphPad Prism 5.0 and non-linear regression (curve fit). Data are presented as means and SEM for
n=5-10 independent experiments. (B) The induction of PLZF mRNA by GC-agonists in the presence and absence of GR-antagonist RU486 (mifepristone) at 4h. Cells were incubated with RU486 (1µM) for 30 min prior to addition of GCs. Results are expressed as fold change from control. Data are presented as means and SEM for n=4 independent experiments; two-way ANOVA with repeated measures, Bonferroni post-hoc tests, ***P<0.001. (C) Representative blot and densitometry analysis of the effect of dexamethasone (Dex) on PLZF protein expression in the presence and absence of GR-antagonist RU486 (mifepristone) (1µM) in BEAS-2B cells at 4h and 24h. β-tubulin was used as loading control. The results are expressed as fold change from vehicle control. Data are presented as means and SEM for n=5 independent experiments; two-way ANOVA with repeated measures, Bonferroni post-hoc tests, **P<0.01, *P<0.05, ###P<0.001.

5.3.3 Validation of siRNA-mediated PLZF protein knock down in BEAS-2B cells

To ascertain whether PLZF is involved in BEAS-2B cell proliferation, cytokine production or in mediating GC effects, we performed small interfering RNA (siRNA)-mediated PLZF gene silencing using 2 different pre-designed, validated PLZF siRNA sequences. An appropriate non-silencing negative control (NC) (scrambled) siRNA sequence was used to distinguish sequence-specific silencing from non-specific effects of the PLZF siRNAs. The basal expression of PLZF protein was previously shown to be extremely low in BEAS-2B cells starved in hydrocortisone-free medium for 24h. Thus, for validation of PLZF knock-down, 20h after transfection process, BEAS-2B cells were treated with dexamethasone (Dex) (10nM), in order to increase the protein expression, and detect the knock down. Cells lysates were collected 4h and 24h following Dex treatment for PLZF protein analysis by western blot. Firstly, the PLZF protein expression remained unchanged in the groups transfected with NC siRNA compared with the non-transfected groups, at both 28h and 48h post transfection process (Figure 5.1). Both PLZF siRNA 1 and PLZF siRNA 2 produced significant PLZF protein knock down 28h after transfection (4h after Dex treatment), with more than 70-80% of reduction in the protein levels compared to the NC siRNA (Figure 5.1A). Although we observed variability in the response to NC siRNA at 48h (24h after Dex treatment), PLZF protein expression remained significantly lower in the groups transfected with PLZF siRNA 1 and 2 (Figure 5.1B).
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5.3.4 The effect of PLZF knock down on BEAS-2B proliferation and metabolic activity

In order to investigate the effect of PLZF knock down on the BEAS-2B cell proliferation, following transfection process, cells were growth-arrested in the incomplete medium with no mitogens. On the following day, cell proliferation was induced by either 2% or 5% fetal calf serum (FCS) for 24h, after which the cell number was determined by examination of total and viable cells. PLZF knock down alone did not have any significant effect on the basal cell number. Whilst 2% FCS alone didn’t induce BEAS-2B cell proliferation, 5% FCS significantly increased
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the cell number. More importantly, groups transfected with PLZF siRNA sequences had considerably higher cell number compared to the groups transfected with NC siRNA. However, this effect was only observed with 5% FCS, and not with 2% FCS. In addition, 30 minutes prior to the addition of FCS, cells were treated with Dex (10nM). Interestingly, Dex did not have any further effect on BEAS-2B proliferation at 24h (Figure 5.12).

![Figure 5.12](image)

**Figure 5.12** The effect of PLZF knock down on BEAS-2B cell number in the presence and absence of dexamethasone and different concentrations of mitogen, fetal calf serum (FCS). BEAS-2B cells were seeded at 25,000 cells/cm² and transfected with either negative control (NC) or 2 different PLZF siRNA. Once the transfection process was quenched, cells were incubated in hydrocortisone- and mitogen-free medium overnight for recovery from transfection. On the next day, cells were treated with either vehicle or Dex (10nM), and after 30 min, cells were exposed to either 2% (A) or 5% FCS (B) for 24h. Panels (A) and (B) share the same vehicle- and Dex-treated groups. Cell number was determined using trypan blue method. Data are presented as means and SEM for n=5 independent experiments; two-way ANOVA with repeated measures, Bonferroni post-hoc tests, **P<0.01, *P<0.05.

Using similar experimental protocol, at 24h we collected total mRNA to analyse gene expression of several proliferation markers, cyclin A2, c-myc and p21 by RT-qPCR. In the groups exposed to non-silencing NC siRNA, cyclin A2 mRNA levels remained unchanged following the addition of FCS. However, in the groups exposed to either PLZF siRNA 1 or PLZF siRNA 2, there was an increase in the expression of cyclin A2 following the addition of 5%FCS. Whilst c-myc mRNA
was significantly induced with both 2% and 5% FCS, PLZF knock down had no further effect on c-myc mRNA levels at this time point. Furthermore, both FCS concentrations substantially decreased levels of p21 mRNA. However, PLZF knock down did not affect this reduction (Figure 5.13A).

Pre-treatment with Dex (10nM) did not change the basal levels of cyclin A2 and c-myc mRNA. However, the expression of cyclin A2 was impaired following Dex treatment in the presence of the PLZF siRNA 2 sequence in both the vehicle and the 5%FCS group. This observation was not replicated with PLZF siRNA 1 sequence. Whilst the group transfected with PLZF siRNA 2 also showed a substantial increase in the c-myc gene expression following incubation with both Dex and 5%FCS, this was not observed in the group transfected with PLZF siRNA 1. Lastly, the expression of p21 mRNA expression was significantly inhibited by Dex. The PLZF knock down had no additional effect on p21 gene expression at given time point (Figure 5.13B). The summary of results is shown in Table 5.1.

To further examine the effect of PLZF knock down on BEAS-2B cell proliferation and metabolic activity, a resazurin-based cytotoxicity assay was performed using different cell densities. Fluorescence readings were carried out at 0.5, 1, 2, 3, 4 and 5h following the addition of resazurin solution and each time point was analysed separately. Firstly, increasing the seeding density in all groups increased the fluorescence signal, with substantial difference observed between densities 18,000 and 37,000 cells/cm². A significantly higher increment in the fluorescence was detected in the positive control group (10%FCS) compared with the non-transfected group treated with 5%FCS across all cell densities at 48h (Figure 5.14A). Interestingly, when seeded at higher densities (37,000 and 50,000/cm²), cells transfected with NC siRNA appeared to have higher fluorescence signal compared with the non-transfected cells. However, the measurements from 3 individual replicates of this experiment didn’t reach the statistical significance. Furthermore, regardless of the initial cell density, no additional effect of PLZF knock down (Figure 5.14A) nor Dex treatment (Figure 5.14B) was observed in this set of experiments.
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A  
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Figure 5.13 The effect of PLZF knock down on the gene expression of the cell-proliferation markers in the presence and absence of dexamethasone and different concentrations of fetal calf serum (FCS). BEAS-2B cells were seeded at 50,000 cells/well of a 24-well plate and transfected with either negative control (NC) or 2 different PLZF siRNA. On the following day, 30 min after Dex (10nM) treatment, cells were exposed to either 2% or 5% FCS for 24h, after which total mRNA was extracted and gene expressions of cyclin A2, c-myc and p21 were determined by RT-qPCR. (A) The effect of 2% and 5% FCS on gene expression. (B) Combined effects of 5% FCS and Dex on gene expressions. Data are presented as means and SEM for n=5 independent experiments; two-way ANOVA with repeated measures, Bonferroni post-hoc tests, **P<0.01, ns=non-significant.

Table 5-2 The effect of the non-silencing siRNA and PLZF siRNAs on the expression of cell-proliferation markers in the presence of dexamethasone (Dex) and/or 5% fetal calf serum (FCS); (−) = no effect on gene expression compared with non-silencing siRNA, vehicle treated cells; (↑) = increase in the expression compared with non-silencing siRNA, vehicle treated cells; (↓) = decrease in the expression compared with non-silencing siRNA, vehicle treated cells.

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*the effect was observed only with PLZF siRNA 2
The role of GC-inducible transcriptional repressor PLZF in the airway epithelium.

Figure 5.14 The effect of PLZF knock down on BEAS-2B metabolic activity. BEAS-2B cells were seeded at different densities (18,000, 37,000 or 50,000 cells/cm²) and transfected with either negative
control or PLZF siRNA. On the next day, transfected cells were treated with Dex (10nM) 30 min before for the addition of 5% FCS for 48h. Appropriate groups were exposed to 10% FCS (positive control) for 48h. Metabolic activity was assessed by resazurin assay. Fluorescence readings were carried out using FlexStation 3 microplate reader in the following time sequence: 0.5, 1, 2, 3, 4 and 5h after addition of resazurin solution. Result is expressed as relative fluorescence units (RFU). (A) Comparison of the metabolic activity between positive control, not-transfected and transfected cells. (B) Comparison of Dex effect in groups transfected with negative control and PLZF siRNA. Data are presented as means and SEM for n=3 independent experiments. Each time point was statistically analysed using two-way ANOVA with repeated measures, Bonferroni post-hoc tests, ***P<0.001, **P<0.01, *P<0.05.

5.3.5 The effect of PLZF knock down on the expression of glucocorticoid receptor (GRα) and GC-inducible genes in BEAS-2B cells

In this set of experiments we also sought to ascertain whether PLZF is involved in GC downregulation of the GRα expression. Following transfection, BEAS-2B cells were maintained in the hydrocortisone-free medium overnight prior to the treatment with Dex (10nM) for 4h and 24h, at which time we analysed GRα mRNA and protein expression by RT-qPCR and western blot, respectively. At 4h, although GRα mRNA levels were significantly impaired by Dex, GRα protein expression remained unaffected (Figure 5.15A). At 24h, we observed Dex-downregulation of both GRα gene and protein expression (Figure 5.15B). The PLZF knock down had no effect on the basal GRα mRNA expression at given time points. Dex-impairment of GRα mRNA appeared to be blocked in the absence of PLZF at 4h. However, this knock down effect was not observed in the GRα mRNA expression at 24h, nor in the GRα protein expression at given time points.

We also examined the impact of PLZF knock down on Dex-induction of GILZ, MKP1, ENaCα and CDKN1C mRNA. Similarly to our previous analyses, following transfection, BEAS-2B cells were maintained in the hydrocortisone-free medium overnight prior to the treatment with Dex (10nM) for 4h and 24h, at which time we measured gene expression by RT-qPCR. Dex robustly induced mRNA of all analysed genes at 4h (Figure 5.16). The PLZF knock down had no effect on Dex-induction of GILZ or MKP-1. The induction of ENaCα was significantly compromised in the absence of PLZF at 4h. However, this effect was not observed at 24h. On the
other hand, Dex-induction of CDKN1C mRNA was substantially higher in the absence of PLZF at both time points.

Figure 5.15 The effect of PLZF knock down on the glucocorticoid receptor (GRα) mRNA and protein expression in BEAS-2B cells, 4h and 24h following dexamethasone (Dex) (10nM) treatment. BEAS-2B cells were transfected with either negative control (NC) or 2 PLZF siRNA sequences for 4h, after which transfection process was stopped by changing the medium to steroid-free incomplete DMEM for overnight recovery. On the following day, cells were treated with either vehicle or Dex (10nM) for 4h (A) and 24h (B), at which time both total GRα mRNA and GRα protein expressions were measured by RT-qPCR and western blot, respectively. Result is expressed as % negative control siRNA vehicle. Data are presented as means and SEM for n=5 independent
experiments; using two-way ANOVA with repeated measures, Bonferroni post-hoc tests, ***P<0.001, **p<0.01, ns=non-significant.
### The Role of GC-Inducible Transcriptional Repressor PLZF in the Airway Epithelium

#### A. Glucocorticoid-inducible leucine zipper mRNA
(Fold Neg. control siRNA vehicle)

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#### B. Glucocorticoid-inducible leucine zipper mRNA
(Fold Neg. control siRNA vehicle)

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#### MAPK Phosphatase 1 mRNA
(Fold Neg. control siRNA vehicle)

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#### Epithelial Sodium Channel α-Subunit
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#### Cyclin-dependent kinase inhibitor 1C mRNA
(Fold Neg. control siRNA vehicle)

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The effect of PLZF knock down on the mRNA expression of glucocorticoid-inducible genes GILZ, MKP-1, ENaCα and CDKN1C in the presence and absence of dexamethasone (Dex) (10nM). BEAS-2B cells were transfected with either negative control or 2 PLZF siRNA sequences for 4h, after which transfection process was stopped by changing the medium to steroid-free incomplete DMEM for overnight recovery. On the following day, cells were treated with either vehicle or Dex (10nM) for 4h (A) and 24h (B), at which time mRNA expression of GC-inducible genes was assessed by RT-qPCR. Result is expressed as % negative control siRNA vehicle and are displayed as a log scale for ease of comparison of baseline and glucocorticoid induced changes. Data are presented as means and SEM for n=4-5 independent experiments; two-way ANOVA with repeated measures, Bonferroni post-hoc tests, ***P<0.001, **P<0.01, *P<0.05.

5.3.6 The effect of PLZF knock down on the cytokine production in BEAS-2B cells

In order to ascertain whether PLZF is involved in the TNFα-induced cytokine production in BEAS-2B cells, 30 min after the treatment with inhaled corticosteroid (ICS) fluticasone propionate (FP) (10nM), BEAS-2B cells were challenged with the pro-inflammatory cytokine TNFα (10ng/mL) for 24h. We examined the production of pro-inflammatory cytokines IL-6, IL-8 and GM-CSF. In cells with reduced PLZF expression, there was a significant reduction in TNFα-induced IL-6 release (Figure 5.17A). By contrast, TNFα-induced IL-8 levels appeared to be higher with one of the PLZF siRNA sequences compared to the NC siRNA (Figure 5.17B). The PLZF knock-down did not affect the TNFα-induced GM-CSF release at 24h (Figure 5.17C). Furthermore, whilst FP remarkably inhibited TNFα-induced production of measured cytokines, PLZF knock down did not modify the inhibition at this time point.
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A

IL-6 level
(% TNFα Vehicle Neg. control)

B

IL-8 level
(% TNFα Vehicle Neg. control)

C

GM-CSF level
(% TNFα Vehicle Neg. control)

- Vehicle
- 10nM Fluticasone Propionate
Figure 5.17 The effect of PLZF knock down on TNFα- induced cytokine production on BEAS-2B cells 24h following the treatment with fluticasone propionate (FP) (10nM). BEAS-2B cells were transfected with either negative control or 2 PLZF siRNA sequences for 4h, after which transfection process was stopped by changing the medium to steroid-free incomplete DMEM for overnight recovery. On the following day, 30 min following the treatment with fluticasone propionate (10nM), cells were challenged with pro-inflammatory cytokine TNFα (10ng/mL) for 24h, at which time levels of pro-inflammatory cytokines IL-6 (A), IL-8 (B) and GM-CSF (C) were assessed in the supernatants by ELISA. Result is expressed as % TNFα vehicle negative control siRNA. Data are presented as means ± SEM for n=4 independent experiments, two-way ANOVA with repeated measures, Bonferroni post-hoc tests, ***P<0.001, **P<0.01.
5.4 Discussion

Studies on the expression and function of PLZF have been so far focused mostly on the haematopoiesis and fetal development, in which PLZF was shown to act as a negative regulator of the cell progression and differentiation of the progenitor cells (Chen et al., 1994, Reid et al., 1995, Yeyati et al., 1999). However, many biological functions of this transcriptional repressor in differentiated cells remain to be established. In this chapter, we demonstrate for the first time PLZF expression in the normal and asthmatic human airway epithelium, and describe its subcellular localisation and substantial induction by glucocorticoids in the human bronchial epithelial cells. We further demonstrate that PLZF is not only implicated in the proliferation of the bronchial epithelial cells, but is also a potential mediator of selected glucocorticoid effects in the bronchial epithelium.

5.4.1 GCs regulate PLZF expression in the human airway epithelium

The PLZF expression and its induction by glucocorticoids has been previously noted in different cell types, including smooth muscle and parenchymal cells (Fahnenstich et al., 2003). However, very little research on PLZF expression has been carried out in the airway epithelium. In this study, IHC staining of large human airway biopsies allowed us for the first time to reveal the distribution of PLZF in the human airway epithelium. Throughout the airway, epithelium consists of many different cell types, including ciliated, goblet, basal and other specialized cells with distinct functions (Walters et al., 2013). Research so far has indicated that PLZF is predominantly expressed in the undifferentiated progenitor cells, whilst its basal expression levels in fully differentiated cells are extremely low (Suliman et al., 2012). In the airway epithelium, PLZF was detected in the nuclei of the basal cells, which are thought to be the progenitors of ciliated and goblet cells (Boers et al., 1998). However, PLZF was also expressed in the differentiated ciliated cells, indicating its potential role in the specialised functions of these cells, including cilia movement and secretion of different cytokines or chemokines (Crystal et al., 2008). As there was no detectable PLZF immunoreactivity (ir-PLZF) in the cup-shaped goblet
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cells, PLZF is most likely not implicated in the mucus production by the epithelial cells in the airways.

All asthmatic patients recruited for MESCA biopsy study, regardless of the severity of the disease were on ICS treatment. Higher PLZF expression at the time of the biopsies was only observed in the group of moderate asthma. Using the BEAS-2B cell line as in vitro epithelial cell model of the airway epithelium, we confirmed previous findings that PLZF is a glucocorticoid-inducible target and that the induction is mediated by glucocorticoid receptor and transactivation (Wasim et al., 2012, Fahnenstich et al., 2003, Peppi et al., 2011). Therefore, higher PLZF expression was also anticipated in the group of severe asthmatics, as these patients were all steroid-requiring, using ICS and a daily maintenance dose of oral prednisolone. However, in most of the analysed biopsies, ir-PLZF was not different to levels observed in the non-asthmatics. One of the potential explanations could be the inhibitory effect of the pro-inflammatory cytokine, TGFβ, in these patients. Whilst it is well established that asthma severity correlates with the levels of TGFβ (Al-Alawi et al., 2014), recent in vitro studies have demonstrated TGFβ impairment of glucocorticoid-transactivation of genes in both immortalised cell lines and the air-liquid interface culture of primary human bronchial epithelial cells (Keenan et al., 2014, Salem et al., 2012).

The design of this immunohistochemistry study was based on the availability of MESCA samples. Given that this was a retrospective study, it is not possible to recruit more subjects into the research, particularly those with severe asthma. These samples were simply a snapshot in time and the patients were 42 when they had their endobronchial biopsy taken. As some of the groups indeed had smaller sample size, it is not clear whether the study has enough statistical power to detect any difference between groups. Nonetheless, this is the first analysis to investigate PLZF expression in normal and asthmatic human airways which suggested some interesting findings that should be further investigated in a study with larger sample size.

5.4.2 The effect of smoke on PLZF expression in the human airway epithelium

The MESCA study suggested higher PLZF expression in the airway epithelium of the smoking asthmatic patients compared with non-smokers. Cigarette smoke exposure is a known
cause of worsening asthma symptoms and the severity of the disease, as well as of glucocorticoid resistance and poor asthma control (Siroux et al., 2000, Chaudhuri et al., 2003, Chalmers et al., 2002). One of the proposed mechanisms by which smoke impairs glucocorticoid sensitivity is through oxidative stress, which has been shown to reduce levels of enzyme histone deacetylase 2 (HDAC2) \textit{in vitro} in primary normal human airway epithelial cells (Ito et al., 2001). Given that HDAC2 is required for the activation of GC-GR transcriptional complex, decrease in HDAC2 levels and activity consequently leads to the impairment of glucocorticoid pathways mediated through glucocorticoid receptor (Tamimi et al., 2012). Our \textit{in vitro} cell model has demonstrated that PLZF induction by glucocorticoids, including ICS, is predominantly mediated through glucocorticoid receptor in the airway epithelium. Considering that all asthmatic patients were on ICS therapy at the time of biopsies, higher PLZF expression in smokers was a rather unexpected result. Therefore, other factors and mechanisms were most likely involved in induction of PLZF in the smokers. There is very little or no research done investigating the specific effect of the nicotine or other components of tobacco smoke on the PLZF expression in the airway epithelium. Stimulation of the primary human bronchial epithelial cells with nicotine or nicotine-derived nitrosamines, such as the tobacco specific carcinogen 4-(methylnitrosamo)-1-(3-pyridyl)-1-butanone (NNK) in pharmacologically relevant concentrations (to the ones reached in the plasma of smokers) was shown to induce the phosphorylation and activation of Akt/PI3K (Protein Kinase B/phosphatidylinositol 3-kinase) pathway. This signal transduction pathway can further phosphorylate and activate a variety of downstream targets (Minna, 2003). One of those targets is a transcriptional factor FOXO3a, which has been shown to directly bind to the promoter of PLZF gene and increase its expression (Cao et al., 2013). Nevertheless, as signalling pathways activated by the cigarette smoke are extremely complex (Spira et al., 2004), additional efforts are required to further explain this particular result.
5.4.3 **PLZF is not exclusively expressed within the nuclear speckles in the BEAS-2B cell line**

The high PLZF expression inside the nuclear speckles (also known as the SC-35 domains) was initially described in CD34+ progenitor cells purified from human bone marrow (Reid et al., 1995). Under basal conditions, PLZF was mostly expressed in the nuclei of BEAS-2B cells. About a half of the nuclear PLZF expression was detected inside the nuclear speckles, whilst other half localised diffusely throughout the nucleus. This observation was in contrast with previous reports in the other cell types, indicating an exclusive expression of PLZF in nuclear speckles (Bernardo et al., 2007, Reid et al., 1995, Doulatov et al., 2009). Nuclear speckles are defined as inter-chromatin regions, functioning as a storage or an assembly/modification compartment for transcriptional factors, essential for numerous aspects of mRNA synthesis and different cytoplasmic processes (Lamond and Spector, 2003). Immunofluorescence tagging of transcriptional factors in the nucleus allows detection of not only those factors that are in inactive pools or at the assembly sites, but also in the active complexes at the sites of gene transcription (Misteli and Spector, 1998). Therefore, the PLZF signal detected in BEAS-2B cells could be both from the protein at the transcriptional sites and its storage in the nuclear speckles. It has been demonstrated that post-translational modifications of PLZF, including acetylation, occur in the nuclear speckles, subsequently leading to its activation (McConnell et al., 2003). Furthermore, nuclear speckles represent dynamic structures, with variable size and shape, which can store transcriptional factors upon transcriptional inhibition, or supply them to the active transcriptional sites when there is an increase in gene transcription (Melcak et al., 2000). Different factors can reduce the accumulation of splicing factors in the nuclear speckles (Spector and Lamond, 2011). In particular, the infection of cells with adenovirus has been shown to increase the shuttling of certain transcriptional factors outside of the speckles (Bridge et al., 1995). Given that BEAS-2B cells represent an immortalised and transformed cell line, which was derived from primary human bronchial epithelial cells (HBECs) using SV-40 adenovirus (Reddel, 1988), increased extra-speckle basal PLZF expression in BEAS-2B cells might be an artefact caused by the viral-transformation of the primary cells. However, IHC staining of the human airway epithelium also

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showed that PLZF was localised diffusely throughout the nucleus of the airway epithelial cells. Thus, confocal analysis should also be carried out in the primary HBECs to further elucidate our observations in BEAS-2B cells.

The expression of nuclear speckles was not affected by Dex treatment. The PLZF expression, on the other hand, was highly induced in BEAS-2B cells at 24h. The increase in PLZF expression was also detected in the speckles, as we observed higher overlapping green and red fluorescence signal on the images. However, co-localisation analysis suggested that Dex treatment didn’t increase the co-localisation of PLZF and nuclear speckles. Furthermore, we observed higher red fluorescence signal outside the nuclei in Dex-treated cells. Cytoplasmic PLZF protein has been also reported as active, with novel regulatory roles (Schefe et al., 2006, Doulatov et al., 2009). This may suggest that, apart from mediating Dex effects in gene transcription in the nucleus, PLZF may also be important in exerting GC effects outside the nucleus in the airway epithelial cells.

5.4.4 The impact of PLZF on BEAS-2B cell cycle and proliferation

Fetal calf serum (FCS) represents a complex mixture of different growth factors and hormones, which is an established mitogen of the bronchial epithelial cells, required in the culture medium (Stewart et al., 2012). We used two different concentrations of FCS to induce BEAS-2B proliferation within 24h and specifically investigate whether PLZF knock down can affect the cell number and gene expression of several regulators of the cell cycle. At 24h, only 5% FCS significantly increased BEAS-2B cell number. More importantly, this increase in cell proliferation was even higher in the presence of PLZF knock down, suggesting that PLZF is involved in the repression of some of the mitogen-activated pathways. Therefore, we analysed the expression of several cell cycle marker genes, previously reported to be transcriptionally regulated by PLZF in different cell types.

One of the first identified gene targets for PLZF was cyclin A2, an important positive regulator of cell cycle progression. In NIH3T3 fibroblast cell line, PLZF directly suppressed cyclin A2 and triggered cell growth arrest in the S phase of the cell cycle. The proposed pathway
included binding of zinc-finger domains in the structure of PLZF to specific sequences contained within the cyclin A2 promoter (Yeyati et al., 1999). In our study, PLZF appeared to have the similar effect on cyclin A2 expression, as PLZF knock down produced a small, but significant increase in cyclin A2 mRNA in BEAS-2B cells. However, similar to the cell number result, this effect was only observed in the cells proliferating with 5%FCS. Cyclin A2 activates cyclin-dependent kinases that further control transition from G1 to S phase of the cell cycle, as well as the entry to cell mitosis (Bertoli et al., 2013). The PLZF-repression of cyclin A2 may therefore be an important pathway involved in regulating mitogen-induced proliferation of the bronchial epithelium.

Additionally, gene expressions of two other cell cycle markers, c-myc and p21 were analysed. In human myeloid cell line U937T, PLZF was shown to directly repress transcription of proto-oncogene c-myc, supressing cell growth (McConnell et al., 2003). On the contrary, PLZF was found to directly repress the levels of the negative regulator of the cell cycle, p21 (CDKN1A) in various cell types in vitro (Choi et al., 2014). In BEAS-2B cells, whilst c-myc gene expression was induced by FCS in the concentration-dependent matter, p21 expression was significantly downregulated. However, PLZF knock down had no further effect on c-myc and p21 mRNA levels at given time point.

We also performed resazurin assay 48h following FCS treatment. Although all the groups following 5%FCS treatment appear to be proliferative and metabolically active after 48h, this assay did not show higher proliferation/metabolic activity of the cells transfected with PLZF siRNA. However, as siRNA transfection of the cells is a transient type of gene silencing, siRNAs are not permanently incorporated into the cellular genome. The duration of this type of gene silencing can be especially short in the rapidly dividing and proliferative cells (Sarkies and Miska, 2014). Therefore, the lack of difference in the proliferation between these groups at 48h may be the consequence of the loss of the siRNA effect and increase in de novo synthesis of the PLZF protein.
5.4.4.1 PLZF knock down and Dex-effect on the FCS-induced BEAS-2B proliferation

Glucocorticoids are an established potent inducers of apoptosis in many different types of tissues (Gruver-Yates and Cidlowski, 2013). Knock down and overexpression experiments in lymphoid cell lines have demonstrated that PLZF is a mediator of glucocorticoid-induced apoptosis (Wasim et al., 2010). Although some studies have demonstrated glucocorticoid-induced apoptosis in the cultured airway epithelial cells (Dorscheid et al., 2001), others have delivered the opposite findings (White, 2011), leaving the role of glucocorticoids on the epithelial cell survival and apoptosis unclear.

In BEAS-2B cells, Dex treatment did not appear to affect FCS-induced cell proliferation. However, some differences in the gene expressions were detected. Whilst cyclin A2 and c-myc mRNA expression remained unchanged following Dex treatment, levels of the negative cell cycle regulator p21 were significantly downregulated. Interestingly, in the lung cancer epithelial cell lines A549 and Calu-1, Dex was shown to have the opposite effect on p21 expression (Greenberg et al., 2002). However, more recent study has demonstrated differential regulation of p21 expression by glucocorticoids in undifferentiated and differentiated epithelium of the mammary glands. In particular, in undifferentiated epithelium, Dex induced the expression of p21, reducing cell proliferation, whereas in differentiated cells Dex inhibited p21 expression, promoting cell survival and proliferation (Hoijman et al., 2012). BEAS-2B cells are immortalised cell line with potential to proliferate, but not to differentiate (Stewart et al., 2012). Through repression of p21, Dex may therefore promote the proliferation of BEAS-2B cells. Nonetheless, PLZF knock down did not modulate the effect of Dex on p21 expression, suggesting that the repression is not mediated through induction of PLZF.

In the cells treated with both FCS and Dex, PLZF knock down using one of the PLZF siRNA sequences consistently produced robust increases in c-myc gene expression. PLZF has been demonstrated to directly transcriptionally repress the proto-oncogene, c-myc in human myeloid cells (McConnell et al., 2003). Furthermore, suppression of c-myc was shown to be implicated in glucocorticoid-induced apoptosis in both lung cancer epithelial and leukemic cell lines.
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(Greenberg et al., 2002, Zhou et al., 2000). However, this observation should be considered with some caution, as both PLZF siRNAs produced similar knock down of PLZF protein, yet only one PLZF siRNA caused an increase in c-myc gene expression, an observation that may be explained by off-target effects of the active PLZF-targeting siRNA sequence.

5.4.5 PLZF regulates gene expression of selected GC-inducible targets

Glucocorticoids exert many of their actions through binding of the glucocorticoid receptor, GRα, which is expressed in the airway epithelium (LeVan et al., 1997). However, many studies have reported the downregulation of the expression of the GRα receptor by glucocorticoids (Oakley and Cidlowski, 2013). One of the recent studies has further demonstrated that downregulation of GR is mediated through the assembly of the GR-NCoR1-HDAC-3 repression complex at the transcriptional start site of the GR gene, subsequently inhibiting GR gene transcription initiation (Ramamoorthy and Cidlowski, 2013).

Given that PLZF can interact with similar NCoR-HDAC repression complexes to transcriptionally repress different targets (Liu et al., 2016), we firstly sought to investigate whether the induction of PLZF by glucocorticoids mediates the repression of the glucocorticoid receptor expression in BEAS-2B cells. Interestingly, the repression of GRα mRNA expression by Dex appeared to be to some extent limited in the absence of PLZF at 4h. However, this effect was transient, as GRα mRNA levels were even more repressed by Dex, at 24h, regardless of PLZF knock down. Furthermore, knock down did not appear to interfere with Dex-repression of GRα protein expression at given time points.

Although GC-GR-dependent transcriptional activity is complex and incompletely characterized, transactivation of target genes by glucocorticoids is one of the most extensively investigated and better understood mechanisms. Upon ligand binding, GRα as a homodimer translocates to the nucleus and binds to glucocorticoid response elements (GREs) directly on DNA both upstream and downstream of the transcription start site of the target gene, further promoting activation of gene transcription (Keenan et al., 2015b). However, GR can also modulate gene transcription through binding to other transcriptional factors, further enhancing...
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(Langlais et al., 2012), or reducing their transcriptional activity, through protein-protein interactions (Adcock et al., 2011). Therefore, PLZF knock down in BEAS-2B cells was also used to ascertain whether PLZF is implicated in the gene transcription of several glucocorticoid-inducible targets: glucocorticoid-inducible leucine zipper (GILZ), MAP kinase phosphatase -1 (MKP-1), epithelial sodium channel alpha subunit α (ENaCα) and cyclin dependent kinase inhibitor 1C (CDKN1C). The basal expression of these genes was not affected by the PLZF knock down. Furthermore, PLZF knock down did not appear to affect Dex-induction of mRNA of the anti-inflammatory factors, GILZ and MKP-1.

In the absence of PLZF, Dex-induction of ENaCα appeared to be significantly compromised, suggesting that PLZF may be implicated in the glucocorticoid-regulation of ENaCα gene expression. Whilst expressed at the apical plasma membrane of the airway epithelial cells, the activity of epithelial sodium channel (ENaC) is essential for the salt and water reabsorption and airway surface liquid clearance (Bhalla and Hallows, 2008). In particular, the expression of α subunit of ENaC is regulated by mineralocorticoid, aldosterone, through induction of serinethreonine serum- and glucocorticoid-induced protein kinase 1 (SGK-1) (Naray-Fejes-Toth et al., 1999). Interestingly, PLZF has been reported as aldosterone-inducible target in the renal epithelial cells. More importantly, the overexpression of PLZF inhibited the activity of ENaC, albeit through transcriptional suppression of two other ENaC subunits - β and γ. The expression of α-subunit remained unaffected by PLZF (Náray-Fejes-Tóth et al., 2008). Furthermore, the ENaCα expression can also be induced by glucocorticoids. However, SGK-1/phosphoinositide 3-kinase (PI3K) pathway was shown to only partially mediate dexamethasone-induction of ENaCα subunit in the human airway epithelial cells, as GR-direct interaction with GRE in the promoter region also activated the transcription of ENaCα gene (McTavish et al., 2009). The activation of GR by glucocorticoids also highly induces PLZF, which mostly acts as transcriptional repressor. Therefore, given that ENaCα gene expression was less induced with Dex in the absence of PLZF, PLZF-mediated repression of the pathways that inhibit ENaCα transcription may contribute to glucocorticoid-induction of ENaCα mediated through glucocorticoid receptor in the bronchial epithelium.
CDKN1C is a gene product that encodes p57kip2, a member of Cip/Kip family and a potent inhibitor of several G1/cyclin-dependent complexes, which control the cell cycle (Malumbres, 2014). The induction of CDKN1C by glucocorticoids has been demonstrated in various studies, and it has also been suggested that this induction may account for the anti-proliferative actions of glucocorticoids (Samuelsson et al., 1999, Bird et al., 2007). Furthermore, glucocorticoid-induction of CDKN1C is mediated through direct interaction of GR with GRE located upstream of the transcription start site in the CDKN1C promoter (Alheim et al., 2003). On the other hand, PLZF has been shown to directly transcriptionally repress other cyclin dependent kinase inhibitors (i.e. CDKN1A (Choi et al., 2014)). However, the effect of PLZF on CDKN1C expression, particularly in the presence of glucocorticoids remains unexplored.

The induction of CDKN1C gene expression by Dex in our study was notably higher in the absence of PLZF, indicating that PLZF may also play a role in the glucocorticoid-regulation of this negative regulator of cell cycle in BEAS-2B cells. However, it is unclear whether repression of CDKN1C by PLZF is directly mediated through its interaction with the CDKN1C promoter, or it is a result of PLZF-repression of pathways that activate CDKN1C transcription. One of the candidate mediators is the transcriptional factor specificity protein 1 (Sp1). PLZF was found to compete with Sp1 and directly bind to Sp1 binding site in the CDKN1A promoter, further inhibiting transcription of the gene (Choi et al., 2014). The Sp1 responsive elements are also present in the CDKN1C promoter (Dauphinot et al., 2001), suggesting that Dex-induced PLZF levels may outcompete Sp1 on its binding site in the CDKN1C promoter, subsequently decreasing gene transcription.

5.4.6 The impact of PLZF on TNFα-induced BEAS-2B cytokine production

The bronchial epithelium can be activated by different inflammatory signals from the environment to produce multiple cytokines, chemokines and peptides, such as GM-CSF, range of interleukins (IL-1, IL-6, IL-8, IL-13), TGFβ and others. These mediators further promote the activation of the immune cells, contributing to the chronic airway inflammation (Erle and Sheppard, 2014). Tumor necrosis factor α (TNFα) is primarily produced by the immune cells in
response to pathogen infection, and bronchial epithelial cells are well known to respond to TNFα stimulation with production of inflammatory cytokines such as IL-6, IL-8 and GM-CSF (Cao et al., 2010). ICS can notably repress transcription of genes encoding these pro-inflammatory cytokines, which results in inhibition of their synthesis and release by the epithelium (Keenan et al., 2015b). In our study, pre-treatment with ICS, fluticasone propionate markedly inhibited TNFα-induced production of cytokines in BEAS-2B cells. Although the repression of cytokines by fluticasone propionate was not altered following PLZF knock down, we observed some differential effects of the PLZF knock down on the TNFα-induced cytokine levels in the absence of the fluticasone propionate.

The TNFα-induced IL-6 production was markedly inhibited in the absence of PLZF. Increased levels of IL-6 in the serum are considered as a marker of inflammation together with TNFα and other cytokines in different inflammatory lung conditions (Kishimoto, 2010). One of the prominent pathways in response to the TNFα-signalling in the inflammation includes the activation of NF-κB, which subsequently leads to induction of the IL-6 release (Lawrence, 2009). However, recent study in the bone marrow-derived macrophages has shown that TNFα could also trigger the acetylation of PLZF by histone acetyltransferase-1 (HAT-1). PLZF could then further form a repression complex with NF-κB p50 subunit, blocking NF-κB activity and IL-6 release (Sadler et al., 2015). Therefore, lower TNFα-induced IL-6 levels in BEAS-2B cells in the absence of PLZF may suggest that PLZF is a part of another signalling pathway regulating TNFα-induced IL-6 production in the bronchial epithelium.

The TNFα-induced levels of IL-8 were higher in the presence of PLZF knock down. IL-8 is a powerful chemokine responsible for the neutrophil activation and recruitment, as particularly observed in COPD (Stockley et al., 2014). In the human bronchial epithelial cells, TNFα was demonstrated to induce transcription of IL-8 promoter via activation of c-Jun amino-terminal kinase (JNK) – activator protein-1 (AP-1) signalling pathways (Li et al., 2002). Furthermore, AP-1 has been reported to be a potent inducer of PLZF expression (Fahnenstich et al., 2003). However, as the increase in IL-8 levels in BEAS-2B cells was not observed with both PLZF siRNA sequences, this may also suggest an off-target effect of the siRNA sequence. Therefore,
to determine whether PLZF can suppress TNFα-induced IL-8 production in the bronchial epithelium, further analysis is required using more appropriate gene-silencing systems.

5.4.7 Immortalised cell line as a model system for genetic manipulation

Primary human bronchial epithelial cells (PHBECs), particularly differentiated into air-liquid interface (ALI) culture, are generally recognised as the most appropriate in vitro model of the bronchial epithelium, strongly resembling physiological conditions and the behaviour of the bronchial epithelium in situ. However, this type of model has various technical limitations. Some of those include short life span with only several cell passages, high cost of reagents necessary for the cell culturing and differentiation into mucociliary ALI cultures, variability between different donors and experiments and, importantly, difficulties with transfections and genetic manipulations (Stewart et al., 2012). Whilst variety of technologies have been developed to deliver viral and non-viral vectors into primary cells (Grzesik et al., 2013, Chu et al., 2015), due to the factors linked to the receptor-binding, membrane-properties and other intracellular signalling mechanisms (Hsu and Uludag, 2012), these cells remain largely resistant to many of the currently available techniques for genetic modulation, with low efficiency of transfection. On the other hand, in various fields of research, the use of immortalised cell lines together with different gene overexpression and silencing techniques has allowed successful investigation of specific targets and their roles in the cells. Immortalised human bronchial epithelial cell line BEAS-2B has proven to be, firstly, not only responsive to inflammatory stimuli, but also to glucocorticoid treatment. Secondly, it has been demonstrated as the cell line which is easy to genetically manipulate, particularly using small interfering RNA gene silencing techniques (Wilson et al., 2009, Rippmann et al., 2005, Dieudonné et al., 2012). Therefore, BEAS-2B cells were chosen as a model system for investigation of the role of transcriptional repressor PLZF in the bronchial epithelium.
5.4.8 Advantages and limitations of siRNA mediated gene silencing

Over the past decade, siRNA and other RNA interference-based gene silencing methods have been a powerful tool enabling the investigation of the functions of different genes and proteins in the cells. Apart from being easy and fast laboratory technique, siRNA gene silencing has a relatively high efficiency in suppressing gene expression \textit{in vitro}. However, while effective, this method has several limitations (Unniyampurath et al., 2016).

Firstly, siRNAs are not permanently incorporated into the cellular genome, allowing the analysis of the resulting genotypic and phenotypic changes only for a limited period of time (Kim and Eberwine, 2010). More importantly, siRNAs have been reported to have nonspecific and off-target effects in the cells. Nonspecific effects may even involve the activation of immune responses, including the induction of subsets of genes mediating interferon response (Persengiev et al., 2004). Furthermore, very common are their off-target effects, which are the consequence of targeting sequences similar to the target of interest. As off-target and non-specific effects are more likely to occur with higher siRNA concentration, selecting the right concentrations of siRNA, as well as using the appropriate negative control (scrambled) sequence are the essential steps in the optimisation of the transfection protocol. In addition, identified phenotypes following siRNA gene knock down should be confirmed with different siRNAs targeting the same transcript (Dorsett and Tuschl, 2004).

In our experiments, we used two different PLZF siRNAs. Both siRNAs produced similar percentage of PLZF knock down, compared with the negative control siRNA. However, in several experiments, the PLZF siRNAs had different effects on the resulting phenotype of BEAS-2B, which complicated the interpretation of the results. Therefore, the use of stable transfected human bronchial epithelial cell line may allow further clarification of the results achieved with transient siRNA transfections. The recent emergence of CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas9 system for gene editing may take the analysis one step further, by introducing the heritable PLZF deletions in the genome of the primary human bronchial epithelial cells, both submerged and differentiated into air-liquid interface cultures (Chu et al., 2015).
In this chapter we have demonstrated the presence of transcriptional repressor PLZF in the human airway epithelium and its substantial induction by both cortisol and synthetic GCs in the bronchial epithelial cells \textit{in vitro}. Whilst this study was the first to compare the expression of PLZF in the airway epithelium of healthy individuals and asthmatic patients, it revealed higher PLZF expression in the smoking asthmatics than in non-smokers.

Furthermore, this was the first study to investigate the role of PLZF in the human airway epithelium using siRNA-mediated PLZF silencing. The basal expression of PLZF appeared to repress cyclin A2 gene expression and proliferation of the BEAS-2B cells. PLZF also interfered with TNF\(\alpha\)-induced IL-6 release. Finally, PLZF was shown to repress GC-induction of the negative regulator of cell cycle CDKN1C (p57\(^{kip2}\)), and also to mediate GC-induction of the mRNA of \(\alpha\) subunit of the epithelial sodium channel (ENaC\(\alpha\)). These data provide the evidence for the novel roles of PLZF, particularly in mediating selected glucocorticoid effects in the airway epithelium.
CHAPTER 6:
GENERAL DISCUSSION AND CONCLUSIONS
6.1 Key findings and their significance

6.1.1 Introduction

Airway epithelial cells regulate the interface with the environment, including maintaining microbial defence, and have well-recognised immune-modulatory functions that shape a proper immune response in the lung. Alterations in these functions can significantly contribute to inflammation in chronic respiratory diseases, including asthma (Lambrecht and Hammad, 2012). Despite the proven efficacy of GCs in the control of chronic inflammatory diseases, GC insensitivity remains a challenging clinical problem in some patients with severe chronic disease. Emerging evidence suggests that physiological GC, cortisol, may act as a partial agonist at the glucocorticoid receptor in the airway epithelium, which prompts the question of whether cortisol can limit the effects of synthetic, therapeutically used GCs.

This thesis examined the impact of cortisol on the actions of synthetic GCs in the airway epithelium using both in vitro and in vivo experimental models. This thesis also focused on characterising the transcriptional repressor, PLZF, as a potential mediator of GC effects in the airway epithelium, in order to gain novel insights and further understanding of mechanisms underlying GC actions.

6.1.2 Evidence that the physiological glucocorticoid (GC), cortisol, limits selected actions of synthetic GCs in the human airway epithelium

Synthetic GCs, including inhaled corticosteroids (ICS), exert anti-inflammatory effects in the airway epithelium by transactivation of genes and by inhibition of the release of pro-inflammatory cytokines, including GM-CSF (Keenan et al., 2015b). This thesis has provided the first evidence that the physiological glucocorticoid (GC), cortisol, acts like a partial agonist at the glucocorticoid receptor (GR) in the airway epithelium, consequently limiting GC-induced GC Receptor-dependent transcription of several genes in the BEAS-2B human bronchial epithelial cell line. Furthermore, cortisol limited the inhibition of GM-CSF release by the synthetic GCs in TNFα-activated BEAS-2B cells. Evidence has also been presented indicating that gene
transactivation by synthetic GCs is compromised by the standard air-liquid interface (ALI) growth medium cortisol concentration of 1.4µM in the ALI differentiated organotypic culture of primary human airway epithelial cells (HBECs).

These findings are significant for a number of reasons. Firstly, through impairment of GC-transactivation of anti-inflammatory factors, GILZ and MKP-1 and the transcriptional repressor PLZF, cortisol may interfere with the GC-inhibition of NF-κB-signalling pathways, leading to the release of pro-inflammatory mediators (Eddleston et al., 2007, Turpeinen et al., 2010, Sadler et al., 2015). Furthermore, through limitation of GC-induction of MKP-1, cortisol may modulate GC effects on the epithelial restitution following epithelial injury in inflammation and infection (White et al., 2005, White, 2011). The inhibition by the synthetic GCs of the TNFα-induced GM-CSF release from BEAS-2B cells was compromised by cortisol (1µM). Moreover, in the presence of cortisol, the GM-CSF levels were maintained in the concentration range known to promote neutrophil numbers and activity (Laan et al., 2003). Given that the GC-resistant asthma phenotype is considered to be neutrophil-predominant (Chambers et al., 2015), these findings highlight the potential pathophysiological relevance of cortisol to the contribution of GC insensitivity in the patients with severe GC-resistant asthma. We additionally examined the transactivation of genes by both the ICS fluticasone propionate, and the systemic, oral GC, dexamethasone in ALI culture of primary bronchial epithelial cells. ALI culture is considered to be a more physiologically relevant model of the airway epithelium, exhibiting a differentiated phenotype with basal, goblet and ciliated cells. As cortisol is essential for differentiation of the bronchial epithelium into ALI cultures (Fulcher et al., 2005), the fact that the standard concentration of cortisol in the growth ALI medium (1.4µM) remarkably constrained the induction of genes by the synthetic GCs, further supports our initial findings in the BEAS-2B cell line and complicates interpretation of studies done under these conditions.

Different physiological, psychological and pathogen-induced stresses activate the hypothalamus-pituitary-adrenal axis (HPA-axis), leading to substantial increases in cortisol levels. Importantly, the selected concentrations of cortisol in the experiments conducted in the thesis were not only within the range encountered physiologically in healthy and asthmatic
individuals (Landstra et al., 2002), but also within ranges reported in stress (Jung et al., 2014, Kudielka et al., 2004). Therefore, our observations suggest that cortisol may also compromise the efficacy of therapeutically used GCs when its levels are elevated by stress (Figure 6.1).

![Graph showing the effect of cortisol on gene expression](image)

**Figure 6.1 The effect of the physiological and stress-induced levels of cortisol on the response to synthetic glucocorticoids in the airway epithelium.** Cortisol acts like a partial agonist at the glucocorticoid receptor in the airway epithelium. In the range of physiological concentrations (which vary with the circadian rhythm and are controlled by the activity of hypothalamus-pituitary–adrenal (HPA)-axis, cortisol-binding globulin (CBG) and enzymes 11β-hydroxysteroid dehydrogenases (11β-HSD)), cortisol may limit GC-induced changes in gene expression, thereby compromising beneficial anti-inflammatory protein induction. Moreover, cortisol may compromise the efficacy of therapeutically useful GCs, particularly when its levels are elevated by the physiological, psychological or pathogen-induced stresses.

### 6.1.3 The mouse tracheal epithelium in vivo is resistant to selected GC effects

Data obtained from the initial *in vivo* study, contrasting gene regulation in the mouse tracheal epithelium and lung tissue (containing alveolar epithelium), provided evidence that the mouse tracheal epithelium is relatively resistant to selected effects of the systemic GC, dexamethasone. In particular, in contrast to the induction of PLZF and the repression of pro-inflammatory cytokine IL-6 in lung tissue, there was no regulation of gene expression of these targets in the tracheal
epithelial cells. The lack of transrepression of IL-6 mRNA by dexamethasone was a particularly important observation, as IL-6 is generally considered to be strongly regulated by glucocorticoids (Strandberg et al., 2008), although there is some evidence in the literature for the impaired GC-regulation of IL-6 (Wine et al., 2013). Given that the epithelial cell types comprising the lung tissue and trachea are expected to be exposed to similar concentrations of the synthetic GCs, the impairment of IL-6 regulation by the synthetic GCs in the murine tracheal epithelium may be attributable to an inhibitory effect of circulating levels of corticosterone, the physiological GC in the murine epithelia. On the other hand, it is important to note that fluticasone propionate-inhibition of TNFα-induced IL-6 protein levels in BEAS-2B cells was substantial and not affected by cortisol. These distinct observations may reflect the different time-course of the exposures and the more complex in vivo microenvironment of the epithelium, including the use of an inflammogen to elevate IL-6 expression in the BEAS-2B experiments. Nonetheless, current findings from the initial mouse study provide further rationale for a more in-depth analysis of the effects of physiological and clinically used synthetic GCs in the airway epithelium in vivo.

6.1.4 Evidence that the transcriptional repressor, PLZF slows the proliferation of the cells and mediates selected GC effects in the bronchial epithelium

Although previous studies have reported GC induction of PLZF in other cells types (Fahnenstich et al., 2003), this thesis has presented the first evidence for the substantial induction of PLZF by different inhalational and systemic, therapeutically used GCs, in the human bronchial epithelial cells. This is also the first study to suggest that PLZF slows the proliferation of the bronchial epithelial cells, potentially through repressing a positive cell cycle regulator, cyclin A2, which has been previously established as a target for PLZF in other cells types (Yeyati et al., 1999).

Moreover, the knock down experiments in this thesis highlight the potential of this transcriptional repressor to mediate selected glucocorticoid effects in the bronchial epithelium. Firstly, PLZF was shown to mediate GC induction of the mRNA of α subunit of the epithelial sodium channel (ENaCα), possibly through repression of an intermediate target. The activity of
epithelial sodium channels is essential for the salt and water reabsorption and airway surface liquid clearance (Bhalla and Hallows, 2008). Endogenous glucocorticoids have been shown to be important for the induction and normal function of these channels, particularly during prenatal period (Olver et al., 2004). Thus, our findings underline the potential importance of PLZF in mediating physiological effects of endogenous cortisol on the normal lung development. Furthermore, PLZF was shown to repress GC-induction of the negative regulator of cell cycle CDKN1C (p57kip2). Whilst the GC GR-mediated induction of CDKN1C has been demonstrated in various studies, it has also been suggested that this induction may account for the anti-proliferative actions of glucocorticoids (Samuelsson et al., 1999, Bird et al., 2007). Our findings may, therefore, be of relevance to the distinct GC effects on the epithelial restitution following inflammation and injury (Figure 6.2).

**Figure 6.2 The summary:** The transcriptional repressor PLZF regulates the expression of GC-inducible targets cyclin dependent kinase inhibitor 1C (CDKN1C)/ (p57kip2) and the α subunit of the epithelial sodium channel (ENaCα) in human bronchial epithelial cells.
6.2 Future directions

6.2.1 Is the effect of cortisol at the GR specific to the airway epithelial cells?

This thesis has focused on the airway epithelium, demonstrating that cortisol acts like a partial agonist at the GR in the airway epithelial cells, consequently limiting selected GR-mediated actions of the synthetic GCs. Although airway epithelial cells are one of the key cell types contributing to the chronic inflammation in the airways, the complex interaction between the inflammatory, immune, epithelial and other structural cells essentially underlies the persistent chronic inflammatory response, particularly observed in asthma and COPD (Barnes, 2008a). Glucocorticoids have effects in almost all these cell types in the airway, which makes the interpretation of their actions on the specific cell types in vivo very challenging. Whilst most cell types express functional GR (Oakley and Cidlowski, 2013), depending on the cell type and context, GCs have been shown to have different GR-dependent molecular effects. For example, GC effects in T lymphocytes are mainly inhibitory, including the induction of apoptosis, and inhibition of the synthesis and release of a variety of pro-inflammatory cytokines (Rhen and Cidlowski, 2005). In particular, the GC repression of NF-κB activated pro-inflammatory pathways is mediated by activation of GR. Additional GR-dependent mechanisms have recently been described, including the GR-dependent transactivation of apoptosis-effector genes or GC-induced GR translocation to mitochondria leading to T cell apoptosis (Smith and Cidlowski, 2010). Glucocorticoids inhibit the production and release of pro-inflammatory cytokines from monocytes and macrophages (Valledor and Ricote, 2004). Furthermore, airway smooth muscle cells are a prominent target of inhaled GCs, which not only act to inhibit their proliferation and migration (Stewart et al., 1995), but also their production of cytokines (Hirst and Lee, 1998).

Our findings, therefore, highlight the need for further investigation of whether the effect of cortisol at the GR in limiting synthetic GC actions, is unique to the airway epithelial cells or expands to other GC-responsive cell types in the airways.
6.2.2 “Heterodimer” hypothesis – further in-depth analysis

In the Chapter 3 of this thesis, we made the hypothesis of the GR “heterodimer” (GR\textsuperscript{(Synthetic GC)}-GR\textsuperscript{(Cortisol)}) formation in the presence of endogenous cortisol and synthetic GC, that has a distinct signalling effect to GR homodimers bound uniquely to either synthetic GC (GR\textsuperscript{(Synthetic GC)}-GR\textsuperscript{(Synthetic GC)}) or cortisol (GR\textsuperscript{(Cortisol)}-GR\textsuperscript{(Cortisol)}). We also implied that such “heterodimers” may have lower transactivation activity than the homodimers, with limited activation of gene transcription, even in the presence of higher concentrations of synthetic GCs. To further ascertain this, additional experiments should be carried out using appropriate in vitro models and immunoprecipitation binding assays.

6.2.3 The in vivo experiments: future directions and challenges

To attempt to restore the epithelial responses to synthetic GCs in vivo, in this thesis we performed pilot studies creating a clinically relevant model of iatrogenic corticosterone insufficiency. However, pilot studies didn’t achieve the required reduction in the serum corticosterone levels. As discussed in the Chapter 4 (Section 4.4.3), in order to optimise this model, various parameters should be investigated, including the duration of the treatment with synthetic GCs, sufficient to produce sustained suppression of the HPA-axis in mice. Further insights may also be available from studies using more robust techniques, including adrenalectomy or the use of different stressors, to explore a wider range of corticosterone levels.

The investigation of the effect of modified levels of endogenous GC on the actions of synthetic GCs in the mouse tracheal epithelium in vivo would certainly be a valuable addition to our human in vitro models. However, it should be noted that, whilst corticosterone is considered the main GC involved in the regulation of stress responses in rodents, the chemical structure of corticosterone is different to cortisol, the main GC in humans, which prompts the question of whether these two endogenous GCs are interchangeable and have similar signalling pathways and tissue effects. Studies in different species have shown that cortisol and corticosterone can differentially bind to plasma globulins (Lowy, 1989) and, more importantly, to their tissue
General Discussion & Conclusions

receptors, exerting different organ-specific effects (Schmidt et al., 2010). This field remains insufficiently characterised in the mouse airway epithelium. Therefore, the suggested mouse studies would require extensive analysis, with the prospect of an uncertain relevance to the responses of human epithelia. Furthermore, as mice often respond to experimental interventions in ways that differ from humans (Seok et al., 2013), we strongly believe that the human cell organotypic models, including the air-liquid interface culture, are more definitive and relevant to the human tissue in situ.

6.2.4 PLZF as a mediator of GC effects in the airway epithelium: further evidence using other gene silencing techniques in a better in vitro model of the airway epithelium

The use of immortalised cell lines generally allows successful investigation of the roles of the specific targets in the cells. Data obtained from experiments using transient transfections and siRNAs to knock down PLZF in the immortalised BEAS-2B epithelial cell line, suggest that PLZF mediates GC-regulation of selected targets, as discussed in Section 6.1.4. Although the BEAS-2B cell line originated from a normal human bronchial epithelium and it is glucocorticoid-responsive, it fails to replicate many of the characteristics of cell differentiation, such as mucus production and development of beating cilia in vitro. Moreover, it does not give insight into the natural biological variation observed between individuals, as it is derived from a single donor. Thus, it remains of crucial importance to confirm our initial findings, using a physiological model exhibiting a differentiated phenotype with goblet and ciliated epithelial cells. Future experiments should, therefore, be focused on assessing the role of PLZF in the air-liquid interface culture of primary human bronchial epithelial cells (HBECs). As transfections using siRNAs are transient (Kim and Eberwine, 2010), and the off-target and non-specific effects of the siRNAs generally complicate the interpretation of the results (Persengiev et al., 2004, Jackson and Linsley, 2010), other gene silencing techniques, including CRISPR/Cas9 system, should be considered to stably knock out PLZF in ALI culture of primary HBECs. Finally, given that the airway epithelium is known to be altered in the asthmatic and COPD lung (Hiemstra et al., 2015), introducing heritable PLZF deletions via CRISPR/Cas9 system into primary HBECs from asthmatic or COPD donors...
and culturing them at air-liquid interface would certainly give us further insights into physiological and pathophysiological roles of PLZF in the airway epithelium.

### 6.3 General conclusions

Data presented within this thesis have demonstrated that endogenous corticosteroids may limit certain actions of synthetic pharmacological glucocorticoids in the airway epithelium and contribute to glucocorticoid insensitivity, particularly when the levels of endogenous corticosteroids are elevated by stress. This study also implicates the transcriptional repressor promyelocytic leukaemia zinc finger (PLZF) as a novel mediator of glucocorticoid effects in the airway epithelium. Given that glucocorticoid insensitivity remains a challenging clinical problem in some patients with severe chronic airway inflammatory diseases, there is a need for a better understanding of the mechanisms underlying their actions and responsiveness *in situ*. The findings within this thesis have provided unique insights into mechanisms of glucocorticoid action and insensitivity, allowing the development of strategies for improved treatment of chronic airway inflammatory diseases.
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Cortisol limits selected actions of synthetic glucocorticoids in the airway epithelium

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ABSTRACT: Cortisol, a physiologic glucocorticoid (GC), is essential for growth and differentiation of the airway epithelium. Epithelial function influences inflammation in chronic respiratory diseases. Synthetic GCs, including inhaled corticosteroids, exert anti-inflammatory effects in airway epithelium by transactivation of genes and by inhibition of proinflammatory cytokine release. We examined the effect of cortisol on the actions of synthetic GCs in the airway epithelium, demonstrating that cortisol acts like a partial agonist at the GC receptor (GR), limiting GC-induced GR-dependent transcription in the BEAS-2B human bronchial epithelial cell line. Cortisol also limited the inhibition of granulocyte macrophage colony-stimulating factor release by synthetic GCs in TNF-α–activated BEAS-2B cells. The relevance of these findings is supported by observations on tracheal epithelium obtained from mice treated for 5 d with systemic GC, showing limitations in selected GC effects, including inhibition of IL-6. Moreover, gene transactivation by synthetic GCs was compromised by standard air–liquid interface (ALI) growth medium cortisol concentration (1.4 μM) in the ALI-differentiated organotypic culture of primary human airway epithelial cells. These findings suggest that endogenous corticosteroids may limit certain actions of synthetic pharmacological GCs and contribute to GC insensitivity, particularly when corticosteroid levels are elevated by stress.—Prodanovic, D., Keenan, C. R., Langenbach, S., Li, M., Chen, Q., Lew, M. J., Stewart, A. G. Cortisol limits selected actions of synthetic glucocorticoids in the airway epithelium. FASEB J. 32, 000–000 (2018). www.fasebj.org

KEY WORDS: partial agonist · steroid resistance · asthma · stress · inhaled corticosteroids

The physiologic glucocorticoid (GC) cortisol is essential for normal growth and differentiation of the airway epithelium (1). The airway epithelial cell layer regulates the interface with the environment, including maintaining microbial defense, and has well-recognized immunomodulatory and barrier functions (2). However, changes in epithelial differentiation and functions can significantly contribute to inflammation in chronic respiratory diseases, including asthma (3).

Psychologic and physiologic stresses activate the hypothalamic–pituitary–adrenal (HPA) axis, increasing levels of cortisol (4–6). Furthermore, pathogen-induced stresses, such as viral and bacterial infections, elevate cortisol levels. These effects occur through activation of immune and structural cells. Subsequent release of proinflammatory cytokines can directly stimulate the HPA axis (7). Stress also increases the levels of adrenaline, which is generally regarded to synergize with cortisol, providing multiple beneficial effects in inflammation and bronchoconstriction (8). However, stress is an established trigger of asthma exacerbations and may also play a role in asthma development in childhood (9). Although several studies have investigated normal and stress-induced cortisol levels in individuals with and without asthma (10–14), they have delivered conflicting findings, leaving the role of cortisol in asthma and asthma development uncertain.

Synthetic GCs, including inhaled corticosteroids (ICSs), exert profound influences on the airway epithelium (15–17). Glucocorticoid anti-inflammatory effects are mediated by transactivation of selected genes, such as glucocorticoid inducible leucine zipper (GILZ) and MAP kinase phosphatase 1 (MKP-1) (18), and by inhibition of synthesis and the release of proinflammatory cytokines, including granulocyte macrophage colony-stimulating

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factor (GM-CSF), IL-6, and IL-8 (19). Glucocorticoid transactivation of GILZ inhibits NF-κB transcriptional activity, suppressing signaling pathways that trigger the release of chemokines (20). The induction of MKP-1 was found to inhibit the production of inflammatory cytokines IL-6, IL-8, and cyclooxygenase-2 by airway epithelial cells (21). MKP-1 activity is also recognized as an important contributor to the restoration of the airway epithelium integrity during wound healing processes (22).

Synthetic GCs are potent inducers of apoptosis in many cell types and tissues (23). Transactivation of cyclin-dependent kinase inhibitor (CDKN1C) and promyelocytic leukemia zinc finger (PLZF) has been shown to mediate antiproliferative and apoptotic actions of GCs in different cell types (24, 25). However, although some studies have reported GC-induced apoptosis in cultured human airway epithelial cells (26), others have suggested a pro-survival effect (27, 28), leaving the effect of GCs on epithelial cell survival and apoptosis less clear.

Despite the proven efficacy of GCs in the control of chronic inflammatory diseases, GC insensitivity in some patients with severe disease remains a challenging clinical problem. Several molecular mechanisms of steroid insensitivity have been elucidated in airway structural and immune cells (18), although mechanisms may vary depending on the context and cell phenotype under consideration. There are no specific GC-sensitizing strategies available, even though combination therapy using ICSS and long-acting β2-adrenoceptor agonists in asthma and chronic obstructive pulmonary disease (COPD) could be considered to meet some of the criteria for such an approach, given the evidence for their synergy (29).

The majority of GC effects are mediated through their binding of GC receptor (GR), which is expressed in airway epithelial cells (30). A recent pharmacodynamic investigation of GR-mediated gene transactivation in human bronchial epithelial cells (HBECs) revealed a lower maximum response to hydrocortisone (cortisol) compared with several other synthetic GCs, suggesting a potential partial agonist activity of cortisol at GR in the bronchial epithelium (31). Considering the well-established potential of partial agonists to behave as antagonists in the presence of a full agonist, we postulated that endogenous corticosteroids may limit the actions of synthetic GCs used to treat patients with chronic inflammatory diseases, including asthma.

Here we investigate the effect of cortisol on GR-mediated transactivation of genes and inhibition of the release of proinflammatory cytokines by synthetic GCs in the airway epithelium using both an immortalized bronchial epithelial cell line and an air–liquid interface (ALI) organotypic culture of primary HBECs, which is widely considered to recapitulate the characteristics of the human airway epithelium.

**MATERIALS AND METHODS**

**Cell culture**

The immortalized human SV40 virus–transformed bronchial epithelial cell line BEAS-2B (American Type Culture Collection, Manassas, VA, USA) was cultured in LHC-9 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 2% fetal calf serum (FCS) and kept at 37°C in a humidified 5% CO2/95% air atmosphere. Cells were growth arrested in FCS and steroid-free DMEM (Thermo Fisher Scientific) 24 h before drug treatments. Cells were then exposed for 4 h to GR or mineralocorticoid receptor (MR) agonists [hydrocortisone (HC) (1 nM–10 μM), fluticasone propionate (FP) (1 μM–100 nM), or aldosterone (10–100 nM); Sigma-Aldrich, Castle Hill, NSW, Australia] alone or 30 min after the addition of vehicle or one of the antagonists mifepristone (RU486) (1 μM) or spironolactone (1 μM; Sigma-Aldrich). In subsequent experiments, BEAS-2B cells were treated with HC, 30 min before incubation with FP, for 4 and 24 h for gene and protein analysis, respectively. After steroid treatment, BEAS-2B cells were stimulated with proinflammatory cytokine and TNF-α (10 ng/ml; R&D Systems, Bradesie, VIC, Australia) for 24 h, at which time supernatants were assayed for cytokine levels. The concentrations and durations of exposure are validated in a number of previous studies from our laboratory (32, 33).

**Air–liquid interface culture of primary human bronchial epithelial cells**

Primary HBECS from healthy donors and from donors with asthma and COPD were cultured using bronchial epithelial cell growth medium (Bulletkit; Lonza, Basel, Switzerland) and differentiated into ALI cultures for 4 wk on Corning Transwell inserts (Sigma-Aldrich, St. Louis, MO, USA) coated with rat tail collagen type I. After visualization of beating cilia and mucus production and measurement of transepithelial electrical resistance, cells were kept in normal ALI growth medium (containing ~1.4 μM HC) or exposed to low-HC medium (0.1 μM HC) 24 h before treatment with FP (10 nM) or dexamethasone (Dex; 100 nM) for 4 h, at which time total RNA was extracted and expression of GC-inducible genes was measured by quantitative RT-PCR (qRT-PCR). Data for the gene expression changes in the low hydrocortisone medium and normal ALI growth medium were pooled from differentiated ALI cultures from individual healthy donors and from donors with asthma and COPD.

**Animal treatments and tracheal epithelial scraping**

Experimental protocols were performed with the prior approval of the Animal Ethics Committee of the University of Melbourne (1212356). Nine-week-old BALB/c mice were obtained from the Animal Resource Centre (Murdoch, WA, Australia), housed under a normal light/dark cycle, and given food and water ad libitum. Mice received Dex (1 mg/kg/d) or vehicle (90% peanut oil and 10% DMSO) by intraperitoneal administration for 5 d. On d 5, mice were euthanized, after which tracheal and whole lung tissue were dissected, epithelial cells were obtained by tracheal scraping, and expression of GC-inducible genes in the tracheal epithelial cells and lungs were measured by qRT-PCR.

**Total mRNA extraction, reverse transcription, and quantitative RT-PCR**

Extraction of total mRNA was performed using Trizol reagent (Invitrogen, Mount Waverley, VIC, Australia) from BEAS-2B cells or an Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare, Little Chalfont, United Kingdom) from ALI cultures and animal tissue according to the manufacturers’
Suitable dilutions of standards and supernatants were PBS/0.1% Tween20, and blocked with 10% FCS in PBS for 1 h. Specific capture antibodies overnight, washed several times in Australia. In brief, 96-well plates were incubated with sandwich ELISA kits (BD Biosciences, North Ryde, NSW, Australia). In this set of experiments, HC induced MKP-1, CDKN1C, and PLZF mRNA levels. However, there was a lower maximum response and lower potency for HC compared with FP across all gene expression measurements (Fig. 1A). The effects of HC (1 μM) and FP (10 nM) were strongly inhibited by the GR antagonist RU486 (mifepristone) (1 μM), indicating that gene induction is mediated by GR (Fig. 1B).

To ascertain whether the HC maximum response may be confounded by the ability of HC to bind both GR and MRs in the bronchial epithelial cells, we treated BEAS-2B cells with the MR antagonist spironolactone (1 μM) 30 min before HC or with the MR agonist aldosterone (10 or 100 nM) to examine the expression of GC-inducible genes. Consistent with the previous experiment, HC induced PLZF, MKP-1, and CDKN1C mRNA levels. However, the induction of these genes was neither activated by aldosterone nor modulated by spironolactone (Supplemental Fig. 1). In this set of experiments, HC induced MKP-1, CDKN1C, and PLZF mRNA levels. However, there was a lower maximum response and lower potency for HC compared with FP across all gene expression measurements (Fig. 1A). The effects of HC (1 μM) and FP (10 nM) were strongly inhibited by the GR antagonist RU486 (mifepristone) (1 μM), indicating that gene induction is mediated by GR (Fig. 1B).

RESULTS

Hydrocortisone has a lower maximum response than fluticasone propionate in activating glucocorticoid receptor–dependent gene transcription in BEAS-2B cells

In BEAS-2B cells, both HC and FP induced PLZF/ZBTB16, MKP-1, and CDKN1C mRNA levels in a concentration-dependent manner. However, there was a lower maximum response and lower potency for HC compared with FP across all gene expression measurements (Fig. 1A). The effects of HC (1 μM) and FP (10 nM) were strongly inhibited by the GR antagonist RU486 (mifepristone) (1 μM), indicating that gene induction is mediated by GR (Fig. 1B).

To ascertain whether the HC maximum response may be confounded by the ability of HC to bind both GR and MRs in the bronchial epithelial cells, we treated BEAS-2B cells with the MR antagonist spironolactone (1 μM) 30 min before HC or with the MR agonist aldosterone (10 or 100 nM) to examine the expression of GC-inducible genes. Consistent with the previous experiment, HC induced PLZF, MKP-1, and CDKN1C mRNA levels. However, the induction of these genes was neither activated by aldosterone nor modulated by spironolactone (Supplemental Fig. 1). In this set of

### Cytokine detection in supernatants by enzyme-linked immunosorbent assay

Supernatants obtained from BEAS-2B cells were assayed for IL-8, IL-6, and GM-CSF cytokine levels using commercial sandwich ELISA kits (BD Biosciences, North Ryde, NSW, Australia). In brief, 96-well plates were incubated with specific capture antibodies overnight, washed several times in PBS/0.1% Tween20, and blocked with 10% FCS in PBS for 1 h. Suitable dilutions of standards and supernatants were incubated with detecting antibodies for 2–3 h at room temperature, after which horseradish peroxidase–conjugated streptavidin complex was added, and visualization was carried out using 3,3′,5,5′-tetramethylbenzidine substrate. Sulfuric acid (0.18 M) was used to stop the reaction, and the absorbance was measured at 450 nm.

### Statistical analysis

All data were statistically analyzed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA) and presented as the mean ± SEM for n individual experiments in the BEAS-2B cell line, n individual donors for primary HBECs grown in ALI cultures, and n mice. Unless otherwise specified, data were analyzed by 2-way ANOVA with Bonferroni post hoc tests and unpaired Student’s t test.

### Sodium dodecyl sulfate-polyacrylamide gel electrophoresis/Western blot analysis

BEAS-2B cells were lysed in lysis buffer [1% Triton X-100, 1 mM EDTA, 100 mM NaCl, 10 mM Tris-HCl (pH 7.4)] containing phosphatase and protease inhibitor cocktail (Sigma-Aldrich). Protein concentration was determined by Bradford assay (Bio-Rad, Gladesville, NSW, Australia). Samples were then resolved using SDS-PAGE on 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis/Western blot analysis was used to confirm the specificity of the amplified products.

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experiments, the expression of the α-subunit of the epithelial sodium channel α -subunit (SCNN1A) was analyzed. The induction of ENaCα by mineralocorticoids and glucocorticoids has been shown to regulate the fluid transport in the epithelium (34, 35). Although HC and FP induction of ENaCα gene expression was inhibited by RU486, the mRNA expression of this gene was neither induced by aldosterone nor modulated by spironolactone (Supplemental Fig. 2).

**Hydrocortisone limits selected actions of fluticasone propionate in BEAS-2B cells**

Pretreatment of BEAS-2B cells with HC (1 μM) impaired the maximum induction of certain genes by FP. This effect was most pronounced in the PLZF gene expression levels, although similar patterns of results were observed in GILZ and MKP-1 gene expression because the addition of HC constrained the maximum response to FP. Furthermore, there was no concentration–response relationship to FP in the presence of HC (Fig. 2). This effect of HC was not observed in the expression of some other GC-inducible genes, including CDKN1C, SCNN1A, and potassium voltage-gated channel subfamily B member (KCNB1) (Fig. 2; Supplemental Fig. 3), suggesting a differential effect of HC on FP induction of different genes in the bronchial epithelium.

To further ascertain whether the limitation of maximum FP induction of PLZF expression was simply related to a common effect of concurrent exposure to a high concentration of GC, we incubated BEAS-2B cells with the potent GR agonist Dex (0.01–1 μM) 30 min before the addition of FP. Although FP alone induced PLZF expression, as in the previous experiment, none of the 3 Dex concentrations reduced the maximum FP induction of PLZF expression. Thus, the inhibitory effect of cortisol is not an effect common to high concentrations of other GCs (Fig. 3).

We performed Western blot analysis to ascertain whether PLZF protein induction by FP was similarly restricted by HC pretreatment because the gene encoding PLZF (ZBTB16) was the most affected of the genes analyzed. Indeed, HC (10 μM) inhibited maximum PLZF protein induction by FP (Fig. 4).

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**Figure 1.** The effect of HC and FP on gene expression and its inhibition by the GR antagonist RU486 (mifepristone) in BEAS-2B cells. A) The effects of the physiologic GC, HC (cortisol), and the ICS FP on PLZF (ZBTB16), MKP-1, and CDKN1C gene expression levels in BEAS-2B are shown after 4 h of incubation. Results are expressed as a percentage of gene levels induced by 10 or 100 nM FP. Data are presented as mean and SEM for 4 or 5 independent experiments. B) Induction of PLZF, MKP-1, and CDKN1C by HC and FP in the presence and absence of the GR antagonist RU486 (mifepristone) at 4 h. Cells were incubated with RU486 (1 μM) for 30 min before addition of HC (1 μM) or FP (10 nM). Results are expressed as fold change from control. Data are presented as means ± SEM for 4 independent experiments (2-way ANOVA with repeated measures, Bonferroni post hoc test). **p < 0.01, ***p < 0.001.
To determine whether HC also limited FP inhibition of the production of proinflammatory cytokines in BEAS-2B cells, 30 min after HC (1 μM) addition cells were treated with FP (10 nM), followed by the simulation of cytokine production with proinflammatory cytokine TNF-α (10 ng/ml) for 24 h. As anticipated, TNF-α significantly induced levels of IL-6, IL-8, and GM-CSF from BEAS-2B cells (Supplemental Table 1). Although the elevated cytokine levels were significantly reduced by either HC or FP alone (P < 0.001), HC (1 μM) before FP treatment markedly reduced FP inhibition of GM-CSF release. FP inhibition of IL-6 and IL-8 release was not significantly affected by HC (Fig. 5).

Hydrocortisone limits gene transactivation by fluticasone propionate and dexamethasone in air–liquid interface cultures of primary human bronchial epithelial cells

Because cortisol is essential for growth and differentiation of bronchial epithelial cells into ALI organotypic culture, we sought to ascertain whether standard concentrations of HC in ALI growth medium would inhibit gene transactivation by the synthetic GC agonists FP and Dex. Primary HBECs from healthy donors and from donors with asthma and COPD were differentiated for 4 wk by culture at an ALI, and cell differentiation was confirmed by microscopic visualization of beating cilia, mucus production, and measurement of transepithelial electrical resistance values (Supplemental Fig. 4). Differentiated cells were maintained in the standard ALI growth medium with 1.4 μM HC or exposed to low-HC medium (containing 0.1 μM HC) for 24 h and treated with Dex (100 nM) or FP (10 nM). In ALI medium containing the lower HC level, Dex or FP significantly induced mRNA expression of all measured genes. However, this gene induction was prevented in the standard growth medium containing the higher concentration of HC (Fig. 6). Moreover, the absence of gene induction by FP or Dex in the presence of the higher HC concentration is not explained by HC (1.4 μM) having induced maximum gene expression levels because gene expression in HC (1.4 μM)/FP (10 nM) or Dex (100 nM) was significantly less than that in HC (0.1 μM)/FP (10 nM) or Dex (100 nM) (Fig. 6). Similar results were observed across all ALI cultures regardless of the disease state, as shown by the comparable effect of different HC concentrations on Dex induction of PLZF and GILZ between individual healthy and diseased donors, suggesting that the cortisol effect was not disease specific (Fig. 7).

Mouse tracheal epithelium is resistant to selected glucocorticoid effects

To establish whether epithelial responses in vivo are limited by the presence of endogenous corticosteroids, we assessed GC-responsive gene expression in tracheal epithelium and whole lung tissue from BALB/c mice treated with Dex (1 mg/kg/d) or vehicle for 5 d. Although IL-6 mRNA levels in the lung were significantly reduced by Dex treatment, no such regulation was evident in the tracheal epithelium. Although PLZF expression levels were lower in the tracheal epithelium compared with whole lung, it appears that Dex treatment had at most only a minor effect on inducing PLZF expression in the tracheal epithelium compared with significant induction in lung tissue (Fig. 8).
DISCUSSION

Alterations in the airway epithelial differentiation and functions can significantly contribute to inflammation in chronic respiratory diseases, including asthma. Synthetic GCs, including ICSs, exert anti-inflammatory effects in the airway epithelium by GR-mediated transactivation of genes and by inhibition of release of proinflammatory cytokines (18). Here we present the evidence that cortisol, the physiologic GC in humans, acts like a partial agonist at GR in the airway epithelium, consequently limiting selected beneficial actions of synthetic GCs.

Conventional molecular GC actions occur through binding of the GR, which is expressed in airway epithelial cells (30). Although GR-dependent transcriptional activity is complex and incompletely characterized, transactivation of target genes by GCs is one of the most extensively investigated and better understood mechanisms. Upon ligand binding, GR as a homodimer translocates to the nucleus and binds to GC response elements on DNA, further promoting activation of gene transcription (36). Structural modifications in different synthetic GC molecules significantly modulate their affinity for GR (37), which influences their potency for transcription of target genes. A recent pharmacodynamic investigation of

Figure 3. The effect of FP on PLZF gene expression in the presence and absence of Dex [(A) 0.01 µM; (B) 0.1 µM; (C) 1 µM] in BEAS-2B cells at 4 h. After 24 h of starvation in steroid-free medium, BEAS-2B cells were treated with either vehicle control (0.01% DMSO) or Dex (0.01–1 µM) 30 min before treatment with FP (0.01–10 nM) for 4 h, after which total RNA was extracted and expression of PLZF was measured by qRT-PCR. Results are expressed as a percentage of gene levels induced by 1 nM FP. Data are presented as mean and SEM for 3 independent experiments. Paired Student’s t test was performed to compare the response to 1 nM FP in the presence and absence of Dex.

Figure 4. The effect of FP on PLZF protein expression in the presence and absence of HC (10 µM) in BEAS-2B cells at 24 h. Representative Western blot (A) and densitometry analysis (B) for PLZF expression at 24 h; BEAS-2B cells were serum-starved in steroid-free medium for 24 h followed by treatment with vehicle control (0.1% DMSO) or HC (10 µM) 30 min before treatment with FP (0.1–10 nM) for 24 h, after which protein lysates were collected and protein expression was analyzed by Western blot. β-Tubulin was used as a loading control. Results are expressed as a percentage of the PLZF levels induced by 1 nM FP. Data are presented as mean and SEM for 4 independent experiments. A paired Student’s t test was performed to compare the response to 1 nM FP in the presence and absence of 10 µM HC. **P < 0.01.
GR-mediated gene transactivation in the HBEC line BEAS-2B revealed a lower maximum response of a transfected reporter construct to cortisol (hydrocortisone) compared with several other synthetic GCs (31), raising the question of whether endogenous cortisol could antagonize synthetic anti-inflammatory GCs.

We compared the potencies and the maximal transactivational effects of the clinically used ICS FP with cortisol (HC) in the BEAS-2B cell line. Both HC and FP robustly induced PLZF, MKP-1, and CDKN1C mRNA in a concentration-dependent manner. Transactivation of these genes is considered important in facilitating GC anti-inflammatory and antiproliferative actions in various types of cells (19, 38, 39). We observed distinct lower maxima to HC compared with FP in inducing these targets. The lower HC maxima could have been due to HC additionally binding MRs (40), a property not shared by FP (41). The mineralocorticoid aldosterone and the GC cortisol bind MRs with high affinity, regulating salt and fluid transport in the epithelium of the kidney and the colon (42). MRs are expressed in BEAS-2B cells (43), establishing the potential of HC agonist actions at MR to confound the GR effect. However, because the MR agonist aldosterone did not induce GR-responsive genes and the MR antagonist spironolactone did not modulate HC actions, we excluded any potential HC actions at MR as an explanation for the reduced HC maxima.

The regulation of the fluid transport by GCs and aldosterone was shown to require direct stimulation of the expression of specific ion transporters, including the epithelial sodium channel (34, 35). We investigated the mRNA expression of the α subunit of ENaC (SCNN1A) in BEAS-2B cells. Although aldosterone stimulates the expression of ENaC in renal epithelial cells (44), no such regulation by aldosterone was evident in BEAS-2B cells. Moreover, SCNN1A induction by HC was completely inhibited by the GR antagonist RU486 but was unaffected by the MR antagonist spironolactone, confirming that this effect required GR binding in airway epithelial cells.

Low-efﬁcacy agonists can bind and activate target receptors to produce lower levels of agonist response that may manifest as reduced maximal responses. These agonists are typically referred to as partial agonists. One predictable characteristic of a partial agonist is competition with a full agonist for the same receptor. In this circumstance the partial agonist is able to limit the effect of the full agonist over a range of concentrations of each ligand (45). In our experiments, HC impaired the maximal induction of PLZF, GILZ, and MKP-1 mRNA by FP but not of other genes, such as CDKN1C, SCNN1A, and KCNB1, implying, perhaps, different receptor reserves for these genes (31). Furthermore, for a given GC response element–regulated gene, the lower the efﬁcacy of HC, the greater was the extent of antagonism of the full agonist, FP. The implication of this finding is that HC-mediated reductions in the maximum effect of ICSs may interfere with some of the beneﬁcial ICS actions in the airway epithelium. Through limitation of ICS induction of GILZ and MKP-1, cortisol may interfere with the ICS inhibition of the signaling pathways, leading to the release of proinﬂammatory cytokines and chemokinases in the airway epithelium (20, 21). Glucocorticoid transactivation of MKP-1 was also shown to inhibit MAPK- and ERK-signaling pathways (46), which may block the airway epithelial cell migration (22). Thus, cortisol may also modulate the GC effects on the epithelial restitution after epithelial injury in inﬂammation and infection. Cortisol appeared to have the greatest inhibitory effect on FP induction of PLZF. Although this transcriptional repressor is predominantly involved in cell cycle and differentiation processes in different types of cells (47), the potential roles of PLZF in the airway epithelium are not well understood. PLZF has been reported to mediate GC antiproliferative and apoptotic actions in lymphocytes (24, 48) and to modulate inﬂammatory responses to pathogens through repression of NF-κB pathways in bone marrow–derived macrophages (49). Limiting induction of PLZF by ICS may therefore impair their anti-inflamatory effects.
particularly those mediated through repression of NF-κB signaling pathways in the airway epithelium.

In response to various signals from inflammatory cells, such as lung macrophages, and in response to activation by pathogens and their associated molecular pattern products (50), the airway epithelium can release GM-CSF, IL-6, IL-8, and other cytokines that initiate and/or amplify inflammation through recruitment of eosinophils and neutrophils (2, 19, 51). ICSs can repress transcription of genes encoding these proinflammatory cytokines, inhibiting their synthesis and release by the airway epithelium (52, 53). GM-CSF is a

Figure 6. The effects of different concentrations of HC in the growth media in ALI cultures of primary HBECs on induction of genes by Dex (A) and FP (B). Cells were treated with vehicle control or Dex (100 nM) (A) or FP (10 nM) (B) for 24 h in low-HC medium (containing 0.1 μM HC) or normal ALI growth medium (1.4 μM HC). Results are expressed as $2^{\Delta\Delta Ct} \times 10^5$ for PLZF, GILZ, and MKP-1 mRNA and as $3^{\Delta Ct} \times 10^7$ for CDKN1C mRNA and are displayed as a log scale for ease of comparison of baseline and GC-induced changes under different growth conditions. Data are presented as mean and SEM for 4–9 individual donors (2-way ANOVA with repeated measures, Bonferroni post hoc test). Ns, not significant. *P < 0.05, **P < 0.01, ***P < 0.001.
colony-stimulating factor and inflammogen that regulates the accumulation and activity of neutrophils at the inflammatory site (54), additionally promoting their survival (55). Asthmatic airway epithelium expresses higher levels of GM-CSF protein than non-diseased airway epithelium (56). Furthermore, IL-17 and TNF-α-induced GM-CSF release from HBECs in vitro promotes neutrophil survival (57).

**Figure 7.** The effect of different HC concentrations on the induction of PLZF and GILZ by Dex in the ALI cultures of primary HBECs from healthy donors and from donors with asthma and COPD. Primary HBECs from normal, healthy donors (NHBE) and from donors with asthma and COPD were seeded onto 0.4-μm-pore Transwell membrane coated with type-I rat collagen and cultured at ALI for up to 27 d. Cells were treated with vehicle control or Dex (100 nM) for 4 h in low-HC medium (containing 0.1 μM HC) or ALI growth medium (1.4 μM). The result is expressed as $2^{ΔΔCt} \times 10^5$. Open symbols represent vehicle; filled symbols represent Dex-treated cells from individual healthy donors (circle), donors with asthma (square), and donors with COPD (triangle).

**Figure 8.** Comparison of the effects of Dex on gene expression in mouse tracheal epithelium and lung tissue. Mice were treated with vehicle control (10% DMSO/90% peanut oil) or 1 mg/kg Dex intraperitoneally for 5 d, after which total mRNA was extracted and gene expression was measured by qRT-PCR in tracheal epithelium and lung. Results are expressed as $2^{ΔΔCt} \times 10^5$ for PLZF mRNA and as $2^{ΔΔCt} \times 10^7$ for IL-6 mRNA and are displayed as a log scale for ease of comparison of baseline and Dex-induced changes between tracheal epithelium and lung tissue. Data are presented as means ± SEM for 4 mice per treatment group (Unpaired Student’s t test). *P < 0.05.
associated asthma phenotype is considered to be neutrophil predominant and steroid resistant (58). In our study, inhibition by FP of the TNF-α–induced GM-CSF release was compromised by HC. Moreover, in the presence of HC, GM-CSF levels were maintained in the concentration range known to promote neutrophil numbers and activity (Supplemental Table 2) (57). Thus, cortisol may contribute to steroid insensitivity and disease severity in patients with asthma.

Although the BEAS-2B cell line originated from a normal human bronchial epithelium and is responsive to inflammatory stimuli, it is adenovirus transformed and fails to replicate many of the characteristics of cell differentiation, such as mucus production and the development of beating cilia in vitro. More importantly, perhaps, because it is derived from one human donor, it does not give insight into the natural biologic variation observed between individuals. To assess a physiologic model exhibiting a differentiated phenotype with goblet and ciliated epithelial cells, we cultured primary HBECs at ALI to form an organotypic culture. Because cortisol is essential for the differentiation of the primary bronchial epithelial cells (1), we used medium containing 1.4 μM HC to generate and maintain the ALI organotypic cultures. Induction of genes by both Dex and FP was remarkably constrained in ALI cultures maintained in the growth medium with 1.4 μM HC compared with those exposed to low–HC (0.1 μM) medium for 24 h, strongly supporting the relevance of the partial agonist activity of cortisol to human bronchial epithelium in situ.

In the airways of humans, the pseudostratified epithelium containing basal and fully differentiated ciliated and goblet cells is distributed from the trachea through the terminal bronchiolar. However, in rodents, the presence of basal cells in the airway epithelium is mostly limited to the trachea (59). Therefore, we obtained tracheal airway epithelial cells and lung tissue containing alveolar epithelium from mice treated for 5 d with Dex to contrast gene regulation in these tissues. In contrast to the induction of PLZF mRNA and the repression of IL-6 in lung tissue, there was no mRNA induction or repression of IL-6 mRNA by Dex in the tracheal epithelium. IL-6 release by both inflammatory and epithelial cells contributes to the pathology of chronic respiratory diseases (60–62). IL-6 is generally considered to be strongly regulated by GCs (63, 64), but several studies show circumstances in which GC regulation of IL-6 is impaired (65, 66). IL-6 mRNA regulation by Dex was observed in the lung but not in the tracheal epithelium, even though the cell types comprising these tissues are expected to be exposed to similar, if not identical, concentrations of Dex. This difference in tissue response was predicted from airway epithelial cell pharmacology and may be attributable to an inhibitory effect of circulating levels of cortisol, the physiologic GC in the murine epithelia. Nevertheless, FP inhibition of TNF-α–induced IL-6 protein levels in BEAS-2B cells was substantial and was not affected by HC. These observations may reflect the different time course of the exposures and the more complex in vivo microenvironment of the epithelium, including the use of an inflammogen to elevate IL-6 expression in the BEAS-2B experiments. Further insights may be available from studies using adrenalectomy or different stressors to explore a wider range of corticosterone levels. We measured corticosterone in mice maintained under standard conditions, including daily handling and injections, to be in the range of 0.5–1 μM/L (Supplemental Fig. 5). These concentrations are similar to those of cortisol used in the human cell experiments, and, because corticosterone and cortisol have similar affinity for and activity on GR, these observations further support the relevance of our findings.

Physiologic total plasma cortisol levels show circadian rhythm ranging from a trough of ~50 nM to peaks of ~0.5 μM in healthy individuals and in individuals with asthma (11) and reaching 1–2 μM in periods of acute stress (67). We selected the concentrations of HC (0.1–10 μM) that are within the ranges encountered physiologically. The highest HC concentration appeared to produce maximum transactivation of the genes encoding PLZF, MKP-1, and CDKN1C. However, bioavailable cortisol levels may differ from the circulating cortisol levels in plasma due to influences such as the activity of the enzymes, 11β-hydroxysteroid dehydrogenases (11β-HSD) type 1 and 2, which control the conversion of bioactive cortisol to and from its inactive metabolites (68). The expression of type 2 11β-HSD, which is principally accountable for inactivation of cortisol and synthetic GCs, has been demonstrated in the airway epithelium of patients with and without asthma (69). Cortisol levels in plasma are also controlled by corticosteroid-binding globulin (CBG), which binds 90% of total cortisol in plasma and limits its delivery to tissues and cells (70). The CBG concentration in the extracellular fluid is lower than in plasma (71). Therefore, because the precise range of cortisol concentrations at the receptors in tissues is not known, we consider it justified to examine a range that includes those reported in health and under stressed conditions. Acute stress activates the HPA axis, doubling total plasma cortisol concentrations (6). Furthermore, pathogen-induced stresses, such as viral and bacterial infections, induce the production of proinflammatory cytokines, including TNF-α, IL-1, and IFNs, that directly activate the HPA axis, leading to elevation of adrenocorticotropic hormone and increased cortisol release (7). Cortisol availability may be further increased during inflammation by the activity of neutrophil elastase and serine proteases, which cleave CBG (72). The HC concentration range used in this study therefore spans the physiologic range. Acute stress also leads to substantial increases in circulating adrenaline levels (73). Adrenaline has been shown to contribute to goblet cell metaplasia though activation of β2-adrenoceptors in a murine model of asthma (74). Because adrenaline and cortisol are elevated simultaneously in vivo, ascertaining their combined influences on ICS sensitivity will be of interest in future studies.

The limiting effect of cortisol on gene transactivation by synthetic GCs may not be completely explained by the classic partial agonist activity of cortisol and GR occupancy. Upon ligand binding, GR monomers form GR–GR homodimers, which translocate to the nucleus and bind to target DNA, activating transcription of genes (36). However, the ability of GR to form both
homo- and heterodimers before their binding to the GC-responsive elements on target DNA may also influence GC signaling through GR. For example, heterodimers formed from different GR isoforms, GRα-GRβ, are known to have limited ability to transactivate genes compared with GRα homodimers (75). GR-MR heterodimers are also shown to have different properties in activating GR-signaling pathways compared with their respective homodimers (76). Our findings prompt the question of whether binding of cortisol and synthetic GC to cytosolic GR monomers may generate a heterodimer GR (GR(Synthetic GC)GR(Cortisol)) that has a distinct signaling effect to GR homodimers bound uniquely to either synthetic GC (GR(Synthetic GC)GR(Synthetic GC)) or endogenous cortisol (GR(Cortisol)GR(Cortisol)) (Fig. 9). Such “heterodimers” may have lower transactivation activity than the homodimers, with limited activation of gene transcription even in the presence of higher concentrations of synthetic GCs.

In summary, our observations suggest that the physiologic GC cortisol acts like a partial agonist at the cortisol limit synthetic GC actions in the airway epithelium.

**Figure 9.** The hypothesis of the unique GR(Synthetic GC)GR(Cortisol) “heterodimer” formation. The limiting effect of cortisol on the gene transactivation by synthetic GCs may not be completely explained by the classic partial agonist activity of cortisol and the GR occupancy. The binding of cortisol and synthetic GC to cytosolic GR monomers may generate a heterodimer GR (GR(Synthetic GC)GR(Cortisol)) that has a distinct signaling effect on GR homodimers bound uniquely to either synthetic GC (GR(Synthetic GC)GR(Synthetic GC)) or endogenous cortisol (GR(Cortisol)GR(Cortisol)) (Fig. 9). “Hetero” here denotes the different shape and electronic properties of the GRα when bound to cortisol compared with the synthetic GC. Such “heterodimers” may have lower transactivation activity than the homodimers, with limited activation of gene transcription, even in the presence of higher concentrations of synthetic GCs.

**Figure 10.** The effect of the physiologic and stress-induced levels of cortisol on the response to synthetic GCs in the airway epithelium. Cortisol acts like a partial agonist at the GR in the airway epithelium. In the range of physiologic concentrations (which vary with the circadian rhythm and are controlled by the activity of HPA axis, CBG, and enzymes 11β-HSD), cortisol may limit GC-induced changes in gene expression, which is beneficial for their anti-inflammatory actions. Moreover, cortisol may compromise the efficacy of therapeutically useful GCs, particularly when its levels are elevated by the physiologic, psychologic, or pathogen-induced stresses.
GR in the airway epithelium, limiting synthetic GC-induced changes in gene expression (Fig. 10). Because some of the beneficial anti-inflammatory effects of ICSs/oral GCs on the epithelium are mediated through these gene expression pathways, cortisol may compromise the efficacy of therapeutically useful GCs, particularly when its levels are elevated by stress.

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AUTHOR CONTRIBUTIONS

D. Prodanovic, C. R. Keenan, and A. G. Stewart designed the research; D. Prodanovic, C. R. Keenan, M. J. Lew, and A. G. Stewart analyzed the data; D. Prodanovic, S. Langenbach, M. Li, and Q. Chen performed the research; and D. Prodanovic and A. G. Stewart wrote the paper.

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