

The Role of Glucocorticoid Receptor Nuclear Trafficking in Oxidative Stress induced Corticosteroid Insensitivity

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ABSTRACT

Corticosteroids are widely used in the treatment of chronic inflammatory diseases. However, in patients with chronic obstructive pulmonary disease (COPD), cystic fibrosis and a subset of patients with severe asthma, corticosteroids fail to improve the underlying inflammation. Recent advances in the cellular and molecular basis of the pathology in asthma and COPD have revealed several biological targets that may contribute to the relative corticosteroid insensitivity observed in these diseases. These targets include impaired glucocorticoid receptor (GR) nuclear translocation, reduced histone deacetylase (HDAC)-2 activity and heightened pro-inflammatory transcription factor activity.

Although *in vitro* and *in vivo* data support a role for impaired GR nuclear translocation as a potential mechanism of corticosteroid insensitivity in asthma, as of yet, no published data has confirmed this in COPD patients. Furthermore, the mechanisms that govern GR nuclear translocation are mostly unknown. There is growing evidence that excess reactive oxygen species (ROS) generated by inflammatory and immune cells of the lung leads to oxidative stress, which plays a crucial role in the amplification of redox-sensitive pro-inflammatory transcription factors that leads to corticosteroid insensitivity.

In this PhD, I investigated the role of GR nuclear trafficking in oxidative stress-induced corticosteroid insensitivity. In a monocytic cell line, U937 cells, we showed that hydrogen peroxide (H₂O₂), an oxidant, induced intracellular and mitochondrial oxidative stress. This increase in oxidative stress was associated with enhanced IL-

1 β -induced NF κ B activity and IL-1 β -induced CXCL8 release, pro-inflammatory markers shown to be elevated in COPD. Pre-treatment with H₂O₂ significantly reduced corticosteroid induced GR nuclear import for budesonide (BUD) and fluticasone propionate (FP). This reduction in GR nuclear import was associated with reduced suppression of CXCL8. In our *ex vivo* study in patients with severe asthma and COPD, we observed similar findings, where BUD was unable to induce GR binding to glucocorticoid response elements (GRE) in patients with severe asthma and also, BUD at a low concentration was unable to suppress CXCL8 release in patients with COPD. The degree of corticosteroid insensitivity observed in our patients with severe asthma and COPD was limited to the above mentioned observations. We then investigated the nuclear transport receptors that govern nucleocytoplasmic shuttling of macromolecules in U937 cells and revealed that importin-7 is essential for corticosteroid-induced GR nuclear import. Further experiments identified that RanGTP, an essential co-factor for importin-7 mediated GR nuclear import, was reduced in our oxidative stress-induced corticosteroid insensitivity model. This novel finding suggests that the importin-7-RanGTP complex may be a potential target to overcome corticosteroid insensitivity in COPD patients.

DECLARATION

This thesis is the result of my own work. The work was carried out at the National Heart and Lung Institute, Airway Disease Section, Imperial College, Faculty of Medicine, London. This work was funded by an educational grant from R&D Department, GlaxoSmithKline plc, Greenford, UK.

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I would like to end with a quote, which has been the source of my encouragement during the highs and lows of my PhD; *'And know that success comes with patience, relief with affliction, and ease with hardship'* (Sayidduna Muhammad).

TABLE OF CONTENTS

	Page
TITLE	1
ABSTRACT	2
ACKNOWLEDGMENTS	5
TABLE OF CONTENTS	6
LIST OF FIGURES	15
LIST OF TABLES	20
ABBREVIATIONS	21
CHAPTER 1: GENERAL INTRODUCTION	25
1.1. Introduction	25
1.2. COPD definition	26
1.3. Burden of disease	26
1.4. Risk factors	27
1.5. Pathology	28
1.6. Comparison of Asthma and COPD	31
1.7. Oxidative stress	33
1.7.1. Cellular-derived ROS	34

1.7.2.	Environmental-derived ROS	35
1.7.3.	ROS-activated inflammation in COPD	36
1.8.	Corticosteroids	38
1.8.1.	Inhaled corticosteroids and long-acting β ₂ -agonist combination therapy	40
1.9.	Molecular mechanisms of corticosteroids	42
1.9.1.	GR nuclear translocation	44
1.10.	Corticosteroid resistance	49
1.10.1.	Why do inhaled corticosteroids fail in COPD?	50
1.11.	Potential mechanisms for corticosteroid resistance	51
1.11.1.	Histone deacetylase-2	51
1.11.2.	Defective GR binding and nuclear translocation	53
1.12.	Hypothesis	55
CHAPTER 2: MATERIALS AND METHODS		57
2.1	U937 cell culture	57
2.2	Compounds	57
2.3	MTT assay	58
2.4	MitoSOX Red fluorescence (Mitochondrial ROS)	58
2.5	DCF assay	58
2.6	Western blotting	59
2.6.1.	Cytoplasmic and nuclear extraction	59

2.6.2.	Bradford assay	59
2.6.3.	Polyacrylamide gel electrophoresis	60
2.6.4.	iBlot dry Western transfer	60
2.6.5.	Protein immunodetection	61
2.6.6.	Band quantification	61
2.6.7.	Primary and secondary antibodies	62
2.7	Enzyme-linked immunosorbent assay (ELISA)	62
2.7.1.	CXCL8 and IL-6	62
2.8	DNA promoter binding assays	63
2.8.1.	GR-GRE assay	63
2.8.2.	Nrf2 DNA binding assay	64
2.8.3.	p65 (NF- κ B) DNA binding assay	64
2.9	Quantitative real time transcription polymerase chain reaction (RT-qPCR)	65
2.9.1.	mRNA expression	65
2.9.2.	Reverse transcription	66
2.9.3.	Quantitative RT-PCR	67
2.10	Transfection (importin-7 knockdown)	68
2.11	Ran activation assay	68
2.11.1.	Cell lysates	68
2.11.2.	GTP γ S/GDP (positive and negative controls)	69
2.11.3.	Ran pull-down assay	69

2.11.4. Western blotting	69
2.12 Blood monocyte extraction	70
2.13 Replicate experiments and statistical analysis	70
CHAPTER 3: COMPARISON OF SHORT- AND LONG-ACTING CORTICOSTEROIDS	72
3.1. Introduction	72
3.2. Experimental protocols	77
3.2.1. GR nuclear translocation	77
3.2.2. CXCL8 ELISA	78
3.2.3. <i>DUSP1</i> mRNA expression	79
3.2.4. Corticosteroid washout	79
3.3. Results	80
3.3.1. Comparison of BUD-, FP- and FF-induced GR nuclear translocation	80
3.3.2. Cytokine secretion	82
3.3.2.1. Corticosteroids suppress CXCL8 secretion with varying potencies	82
3.3.2.2. LABAs enhance corticosteroid suppression Of CXCL8	83
3.3.3. <i>DUSP1</i> expression	86
3.3.4. Corticosteroid washout	87

3.3.4.1. FF maintains GR nuclear localisation	87
Following 20 h washout	
3.3.4.2. FF maintains IL-1 β -induced CXCL8	88
Suppression following 20 h washout	
3.3.5 LMB enhances FF-induced GR nuclear localisation	89
3.3.6 LMB enhances FF-induced GR-GRE binding	91
3.3.7 LMB enhances BUD-induced GR nuclear localisation	91
3.3.7.1 Continual drug contact	91
3.3.7.2 Drug washout	93
3.4. Discussion	94
 CHAPTER 4: OXIDATIVE STRESS-INDUCED	 100
 CORTICOSTEROID INSENSITIVITY	
4.1. Introduction	100
4.2. Experimental protocols	103
4.2.1. Intracellular ROS	103
4.2.2. Mitochondrial ROS	105
4.2.3. Involvement of oxidative stress in cytokine synthesis and secretion	105
4.2.4. NF- κ B DNA-binding	105
4.2.5. Oxidative stress on corticosteroid activity and function	106

4.3. Results	107
4.3.1. H ₂ O ₂ causes oxidative stress	107
4.3.1.1. H ₂ O ₂ induces intracellular ROS	107
4.3.1.2. H ₂ O ₂ induces mitochondrial ROS	108
4.3.2. Nrf2 activation	109
4.3.3. H ₂ O ₂ enhances markers of inflammation	110
4.3.3.1. H ₂ O ₂ enhances IL-1 β -induced CXCL8	110
4.3.3.2. H ₂ O ₂ enhances IL-1 β -induced NF- κ B activation	111
4.3.4. Oxidative stress impairs BUD- and FP-driven suppression of IL-1 β -induced CXCL8	112
4.3.5. Oxidative stress impairs BUD- and FP-induced GR nuclear localisation	115
4.3.6. Oxidative stress impairs FP-induced GR-GRE binding	117
4.3.7. Oxidative stress on BUD- and FP-induced <i>DUSP1</i> mRNA expression	118
4.4. Discussion	120
CHAPTER 5: IMPORTIN-7 MEDIATED GR NUCLEAR IMPORT	125
5.1. Introduction	125
5.2. Experimental protocol	128

5.2.1.	Importin-7 knockdown	128
5.2.2.	The role of importin-7 on corticosteroid-induced GR nuclear translocation	128
5.2.3.	The role of importin-7 on corticosteroid-induced GR nuclear translocation	129
5.2.4.	Importin-7 & RanGTP subcellular localisation under oxidative stress	129
5.2.5.	Corticosteroid-induced GR nuclear localisation under nuclear export inhibition	130
5.3.	Results	132
5.3.1.	Importin-7 siRNA reduced both cytoplasmic and nuclear levels of importin-7	132
5.3.2.	Importin-7 siRNA reduced corticosteroid-induced GR nuclear localisation	133
5.3.3.	Importin-7 siRNA reduces corticosteroid -induced suppression of CXCL8	134
5.3.4.	Effects of oxidative stress on cytoplasmic importin-7	135
5.3.5.	Oxidative stress reduces nuclear RanGTP levels	136
5.4.	Discussion	138

CHAPTER 6: CORTICOSTEROID SENSITIVITY IN BLOOD MONOCYTES FROM SEVERE ASTHMATICS AND COPD PATIENTS

6.1. Introduction	141
6.2. Experimental protocols	143
6.2.1. Screening visit	143
6.2.2. Study design	143
6.2.3. Monocytes from blood	144
6.2.4. Corticosteroid-induced GR activation	144
6.2.5. Enzyme-linked immunosorbent assay (ELISA)	145
6.2.6. Statistical methods	145
6.3. Results	146
6.3.1. Patient demographics	146
6.3.2. Corticosteroid-induced GR activation in blood monocytes	147
6.3.2.1. Comparison of BUD- and FF-induced GR-GRE binding	147
6.3.2.2. BUD and FF-induced GR-GRE binding in severe asthma and COPD	148
6.3.3. Pro-inflammatory cytokine secretion in monocytes	149
6.3.4. Corticosteroid-mediated suppression of CXCL8	152
6.3.4.1. Comparison of BUD and FF in monocytes from healthy volunteers	152

6.3.4.2. Corticosteroid suppression of CXCL8 in severe asthma and COPD	154
6.3.5. Corticosteroid suppression of IL-6 in severe asthma and COPD	157
6.3.6. LABA effects on corticosteroid-mediated suppression of CXCL8	160
6.4. Discussion	163
CHAPTER 7: GENERAL DISCUSSION & FUTURE DIRECTIONS	167
REFERENCE	177
PUBLICATIONS	204

LIST OF FIGURES

Figure	Description	Page
1.5	The cellular and molecular profile of COPD	31
1.8	Pharmacotherapy management of COPD	40
1.9a	Ligand binding domain of the glucocorticoid receptor (GR)	42
1.9b	Corticosteroid suppression of pro-inflammatory genes	44
1.9.1a	Potential mechanism of GR nuclear import	47
1.9.1b	Potential mechanism for GR nuclear export	49
1.12	Potential mechanisms of reduced GR nuclear translocation	56
3.1	Comparison of the structures and GR affinities of budesonide, fluticasone propionate and fluticasone furoate	76
3.2.1.1	Effect of DMSO (0.25%) on GR nuclear translocation	77
3.2.1.2	Effect of varying concentrations of fluticasone propionate (FF), 10^{-11} - 10^{-7} M, on GR nuclear localization	78
3.2.2	Effect of IL-1 β on CXCL8 secretion in U937 monocytes	79
3.3.1a	Effect of budesonide (BUD), fluticasone propionate (FP) and fluticasone furoate (FF), all at 10^{-9} M, on GR nuclear translocation.	81
3.3.1b	Effect of budesonide (BUD), fluticasone propionate (FP) and fluticasone furoate (FF), all at 10^{-9} M, on GR nuclear translocation	82
3.3.2.1	Effect of budesonide (BUD), fluticasone propionate (FP) and fluticasone furoate (FF) on IL-1 β -induced CXCL8	83

3.3.2.2	Effect of budesonide (BUD), fluticasone propionate (FP) and fluticasone furoate (FF), alone and in combination with their respective LABAs on IL-1 β -induced CXCL8.	85
3.3.3	Effect of budesonide (BUD), fluticasone propionate (FP) and fluticasone furoate (FF) on DUSP-1 mRNA induction.	86
3.3.4.1	Effect of budesonide (BUD), fluticasone propionate (FP) and fluticasone furoate (FF) on GR nuclear localisation	88
3.3.4.2	Effect of budesonide (BUD), fluticasone propionate (FP) and fluticasone furoate (FF) on IL-1 β -induced CXCL8.	89
3.3.5.1	Effect of leptomycin B (LMB) on glucocorticoid receptor (GR) subcellular localisation.	90
3.3.5.2	Effect of leptomycin B (LMB) on fluticasone fuorate (FF)-induced GR nuclear localisation.	90
3.3.6	Effect of leptomycin B (LMB) on fluticasone fuorate (FF)-induced GR-GRE binding	91
3.3.7.1	Effect of leptomycin B (LMB) on budesonide (BUD)-induced GR nuclear localisation.	92
3.3.7.2	Effect of leptomycin B (LMB) on budesonide (BUD)-induced GR nuclear localisation post 20 h washout.	93
4.2.1	Effect of hydrogen peroxide (H ₂ O ₂) on cell viability.	103
4.2.2	Effect of hydrogen peroxide (H ₂ O ₂) on cell viability	104
4.2.5	Effect of hydrogen peroxide (H ₂ O ₂) and IL-1 β on glucocorticoid receptor (GR) subcellular localisation in resting cells.	106
4.3.1.1	Effect of hydrogen peroxide (H ₂ O ₂) on intracellular ROS.	107

4.3.1.2	Effect of H ₂ O ₂ on mitochondrial superoxide levels	109
4.3.2	Effect of H ₂ O ₂ in combination with IL-1 β on Nrf2 transactivation.	110
4.3.3.1	Effect of H ₂ O ₂ on IL-1 β -induced CXCL8.	111
4.3.3.2	Effect of H ₂ O ₂ on IL-1 β -induced NF- κ B transactivation.	112
4.3.4.1	Effect of H ₂ O ₂ on IC ₅₀ values for corticosteroid-driven suppression of IL-1 β -induced CXCL8.	113
4.3.4.2	Effect of H ₂ O ₂ on corticosteroid suppression of IL-1 β -induced CXCL8.	114
4.3.5.1	Effect of H ₂ O ₂ or IL-1 β alone on corticosteroid-induced GR nuclear localisation.	116
4.3.5.2	Effect of H ₂ O ₂ in combination with IL-1 β on corticosteroid-induced GR nuclear localisation.	117
4.3.6	Effect of H ₂ O ₂ on FP-induced GR-GRE binding.	118
4.3.7	Effect of H ₂ O ₂ and IL-1 β on corticosteroid-induced <i>DUSP1</i> mRNA expression.	119
5.1	Proposed mechanism of reduced corticosteroid-induced GR nuclear translocation.	127
5.2.1	Effect of electroporation with siRNA on cellular activity	128
5.2.4	Intracellular localisation of Importin-7.	129
5.2.5.1	Effect of 4 h Leptomycin B (LMB) treatment on cell viability. Serum	130
5.2.5.2	Effect of 4 h leptomycin B (LMB) treatment on cell viability	131
5.3.1	Effect of importin-7 siRNA transfection.	133

5.3.2	Effect of importin-7 siRNA transfection on FP-induced GR nuclear localisation.	134
5.3.3	Effect of importin-7 siRNA transfection on FP-mediated suppression of IL-1 β -induced CXCL8.	135
5.3.4	No effect of H ₂ O ₂ and IL-1 β alone or in combination on cytoplasmic importin-7 levels.	136
5.3.5	Effect of H ₂ O ₂ and IL-1 β on nuclear RanGTP levels.	137
6.2.3	Fluticasone furoate (FF)-induced GR-GRE binding	145
6.3.2.1	Comparison of budesonide (BUD)- and fluticasone furoate (FF)-induced GR-GRE binding blood monocytes derived from blood of healthy volunteers	147
6.3.2.2.1	Comparison of budesonide (BUD)-induced GR-GRE binding monocytes derived from blood of healthy volunteers, severe asthmatics and COPD patients	149
6.3.2.2.2	Comparison of fluticasone furoate (FF)-induced GR-GRE binding monocytes derived from blood of healthy volunteers, severe asthmatics and COPD patients.	149
6.3.3.1	Basal and IL-1 β -induced CXCL8 secretion from monocytes.	151
6.3.3.2	Basal and IL-1 β -induced IL-6 secretion from monocytes.	151
6.3.4.1.1	Comparison of budesonide (BUD)- and fluticasone furoate (FF)-mediated suppression of IL-1 β -induced CXCL8 secretion from monocytes	153
6.3.4.1.2	Comparison of Budesonide (BUD) and fluticasone furoate (FF)-mediated suppression of IL-1 β -induced IL-6 secretion from	153

	monocytes.	
6.3.4.2.1	Budesonide (BUD)-mediated suppression of IL-1 β -induced CXCL8 in blood from healthy volunteers, severe asthmatics and COPD patients.	155
6.3.4.2.2	Fluticasone furoate (FF)-mediated suppression of IL-1 β -induced CXCL8 in blood monocytes from healthy volunteers, severe asthmatics and COPD patients.	156
6.3.4.2.3	Comparison of Budesonide (BUD)- and fluticasone furoate (FF)-mediated suppression of IL-1 β -induced CXCL8 in healthy volunteers, severe asthmatics and COPD patients.	157
6.3.5.1	Budesonide (BUD)-mediated suppression of IL-1 β -induced IL-6 in blood monocytes from healthy volunteers, severe asthmatics and COPD patients.	158
6.3.5.2	Fluticasone furoate (FF)-mediated suppression of IL-1 β -induced IL-6 in blood monocytes from healthy volunteers, severe asthmatics and COPD patients.	159
6.3.5.3	Comparison of Budesonide (BUD)- and fluticasone furoate (FF)-mediated suppression of IL-1 β -induced IL-6 in healthy volunteers, severe asthmatics and COPD patients.	159
6.3.6.1	Comparison of Budesonide (BUD) alone and in combination with formoterol (FORM).	162
6.3.6.2	Comparison of Fluticasone furoate (FF) alone and in combination with vilanterol trifenate (VI).	162
7.1	Potential role of importin-7 pathway in oxidative stress-induced corticosteroid insensitivity.	173

LIST OF TABLES

Table	Description	Page
1.1	Comparison of the airway inflammation in patients with asthma, COPD and in smoking asthmatics	33
2.1	Primary and secondary antibodies	62
2.2	Genomic DNA elimination components	66
2.3	reverse-transcription master mix components	66
2.4	List of assay probes used	67
2.5	Details of anti-Ran antibody	70
6.1	Patient demographics	146

ABBREVIATIONS

α-1-AT	Alpha-1-antitrypsin
AP-1	Activator protein-1
AR	Androgen receptor
β-ME	β -mercaptoethanol
BAL	Bronchoalveolar lavage
BSA	Bovine serum albumin
BUD	Budesonide
Ca²⁺	Calcium
cDNA	Complementary DNA
COPD	Chronic obstructive pulmonary disease
DBD	DNA binding domain
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescent
ELISA	Enzyme linked immunosorbent assay
ERK	Extracellular-signal-regulated kinase
FCS Foetal Calf Serum	Foetal Calf Serum
FEV₁	Forced expiratory volume in one second
FF	Fluticasone furoate
FP	Fluticasone propionate
FORM	Formoterol
FVC	Forced vital capacity
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GILZ	Glucocorticoid-induced leucine zipper
GINA	Global Initiative for Asthma

GR	Glucocorticoid receptor
GM-CSF	Granulocyte macrophage-colony stimulating factor
GOLD	Global Initiative for Obstructive Lung Disease
GRE	Glucocorticoid response element
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
HBSS	Hanks balanced salt solution
HDAC	Histone deacetylase
HRP	Horse radish peroxidase
HSP	Heat shock protein
IκB	Inhibitor of NF- κ B
ICS	Inhaled corticosteroids
IC₅₀	Inhibitory concentration that gives 50 % suppression
IL-1β	Interleukin-1 beta
IL	Interleukin
IP	Immunoprecipitation
JNK	Jun amino-terminal kinases
KD	Knockdown
LABA	Long-acting β 2-agonists
LBD	Ligand binding domain
LMB	Leptomycin B
MAPK	Mitogen-activated protein kinase
MKP-1	MAPK phosphatase-1
mRNA	Messenger RNA

NADPH	Nicotinamide adenine dinucleotide phosphate
nGRE	Negative GRE
NF-κB	Nuclear factor- κ B
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PD	Pharmacodynamic
PK	Pharmacokinetic
PEF	Peak expiratory flow
RANTES	Regulated upon activation normal T cell expressed and secreted
Ran	RAs-related Nuclear protein
ROS	Reactive oxygen species
RNA	Ribonucleic acid
RT	Reverse transcription
RT-PCR	Real time polymerase chain reaction
SABA	Short-acting β_2 -agonists
SAMA	Short-acting muscarinic receptor-antagonists
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
siRNA	Small interfering (or silencing) RNA
SIRT	Sirtuin
SLPI	Secretory leukocyte peptidase inhibitor
SLM	Salmeterol
TBS	Tris-buffered saline
TBST	Tris-buffered saline-tween

TGFβ	Transforming growth factor-beta
TNFα	Tumour necrosis factor alpha
WHO	World Health Organisation

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction:

Asthma and chronic obstructive pulmonary disease (COPD) are common chronic inflammatory lung diseases that are each characterised by different types of inflammation. Interestingly, some patients with asthma that smoke share a similar pathology to patients with COPD. Differences in the inflammatory patterns between these lung diseases are predominantly determined by the type of immune and inflammatory cells involved. These cells drive the inflammatory cascade that gives rise to the distinct patterns of structural changes between these diseases. Structural changes in the respiratory tract result in airways obstruction and airflow limitation, which is usually reversible in asthma, but is progressive and mainly irreversible in COPD.

Corticosteroids are widely used in the management of asthma and COPD to treat the underlying inflammation. However, in sharp contrast to patients with asthma, they are less effective in COPD patients. These differences in corticosteroid response between asthma and COPD may be partly due to pathological differences at the cellular and molecular level. Recent evidence suggests several molecular mechanisms may contribute to corticosteroid insensitivity in COPD patients. These include reduced histone deacetylase-2 (HDAC2) activity, impaired glucocorticoid receptor (GR) nuclear translocation and heightened pro-inflammatory transcription factor activity. Recent evidence in corticosteroid-resistant asthmatic patients

suggests GR nucleocytoplasmic shuttling, essential for corticosteroid activity, may also be impaired. The purpose of this work is to determine the role of GR nuclear trafficking in corticosteroid insensitivity.

1.2 COPD Definition

The Global initiative for chronic Obstructive Lung Disease (GOLD) defines COPD as a common preventable and treatable disease, which is characterised by persistent airflow limitation that is usually progressive and associated with an enhanced inflammatory response in the airways and the lung to noxious particles and gases. Exacerbations and co-morbidities contribute to the overall severity in individual patients (GOLD).

1.3 Burden of disease

COPD is the fourth most common cause of mortality, and projected to rank fifth in global burden of disease according to the World Health Organization (Barnes, 2007, Rabe et al., 2007). The general prevalence of COPD is 10%, although there are reports of a much higher disease prevalence (Mannino and Buist, 2007). Prevalence surveys conducted in numerous countries suggest approximately one quarter of adults aged over 40 years may have COPD (Menezes et al., 2005, Chapman et al., 2006, Lopez et al., 2006b). Approximately one in four individuals during their lifetime are likely to be diagnosed with COPD. In addition, 50% of smokers who reach 80 years of age will develop COPD (Gershon et al., 2011). Furthermore, a meta-analysis of several studies conducted in 28 countries revealed that the prevalence of COPD was considerably greater amongst smokers and ex-smokers than in non-smokers (Halbert et al., 2006).

COPD is a major global cause of morbidity and mortality. In the year 2000, there were approximately 8 million outpatient and 1.5 million emergency department COPD visits, with more than 600,000 hospitalisations in the US. In most parts of the world, outpatient COPD consultations considerably outnumbered those for asthma, lung cancer, pneumonia and tuberculosis (Schellevis et al., 1994). The increasing epidemic of smoking and changing population demographics has projected COPD to become the third leading cause of death worldwide by 2020. The increasing burden of COPD has also presented a major economic and social burden where, in the European Union, COPD accounts for more than 50% (>€38 billion Euros) of the total costs of respiratory disease (Loddenkemper et al., 2003). Furthermore, with increasing severity of COPD disease; the cost of care has been shown to increase considerably (Rabe et al., 2007).

1.4 Risk Factors

Globally, cigarette smoke is the most common risk factor and cause of COPD. Smokers have a higher prevalence of respiratory symptoms and lung function abnormalities than non-smokers (Rabe et al., 2007, Eisner et al., 2005). Moreover, passive exposure to cigarette smoke can also lead to the development of COPD, due to an increase in total burden of inhaled particles and gases in the lungs (Dayal et al., 1994, Eisner et al., 2005). In a study involving 4,197 never-smoking adults, individuals subjected to passive smoking had an elevated risk of dyspnoea and wheezing and, an increased risk of symptoms associated with chronic bronchitis. Furthermore, the risk of these respiratory symptoms was elevated with increased passive smoking exposure (Leuenberger et al., 1994).

In developing countries, exposure to indoor pollution due to the burning of biomass fuels is also associated with the development of COPD (Torres-Duque et al., 2008). COPD is a polygenic disease and risk factors for its development mainly arise from a gene-environment interaction and due to differences in genetic predisposition to COPD. Where, one individual with a similar smoking history may develop COPD and the other may not (Sadeghnejad et al., 2007).

1.5 Pathology

Clinically, the hallmark of COPD is progressive airflow limitation that is not fully reversible, which manifests itself primarily as dyspnoea and exercise limitation. These physiological symptoms arise from pathological and structural changes that take place in the proximal and peripheral airways, lung parenchyma and pulmonary vasculature (Hogg et al., 2004). Biologically, COPD is an inflammatory disease of the lungs, characterised by an abnormal inflammatory response to noxious particles and gases, where the extent of the pulmonary inflammation correlates to COPD disease severity (Turato et al., 2002). Inhaled cigarette smoke, and other noxious particles which are rich in reactive oxygen species (ROS), cause lung inflammation and this is amplified in COPD patients (GOLD).

Oxidative stress is an important feature in COPD, where an imbalance between the production of harmful ROS and the ability to repair the resulting damage is believed to be important (Lee and Yang, 2012, Rahman, 2005). It is also recognised there are significant extra-pulmonary effects and co-morbidities in COPD patients that contribute to the severity of the disease (GOLD). However, most clinical trials have

shown that existing medications do not reverse the decline in lung function, which is the key feature of the disease (Barnes, 2000a, Suissa and Barnes, 2009). According to the GOLD guidelines, patients with COPD should be treated with bronchodilators at all stages of the disease, with the addition of inhaled corticosteroids (ICS) in severe (stage III) to very severe (stage IV) COPD patients (Rabe et al., 2007).

The cellular composition of the chronic inflammation in COPD is characterised principally by macrophages, neutrophils and T-lymphocytes. These inflammatory cells are found in elevated levels in the bronchial tree, small airways and lung parenchyma from COPD patients (Hogg et al., 2004). They release a variety of chemotactic factors, pro-inflammatory cytokines and growth factors that further amplify the abnormal inflammatory response in COPD patients (Barnes et al., 2003). Of the cells identified, macrophages play an important role in the pathogenesis of COPD, and are derived from circulating monocytes that migrate in response to chemoattractants such as CCL1 and CCL2. Macrophages are markedly increased in the alveolar space and lungs of patients with COPD. Macrophages release a plethora of inflammatory proteins that further drive the chronic inflammation observed in COPD and give rise to many of the structural changes characteristic of COPD (Barnes, 2004). Historically it has been shown that alveolar macrophages from COPD patients show a degree of corticosteroid insensitivity (Barnes, 2004, Keatings et al., 1997).

In addition to an elevated number of macrophages, COPD patients also have elevated levels of neutrophils and mediators such as tumour necrosis factor-alpha (TNF- α) and chemokine (C-X-C motif) ligand 8 (CXCL8) secretions compared to smoking and non-smoking subjects. Levels of CXCL8 but not TNF- α have been

shown to be significantly higher in sputum from COPD patients compared to patients with asthma (Keatings et al., 1996, Hodge et al., 2011). CXCL8 is a chemoattractant for neutrophils which acts on the CXCR2 receptor that predominantly resides on neutrophils (Pignatti et al., 2005).

Similar to observations in patients with asthma, the transcription factor nuclear factor- κ B (NF- κ B) is found in elevated levels in COPD patients and regulates the secretion of many of the inflammatory mediators. In a study involving 11 active smokers with moderate COPD, p65 (a subunit of NF- κ B) nuclear expression in sputum macrophages, was found to be markedly elevated during clinical exacerbations of the disease compared to baseline levels (Caramori et al., 2003). The activation of NF- κ B is regulated by the I κ B kinase (IKK) complex. In peripheral blood mononuclear cells (PBMCs) of COPD patients, the expression and activation of the alpha isoform of I κ B kinase (IKK) was higher than in asthmatics. Both asthmatics and COPD patients had greater levels of the beta isoform of IKK than controls. Fluticasone propionate (FP) inhibited IKKbeta to a greater extent than IKKalpha (Gagliardo et al., 2011). These differences in the regulation of NF- κ B in asthmatics and COPD patients may partly explain the different responses to corticosteroids in these patients (Barnes, 2010b).

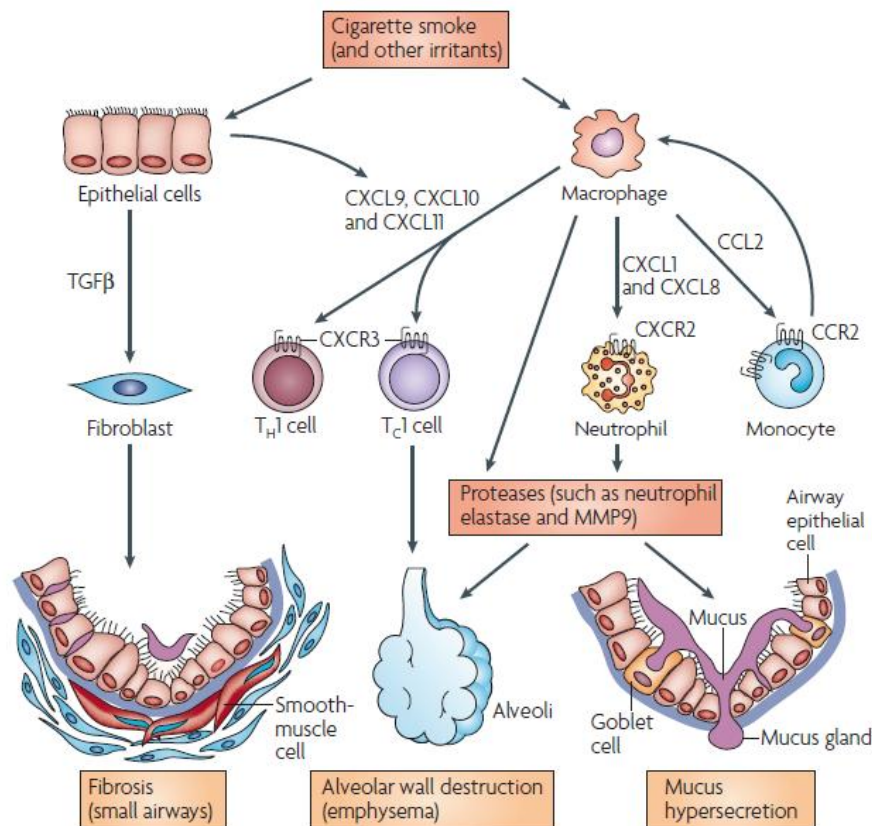


Fig. 1.5 The cellular and molecular profile of COPD (Barnes, 2008).

1.6 Comparison between Asthma & COPD

Chronic inflammation in asthma and COPD is characterised and orchestrated by distinct inflammatory cells, cytokines, chemokines, adhesion molecules, enzymes and receptors. The inflammation in asthma is often described as eosinophilic-led, whereas the disease in COPD is described as neutrophilic-led inflammation. Recent advances in the underlying immunopathology of asthma and COPD have highlighted the importance of different subsets of T cells that may initiate different inflammatory profiles and consequently different structural changes (Brusselle et al., 2011, Lloyd and Hessel, 2010).

However, there are shared similarities in the inflammatory profile and clinical features between these diseases and this has led to the development of the Dutch

hypothesis that proposes asthma and COPD are different forms of the same disease (Postma and Boezen, 2004). In contrast, based on the very distinct cellular and inflammatory profiles observed between the diseases, an opposing hypothesis, the British hypothesis, proposes they are separate entities (Kraft, 2006, Barnes, 2006a). Moreover, asthma is mainly a disease of the large conducting airways, although involvement of the smaller airways in severe forms is well documented, whereas COPD is characteristically a disease of the small airways and lung parenchyma (Barnes, 2000b, Jeffery, 2000). It is important to note that a sub-population of patients with asthma are corticosteroid-insensitive. These corticosteroid-insensitive patients with asthma can resemble some aspects of the inflammatory profile observed in COPD (Wenzel and Busse, 2007, Jatakanon et al., 1999).

In a prospective study over 7.5 years in patients with severe asthma, the annual decline in post-bronchodilator FEV₁ was far greater than the decline in pre-bronchodilator FEV₁ (indicating loss in reversibility) and this was strongly associated with an influx of CD8⁺ T cells ($r = 0.39$, $p = 0.032$) (Kurashima et al., 2006, van Rensen et al., 2005, Cho et al., 2005). Interestingly, it has also been observed that asthmatic patients who smoke also exhibit a degree of corticosteroid resistance and have a similar pathology to COPD patients (Table 1.1). Bronchial biopsies in asthmatic patients who smoke have shown remodelling of the bronchial mucosa, squamous cell metaplasia, and increased expression of subepithelial neutrophil elastase, IFN- γ and intraepithelial CXCL8, similar to that observed in patients with COPD (St-Laurent et al., 2008). Recently, increased expression of arginase I, which is involved in the regulation of airway smooth muscle tone, has also been shown in the airways of smoking asthmatics, which may also contribute to the COPD-like airway changes observed in this disease group (Bergeron et al., 2007).

Cell type	Asthma (mild-to-moderate)	COPD	Smoking Asthmatics
Neutrophils	-	++	+
Eosinophils	+	-	
Macrophages	+	+++	++
T-lymphocytes	Th2++	Tc1+	++
Chemokines	CXCL8	CXCL8+	CXCL8+
Cytokines	IL-4, IL-5 & IL-13	TNF- α	TNF- α
Oxidative stress	-	++	++
Steroid response	++++	-	-

Table 1.1 Comparison of the airway inflammation in patients with asthma, COPD and in smoking asthmatics (St-Laurent et al., 2008, Saetta et al., 2001, Barnes, 2008).

1.7 Oxidative stress

The major environmental risk factor for developing COPD is cigarette smoke, which contains abundant ROS. Cigarette smoke induces oxidative stress in the lungs of smokers and COPD patients, which subsequently leads to amplification of the underlying inflammation (Rahman, 2005). Neutrophils of smokers and COPD patients during exacerbations release more of the free radical superoxide anion ($O_2^{\cdot-}$) (Rahman et al., 1996). Furthermore, the lungs of COPD patients have elevated levels of 4 hydroxy-2-nonenal (4-HNE), a highly reactive product of lipid peroxidation and a key mediator of oxidant-induced cell signalling. In a study involving 23 current or ex-smokers with COPD, 4-HNE levels were higher in airway and alveolar epithelial cells, and neutrophils (Rahman et al., 2002). It was clearly noted that the elevated levels of 4-HNE inversely correlated with FEV₁. Another product of lipid peroxidation, 8-isoprostane, a very biologically active mediator, is also found in

elevated levels in the plasma and bronchoalveolar lavage of patients with COPD and in chronic smokers compared to healthy subjects (Montuschi et al., 2000). Auto-antibodies against carbonyl-modified protein, as a result of oxidative stress, are also elevated in COPD (Kirkham et al., 2011). This suggests oxidative stress may also drive an autoimmune response in COPD.

Fundamentally, the lungs are exposed to either cellular- or environmental-derived ROS (Rahman, 2005, Lopez et al., 2006a). Cellular-derived ROS are enzymatically produced by epithelial and inflammatory cells, under normal physiological conditions of the inflammatory-immune response towards an irritant or pathogen (Rochelle et al., 1998). Cellular-derived ROS is predominantly generated from nicotinamide adenine dinucleotide phosphate oxidase (NAPDH oxidase) and mitochondria. In contrast, environmental sources of ROS include airborne particulate matter from diesel car exhausts, cigarette smoke and oxidant gases, and nitrogen dioxide (Rahman, 2005).

1.7.1 Cellular-derived ROS

In the airspace of COPD patients, activation by inflammatory mediators and subsequent infiltration of inflammatory cells results in the release of intracellular ROS, such as $O_2^{\cdot-}$, which plays an important role as a catalyst for the generation of further cell damaging ROS. Superoxide dismutase (SOD) rapidly converts $O_2^{\cdot-}$ into hydrogen peroxide (H_2O_2). The highly damaging hydroxyl ion ($OH^{\cdot}$) is formed in the presence of Fe^{2+} . Cellular-derived ROS can be generated from several intracellular sources; mitochondrial respiration, the NADPH oxidase system, and xanthine/xanthine oxidase (XO).

Mitochondria play a crucial role in the pathological generation of ROS in disease (Pacher et al., 2007, Kirkham and Rahman, 2006, Al Ghouleh et al., 2011). In a study involving 152 samples from patients who underwent surgical resection for various pulmonary diseases, the frequency of the 4,839 base pair (bp) mitochondrial DNA deletion correlated positively with smoking pack-years history, with current smokers having significantly greater rates of incidence and proportion of the mitochondrial DNA mutation than non-smokers. Moreover, changes in mitochondrial DNA in smokers in comparison to non-smokers were associated with increased levels of lipid peroxidation. Multiple regression analysis revealed smoking history, lung tissue lipid peroxidation, and the FEV₁/FVC ratio correlated positively with the level of the mitochondrial DNA mutation (Fahn et al., 1998). Abnormal mitochondria function has also been reported in mitochondria in muscle biopsies of the vastus lateralis and external intercostalis from COPD patients (Puente-Maestu et al., 2009), where it has been noted that muscle fatigue and weakness worsens with disease (Polkey, 2003).

1.7.2 Environmental-derived ROS

Each puff of cigarette smoke contains more than 10^{15} oxidants. In this myriad, oxidants may have a short half-life such as $O_2^{\cdot -}$ or a relatively long half-life such as tar-semiquinone, which can lead to $\cdot OH$ and H_2O_2 generation (Church and Pryor, 1985, Nakayama et al., 1989). Consequently, H_2O_2 in the presence of Fe^{2+} can lead to the production of the highly damaging $\cdot OH$ via the Fenton reaction. It has been shown that smokers have significantly higher levels of iron in their lungs than non-smokers, potentially predisposing them to increased ROS burden (Ghio et al., 1997).

The oxidant H_2O_2 and other biomarkers of oxidative stress, such as 8-isoprostane, are found in elevated levels in exhaled breath condensate, sputum and in the systemic circulation of patients with COPD (Makris et al., 2008, Dekhuijzen et al., 1996). Furthermore, COPD patients during exacerbations have increased levels of oxidative stress. In the study by Dekhuijzen and colleagues it was observed that patients with stable COPD had significantly higher levels of exhaled H_2O_2 ($0.205 \pm 0.054 \mu\text{M}$) compared to healthy volunteers ($0.029 \pm 0.012 \mu\text{M}$) which was further raised in patients with an exacerbation ($0.6 \pm 0.075 \mu\text{M}$, $p < 0.01$) (Dekhuijzen et al., 1996). Elevated levels of H_2O_2 may also be partly derived from the increased release of $\text{O}_2^{\cdot -}$ from alveolar macrophages, and this has been shown by Rahman and colleagues where, elevated levels of H_2O_2 were observed from circulating neutrophils from COPD patients during exacerbations (Rahman and MacNee, 1996, Rahman et al., 1996).

1.7.3 ROS-activated inflammation in COPD

Inhaled cigarette smoke and other noxious particles acutely induce pulmonary inflammation through the activation of pro-inflammatory redox-sensitive transcription factors such as NF- κ B and activator protein-1 (AP-1) and, through signalling pathways such as p38 mitogen-activated protein kinases (p38 MAPK) and phosphoinositide-3-kinase (PI3K) (Di Stefano et al., 2002, Lee et al., 2012, Isajevs et al., 2011). In a clinical study to compare patterns of inflammation between the large and small airways in patients with COPD, it was shown that both the large and small airways had greater levels of NF- κ B expression than non-smokers and smokers without COPD (Isajevs et al., 2011). This was associated with higher levels of macrophages, CD8+ T lymphocytes and neutrophils. It was also observed that the

small airways of COPD patients had fewer macrophage numbers than the large airways, whereas more neutrophils were present in the small airways. In a separate study in PBMCs from patients with asthma and COPD, Gagliardo and colleagues observed that different isoforms of IKK, which drives the NF- κ B activation, dominated in asthma and COPD (Gagliardo et al., 2011). NF- κ B has been shown to upregulate the transcription of many inflammatory genes, such as CXCL8, TNF- α and IL-6, which are highly expressed in COPD patients (Keatings et al., 1996).

The sputum of COPD patients have been shown to have significantly higher levels of TNF- α and CXCL8 than smokers and non-smokers (Keatings et al., 1996). Furthermore, Rusznak and colleagues noted that bronchial epithelial cells from COPD patients had greater levels of the pro-inflammatory mediator IL-1 β than smokers upon cigarette smoke exposure (Rusznak et al., 2000). This increase in inflammatory mediators leads to activation and induction of non-specific inflammatory cells, macrophages and neutrophils, and adaptive immune cells, T-lymphocytes, in the bronchial mucosa and small airways.

The increased burden of inhaled oxidants is enhanced by activated macrophages and neutrophils in the lungs of smokers (Rahman and MacNee, 1996, Saetta et al., 1993). In retinal pigment epithelial cells, cigarette smoke increased mitochondrial membrane potential which was associated with an increase in superoxide levels (Bertram et al., 2009). Macrophages and neutrophils generate O₂^{•-} which is rapidly converted to H₂O₂ by SOD. This abnormal inflammatory response to noxious particles and gases, orchestrated by a cascade of inflammatory cells and mediators, leads to narrowing and structural changes of the small airways and destruction of the

lung parenchyma; the latter is a characteristic of the pathological and radiological findings of emphysema. These structural changes include increased bronchial wall thickness and smooth muscle tone, mucus hypersecretion and loss of elastic structure (Roche et al., 2011) and it is these pathological changes of the lungs that lead to progressive airflow limitation, which is the hallmark of COPD (Usmani and Barnes, 2012).

1.8 Corticosteroids

Inhaled corticosteroids (ICS) are widely used in the treatment of COPD patients. However, in sharp contrast to asthmatic patients they provide little clinical benefit (Barnes, 2010a). The addition of corticosteroids is recommended for severe COPD patients at GOLD stage III (Rabe et al., 2007) (Fig. 1.8). However, corticosteroids fail to prevent disease progression or mortality in COPD patients (Suisa and Barnes, 2009, Barnes, 2000a). In a two-year double-blind, randomized, placebo controlled trial, high dose ICS had no effect on the decline in the forced expiratory volume in one second (FEV₁) in COPD patients (Renkema et al., 1996).

More recently, several long-term clinical studies in COPD patients have shown that high dose ICS had no effect on the annual decline in FEV₁, in comparison to placebo (Pauwels et al., 1999, Burge et al., 2000). In a randomised three-year, placebo-controlled study conducted by Vestbo and colleagues, high dose budesonide had no effect on respiratory symptoms and no significant reduction in the number of disease exacerbations in 145 COPD patients. Of the 316 exacerbations that occurred during the study, 155 exacerbations were in the budesonide-treated group with the remaining 161 in the placebo-treated group (Vestbo et al., 1999). In contrast, a

reduction in the number of disease exacerbations has been observed in two studies. In a six-month randomised, double blind, placebo controlled study involving 13 European countries, New Zealand and South Africa, patients with COPD receiving fluticasone propionate 500 micrograms twice daily had significantly less moderate or severe exacerbations compared to a placebo group, 86% vs 60%, respectively (Paggiaro et al., 1998). However, there was no difference in the primary endpoint; the number of patients who had at least one exacerbation by the end of treatment. A meta-analysis of six long term studies involving 3571 COPD patients, where the rate of decline in FEV₁ was the primary endpoint, failed to show any effect of ICS on the rate of FEV₁ decline (Highland et al., 2003). In a more recent review of 55 studies involving 16,154 subjects, the long-term use of inhaled corticosteroids did not consistently reduce the rate of decline in FEV₁ in patients with COPD (Yang et al., 2012).

The Toward a Revolution in COPD Health (TORCH) study investigated all-cause mortality as its primary endpoint between four treatment arms of; fluticasone propionate alone, salmeterol alone, salmeterol in combination with fluticasone propionate and, placebo (Vestbo, 2004). Post-hoc analysis showed a small but significant reduction of 13 ml (millilitres)/year (95% CI, 5- 22; p= 0.003) in the annual rate of FEV₁ decline by fluticasone propionate compared to placebo (Celli et al., 2008). However, meta-analysis of all these studies, involving more than 13,000 COPD patients has revealed no significant effect of ICS on the rate of FEV₁ decline, although the number of disease exacerbations are reduced by approximately 25% (Yang et al., 2007).

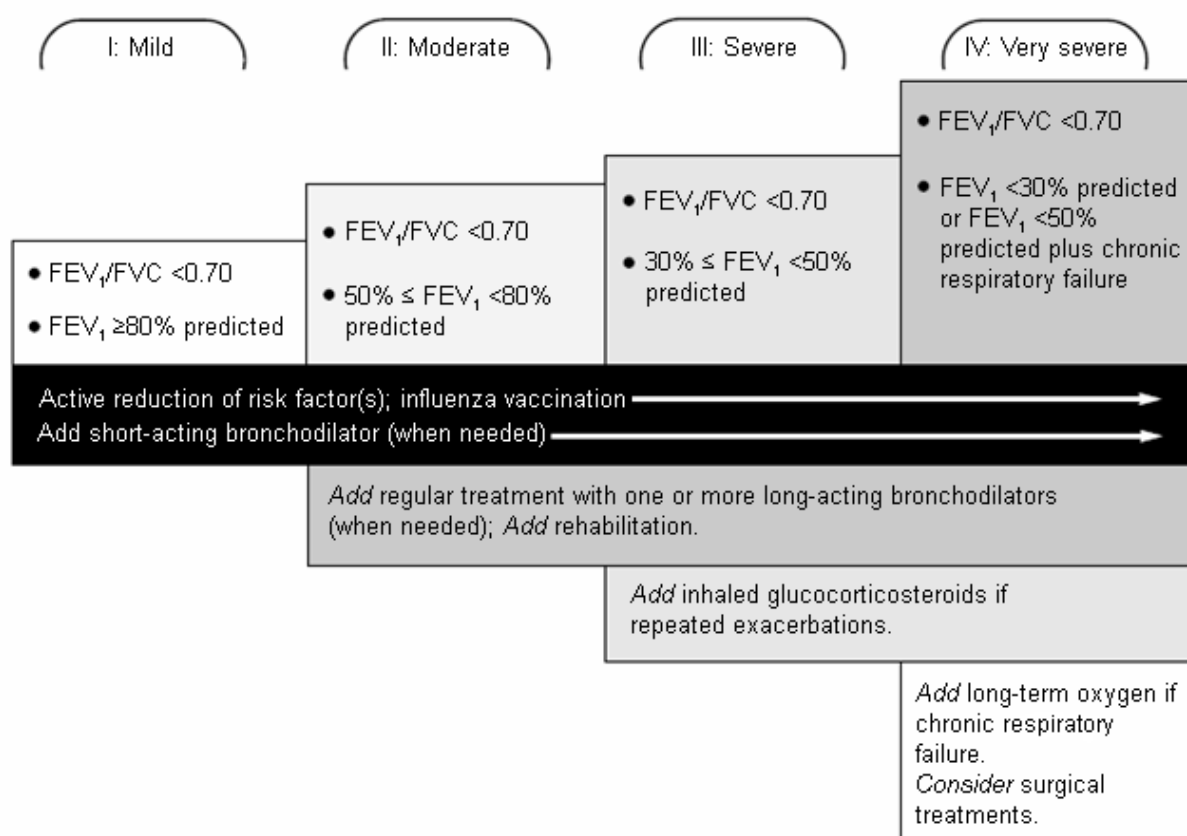


Fig. 1.8 Pharmacotherapy management of COPD (Rabe et al., 2007). Definition of abbreviation: FEV_1 = forced expiratory volume in 1 second; FVC = forced vital capacity.

1.8.1 Inhaled corticosteroid and long-acting β_2 -agonist combination therapy

In patients with asthma, the addition of long acting β_2 -agonists (LABA) to low dose ICS have been shown to be more clinically beneficial than the use of high dose ICS, allowing a reduction in ICS dose and minimising adverse side effects of corticosteroids (Greening et al., 1994, Pauwels et al., 1997, Juniper et al., 1999). Subsequent biological studies have been undertaken to understand the cellular mechanisms of this complementary interaction (Usmani et al., 2005b, Roth et al., 2002). In the management of patients with COPD, combination therapy with ICS and LABA is considered for severe diseased patients at GOLD stage III & IV and those with frequent exacerbations (Rabe et al., 2007).

Several clinical studies investigating the combination of corticosteroid and LABA in patients with COPD have shown beneficial effects on symptoms and lung function in comparison to corticosteroid alone. However, the improvement in disease exacerbations of COPD with combination therapy have not been statistically greater than the effects of LABA treatment alone (Calverley et al., 2007, Mahler et al., 2002, Calverley et al., 2003, Kardos et al., 2007). A 2x2 factorial study analysis of the TORCH study, where the corticosteroid was fluticasone propionate used in combination with the LABA salmeterol, showed that the reduction in all-cause mortality in the combination treatment group was entirely due to the LABA component of salmeterol (Suisse et al., 2008, La Vecchia and Fabbri, 2007).

A recent Cochrane database meta-analysis of eleven studies reviewed the effect of the two combination therapies of fluticasone propionate/salmeterol and budesonide/formoterol on disease exacerbations, mortality and pneumonia. This showed that COPD disease exacerbations were less frequent compared to placebo (Nannini et al., 2007). Furthermore, the analysis showed an overall reduction in mortality, which did not quite reach statistical significance ($p=0.052$), and this outcome was greatly influenced by the results of the TORCH study where a reduction in all-cause mortality was shown with fluticasone propionate/salmeterol. Of note, however, in a post-hoc analysis of the TORCH study, there was an observed increased risk of pneumonia with combination therapy, primarily associated with ICS use (Crim et al., 2009).

1.9 Molecular mechanisms of corticosteroids

Corticosteroids exert their anti-inflammatory effects primarily through binding and subsequent activation of the glucocorticoid receptor (GR), a ligand-inducible transcription factor. GR is a member of the superfamily of steroid receptors and is fundamental for the regulation of many physiological and metabolic processes. These processes include inflammatory and immune responses, cell proliferation and metabolic homeostasis (Beato and Klug, 2000, Whitfield et al., 1999). Like many transcription factors, GR undergoes nucleocytoplasmic shuttling upon activation (Deroo et al., 2002). GR is made up of three major functional domains, the N-terminal domain (BTD), the central DNA-binding domain (DBD) and the C-terminal ligand-binding domain (LBD) (Fig. 1.9a), that facilitate GR nucleocytoplasmic shuttling and binding to DNA promoter regions. Furthermore, a ligand-dependent transcriptional activation function (AF)-2 is located in the LBD. A hinge junction between DBD and LBD also facilitates GR nuclear translocation (Vandevyver et al., 2012).

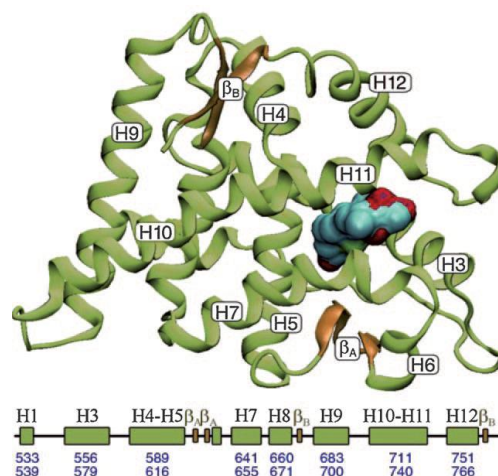


Fig. 1.9a Ligand binding domain of the glucocorticoid receptor (GR) (Veleiro et al., 2010). The ligand bound to the active site of GR is dexamethasone.

In the resting state, the inactive GR is bound to a protein complex that includes heat shock protein (hsp)-90, acting as molecular chaperones to prevent the nuclear localisation of unoccupied GR. Upon activation, GR translocates into the nucleus where it affects gene transcription via DNA-binding dependent and independent mechanisms (Fig. 1.9b). The DNA-binding dependent mechanism occurs via direct binding to the promoter regions of corticosteroid-sensitive genes, at glucocorticoid response elements (GRE). The consensus GRE sequence is 5-AGAACA_{nnn}TGTTCT-3 (Strahle et al., 1987), which may vary among different promoters (Meijsing et al., 2009). Recently global chromatin immunoprecipitation (ChIP)-sequencing data has revealed that GR binds to DNA mostly via the aforementioned consensus sequence (Voss et al., 2011). Binding of activated GR to GRE leads to the transcriptional induction of anti-inflammatory genes such as dual specificity protein phosphatase 1 (*DUSP1*) (Tchen et al., 2010). Whilst the DNA-binding independent mechanism involves activated GR binding to transcription factors and coactivators, which in turn bind to DNA (Muzikar et al., 2009). Binding of activated GR to AP-1 and NF- κ B may lead to gene transrepression of genes regulated by these transcription factors (McKay and Cidlowski, 1998, De Bosscher et al., 2003, Heck et al., 1994). Recent evidence suggests that activated GR may form a multi-protein repression complex with the enzyme O-linked β -N-acetylglucosamine transferase (OGT), which leads to the suppression of NF- κ B transcriptional activity (Li et al., 2012). In this study, depletion of OGT abolished GR repression of NF- κ B transcriptional activity, whereas overexpression potentiated it.

Activated GR may also lead to the recruitment of histone deacetylase-2 (HDAC2) to the p65 subunit of NF- κ B bound to the promoters of several inflammatory genes and

inhibit the transcription of a wide range of pro-inflammatory cytokines, by preventing local histone acetylation (Fig. 2.0) (Barnes, 2006b, Barnes, 2011, Garside et al., 2004). By this process of transrepression by tethering, corticosteroids reduce pro-inflammatory cytokines such as TNF- α and CXCL8 in asthmatic patients, whereas they are far less effective in COPD patients (Barnes, 2008). Furthermore, activated GR is deacetylated by HDAC2 to enable binding to the NF- κ B complex that leads to the subsequent suppression of NF- κ B induced genes. A reduction in HDAC2 activity has been shown to increase GR acetylation, which prevents GR from associating with the p65 subunit of NF- κ B and thereby less inhibition of NF- κ B induced inflammation (Ito et al., 2006).

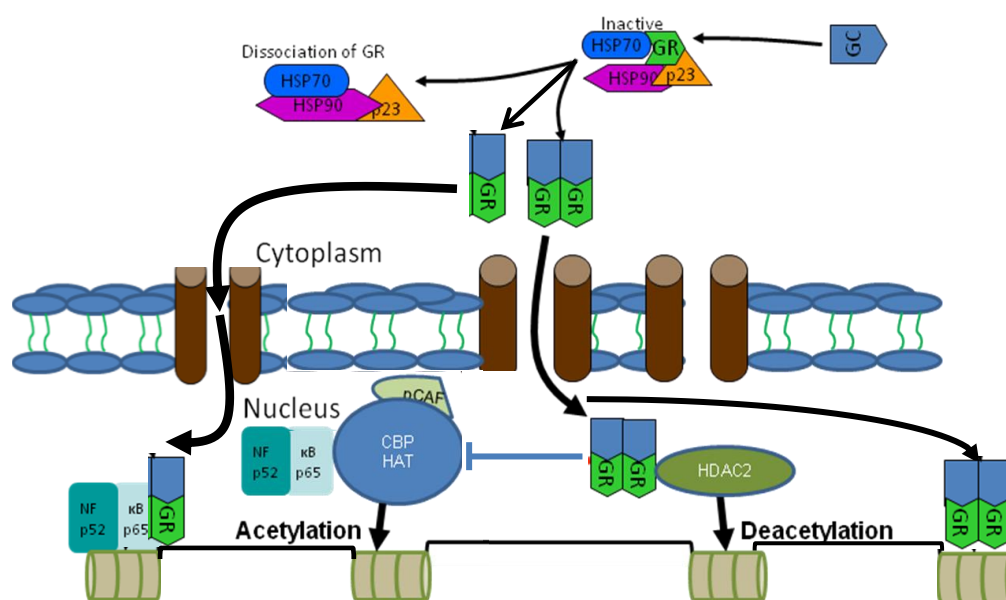


Fig. 1.9b Corticosteroid suppression of pro-inflammatory genes. In the resting state, inactive glucocorticoid receptor (GR) is bound to a complex of molecular chaperones. Upon corticosteroid binding, activated GR translocates into the nucleus where it affects gene transcription via DNA-binding dependent and –independent mechanisms.

1.9.1 GR nuclear translocation

Nucleocytoplasmic shuttling of proteins across the nuclear pore complex (NPC) is a dynamic process regulated by nuclear import and export complexes requiring

several sequential steps. These steps involve retrograde movement through the cytoplasm, recognition of nuclear localisation signals (NLS) on cargo proteins by nuclear import proteins, docking to the nuclear pore, translocation across the nuclear compartment, binding to and release from nuclear sites of action, recognition of nuclear export signals (NES) on cargo proteins by nuclear export proteins and finally, anteretrograde cytoplasmic movement (Galigniana et al., 2010).

Nuclear importin and exportin receptors, containing a Ran-GTP binding domain, mediate RanGTPase-dependent transport across the nuclear envelope (Nakielny and Dreyfuss, 1999, Strambio-De-Castillia et al., 2010). The RanGTPase system is composed of a Ran gradient across the NPC, where RanGDP is predominantly found in the cytoplasm, whereas RanGTP mainly resides in the nucleus. This gradient is maintained by 2 enzymes; cytoplasmic RanGTPase-activating protein (RanGAP) which hydrolyses RanGTP to RanGDP and the nuclear RanGDP/GTP-activating protein (RANGEF) which hydrolyses RanGDP to RanGTP (Poon and Jans, 2005, Wagstaff and Jans, 2009).

Although the mechanisms that govern GR nucleocytoplasmic shuttling remain largely unknown, evidence over the previous decade suggests that specific nuclear import and export receptors may mediate GR nucleocytoplasmic shuttling, essential for corticosteroid regulation of gene transcription (Freedman and Yamamoto, 2004, Vandevyver et al., 2012). In 1999, a group of researchers discovered two distinct nuclear localisation signals, NLS1 and NLS2, located on GR. The NLS1 is located within the DBD, whereas NLS is located in the LBD (Savory et al., 1999). In this study NLS1 was responsible for rapid GR nuclear translocation (4-6 min) and NLS2 mediated relatively slower GR nuclear translocation (45 min to 1h) (Savory et al.,

1999). Importantly, nuclear localisation of fusion proteins containing either the full-length receptor or the NLS2 alone was hormone-dependent. Furthermore, the rates of hormone induced nuclear localisation of both the full-length receptor and NLS2 were similar (Picard and Yamamoto, 1987, Cadepond et al., 1992).

More recently, several nuclear importin receptors, importins-7, -8, -13 and the α/β heterodimer have been shown to interact with, and mediate, GR nuclear translocation (Freedman and Yamamoto, 2004, Vandevyver et al., 2012). In a study investigating the role of importins on GR trafficking, importin-7 fused to glutathione S-transferase (GST) bound directly to the GR purified from rat liver. Furthermore, immunoprecipitation of the S-methionine-labelled fragments of GR in reticulocyte lysate, revealed that importin-7 binds to both NLS1 and NLS2, suggesting multiple sites for importin-7 binding on GR. Finally, importin-7 and GR binding was impaired by RanQ69L, a GTPase-deficient Ran mutant (Freedman and Yamamoto, 2004). It is not known whether these different importins interact in combination with each other or individually to facilitate GR nuclear translocation. It would be important to characterise the role of importin-7 on GR nucleocytoplasmic shuttling in inflammatory cells following corticosteroid challenge.

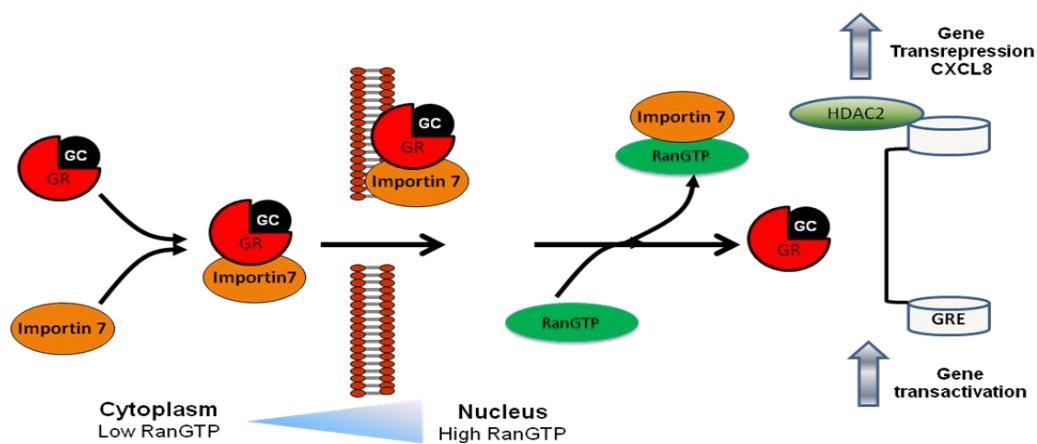


Fig. 1.9.1a Potential mechanism of glucocorticoid receptor (GR) nuclear import. Corticosteroid-induced GR nuclear translocation is essential for corticosteroid-mediated suppression of pro-inflammatory mediators. Importin-7 binds to and mediates nuclear translocation of the GR. Once in the nucleus, nuclear RanGTP binds to importin-7 resulting in the dissociation of the importin-7 and GR complex. Consequently, the GR is able to move through the nucleus and either binds to glucocorticoid response elements on corticosteroid-inducible genes or recruits histone deacetylase (HDAC)-2 to prevent the gene transcription of pro-inflammatory mediators. Activated GR may also prevent gene transcription by binding as a monomeric unit to other transcription factors.

Similarly, nuclear export of proteins is also regulated by the RanGTPase system. A nuclear export signal (NES) located on the cargo protein is recognised by exportins. Recognition of the NES of a protein results in the formation of the exportin-cargo protein complex and subsequent nuclear export of the complex. Some studies have shown several nuclear export pathways may be implicated in GR nuclear export. Of the nuclear export pathways studied, exportin-1 (CRM-1) is the best characterized. Leptomycin B (LMB), a specific inhibitor of CRM-1 mediated nuclear export, has been shown to inhibit GR nuclear export, leading to enhanced unliganded and hormone-treated GR nuclear localisation (Savory et al., 1999). However, other studies have suggested GR nuclear export is governed by a pathway other than the CRM-1 mediated transport mechanism, namely the nuclear export receptor calreticulin (CRT).

Similar to exportin receptors, CRT-mediated nuclear export is Ran-GTPase-dependent and forms a trimeric CRT-cargo protein-RanGTP complex (Galigniana et al., 2010). Using a combination of microinjection and transfection approaches, Holaska and colleagues have shown that CRT induced GR nuclear export. CRT binds to a 15 amino acid sequence located between the two zinc fingers of the GR DBD which contains the GR NES (Holaska et al., 2001). Subsequently, the same group reported that this effect of CRT on binding to the GR DBD and induction of GR nuclear export was Ca^{2+} dependent (Holaska et al., 2002). These differences in pathways that govern GR nuclear export may be cell specific. It would be important to characterise the role of CRM-1 mediated transport on GR nuclear export in inflammatory cells important in asthma and COPD. Unravelling the mechanisms that govern GR nuclear export would allow one to potentially increase the pharmacokinetic profile of a corticosteroid, as well as more importantly, the duration of the anti-inflammatory effects of the corticosteroid.

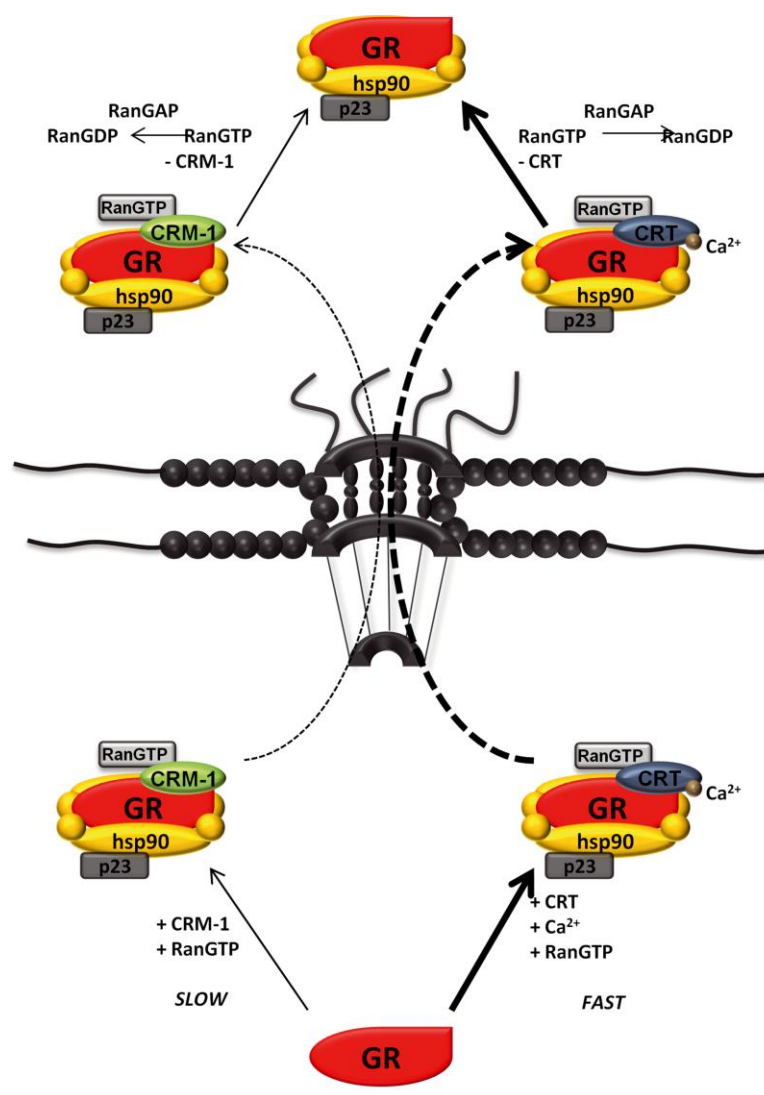


Figure 1.9.1b Potential mechanism for glucocorticoid receptor (GR) nuclear export (Vandevyver et al., 2012). Calreticulin (CRT) and exportin-1 (CRM-1) are two potential nuclear export receptors that may govern GR nuclear export.

1.10 Corticosteroid resistance

As highlighted above, unlike mild-to-moderate asthmatics, subsets of severe asthmatics and asthmatic patients who smoke show a degree of corticosteroid resistance similar to that observed in COPD patients (Table 1.1) (Suissa and Barnes, 2009, Thomson and Spears, 2005, Barnes, 2010a). Asthmatic patients who smoke as well as those patients with severe asthma, require high doses of ICS or even oral corticosteroids to achieve relative control of their disease, which still remains suboptimal on treatment (Thomson and Spears, 2005). COPD patients are also

recommended high dose ICS, despite lack of their clinical benefit (Suissa and Barnes, 2009). Corticosteroid resistance persists even after smoking cessation and markers of oxidative stress and inflammation continue to be elevated in COPD patients (Gamble et al., 2007).

1.10.1 Why do inhaled corticosteroids fail in COPD?

There are several well established clinical trials which have shown that high doses of ICS fail to suppress markers of inflammation in sputum and bronchial biopsies from COPD patients compared to asthmatics (Keatings et al., 1997, Culpitt et al., 1999, Hattotuwa et al., 2002). In a two-week clinical study in 13 patients with severe COPD, inhaled budesonide 800mg twice daily did not suppress the expression of TNF- α and CXCL8 which play pivotal roles in propagating the inflammatory response (Keatings et al., 1997). Additionally, there was no improvement in lung function or symptom scores. This group also investigated whether this effect could be due to poorly inhaled drug delivery not reaching the distal airways in COPD patients, yet similarly observed, that systemically delivered oral prednisolone also did not suppress inflammatory markers (Keatings et al., 1997). In a double-blind, placebo-controlled study involving 30 COPD patients, fluticasone propionate 500 micrograms twice daily did not suppress inflammatory markers in bronchial biopsies including CD8+ cells, CD68+ cells or neutrophil levels, which are highly expressed in COPD (Hattotuwa et al., 2002).

Better understanding of the mechanism(s) that may cause corticosteroid insensitivity are greatly needed to effectively control the underlying causes of COPD and symptoms of the disease. Additionally, drug development in COPD has been rather slow due to several factors. Traditionally, the high incidence of co-morbidities and

inadequate information on biomarkers to monitor the short and long-term potential of new treatments (Usmani and Barnes, 2012), as well as discrepancies between animal models of COPD and patients, has made it difficult for drug development in COPD (Barnes, 2010c). Also, there has been difficulty in targeting inhaled therapies to the lower airways of the lungs (De Backer et al., 2010, Usmani et al., 2005a).

However, recent advances in the cellular and molecular mechanisms of COPD have revealed several potential targets that may reverse some components of the pathology of COPD, as well as reverse corticosteroid insensitivity (Barnes, 2008). Potential mechanisms in severe asthmatics and COPD patients that may give rise to corticosteroid insensitivity include reduction in HDAC2 activity, impaired GR nuclear translocation, and increased pro-inflammatory transcription factors (Barnes, 2011). Profiling of the gene expression in primary lymphocytes identified bone morphogenetic protein receptor (BMPRII) as a potential modulator of corticosteroid insensitivity (Donn et al., 2007).

1.11 Potential mechanisms for corticosteroid resistance

1.11.1 *Histone Deacetylase-2*

The expression of the enzyme HDAC2, which is recruited by corticosteroid activated-GR to switch off the transcription of pro-inflammatory genes, is reduced in patients with COPD (Lin, 2005). In the lung tissue of COPD patients, reduced HDAC2 activity correlated with the severity of COPD disease. Furthermore, mRNA and protein expression of HDAC2 were significantly lower in COPD patients (Ito et al., 2005). In a preclinical study using BEAS-2B lung epithelial cells, H₂O₂ reduced HDAC2 activity

(Ito et al., 2004). The investigators showed that the reduction in HDAC2 activity was associated with tyrosine nitration status.

In mice exposed to cigarette smoke, phosphoinositide-3-kinase- δ (PI3K δ) dead knock-in mice had reduced HDAC2 tyrosine nitration compared to wild type mice (Marwick et al., 2009). In PI3K δ knockout mice exposed to smoke, HDAC2 and corticosteroid activity were not reduced in comparison to the wild type group. In PBMCs from COPD patients, it has been shown corticosteroid insensitivity and reduced HDAC2 activity were reversed by a non-selective PI3K inhibitor theophylline (To et al., 2010). Furthermore, theophylline also inhibited PI3K δ which was up-regulated in COPD patients. This suggests PI3K δ inhibition may serve as a potential therapeutic target to overcome corticosteroid insensitivity due to cigarette smoke.

Upon activation, GR is deacetylated by HDAC2 to enable binding to the NF- κ B complex that leads to the subsequent suppression of NF- κ B-induced genes. A reduction in HDAC2 activity has been shown to increase GR acetylation, which prevents GR from associating with the p65 subunit of NF- κ B and thereby less inhibition of NF- κ B-induced inflammation (Ito et al., 2006). In alveolar macrophages of COPD patients, HDAC2 knockdown by RNA interference resulted in reduced dexamethasone suppression of IL-1 β -induced GM-CSF (Ito et al., 2006). This reduction in dexamethasone suppression was not associated with reduced GR nuclear translocation or binding to DNA promoter regions of corticosteroid-sensitive genes, but via impaired association between GR and NF- κ B. Site directed mutagenesis of GR to decrease GR acetylation, reduced the ability of HDAC2 to inhibit transcription of NF- κ B sensitive genes. Therefore, a reduction in HDAC2

activity plays a pivotal role in corticosteroid insensitivity through reduced suppression of NF- κ B induced pro-inflammatory mediators (Ito et al., 2006). A recent study in alveolar macrophages from patients with COPD demonstrated that nitrosylation of HDAC2 was increased, which led to corticosteroid insensitivity (Malhotra et al., 2011). In this study, treatment with sulphoraphane, a small activator of nuclear factor erythroid 2-related factor 2 (Nrf2), reduced nitrosylation of HDAC2 which was associated with restored dexamethasone sensitivity.

1.11.2 Defective GR binding and nuclear translocation

The data on impaired HDAC2 activity in COPD is extensive and supported by several clinical trials. Unfortunately, not much is known about whether GR nucleocytoplasmic shuttling, essential for HDAC2 recruitment and subsequent downregulation of pro-inflammatory gene transcription, is impaired under oxidative stress and whether it contributes to corticosteroid insensitivity in COPD patients. There is some published data in corticosteroid resistant asthmatics to suggest GR nucleocytoplasmic shuttling is impaired. In a study involving corticosteroid resistant asthmatic patients, reduced dexamethasone suppression of TNF- α was associated with impaired GR nuclear translocation in PBMCs (Matthews et al., 2004). In another study in PBMCs from severe asthma patients, GR ligand-binding affinity was reduced in the nucleus (Irusen et al., 2002). Furthermore, the combination of the cytokines interleukin (IL)-2 and IL-4, which show increased expression in the airways of corticosteroid resistant asthmatics, led to a reduction in GR nuclear translocation and ligand-binding affinity. This reduced GR nuclear translocation was restored with a p38 MAPK inhibitor (Mercado et al., 2012). Greater degree of p38 MAPK activation in corticosteroid insensitive asthma patients supports the role of p38 MAPK in

corticosteroid insensitivity. It has been postulated that p38 MAPK phosphorylates GR, which may alter its binding and nuclear translocation (Barnes, 2010b).

At the *in vitro* level, it has been shown GR nuclear translocation is redox sensitive. In human GR-expressing CHO_{MTGR} cells, H₂O₂ inhibited GR nuclear translocation, in addition to partially inhibiting ligand-dependent dissociation of Hsp90 from GR. GR nuclear translocation was reversed with *N*-acetyl-L-cysteine (Okamoto et al., 1999). Another potential mechanism that may contribute to defective GR nuclear translocation is increased expression of GR β . It has been shown that knockdown of GR β in alveolar macrophages of asthma patients resulted in increased GR α nuclear translocation, the GR isoform that corticosteroids bind to, and increased corticosteroid response (Goleva et al., 2006).

Nucleocytoplasmic shuttling of proteins has also been shown to be sensitive to oxidative stress (Kodiha and Stochaj, 2012). H₂O₂ at exceptionally high concentrations has been shown to impair importin- β -mediated nuclear translocation; importin- β being a member of the same family as importin-7. In growing yeast *Saccharomyces cerevisiae* cells, heat, shock and H₂O₂ significantly impaired classical nuclear import of NLS fused to green fluorescent protein (NLS-GFP). This was associated with a redistribution of the small GTPase, Gsp1p, from the nucleus into the cytoplasm (Stochaj et al., 2000). In HeLa cells, several components of the classical nuclear import pathway were affected by extremely high concentrations of H₂O₂. This resulted in the redistribution and degradation of RanGTP, essential for nuclear import of cargo proteins, and relocalisation of importin- β , resulting in impaired nuclear import (Kodiha et al., 2004).

1.12 Hypothesis

As described above, unlike mild-to-moderate asthmatics who respond well to ICS, ICS fail to control the underlying inflammation and reverse the decline in lung function in COPD patients. Several mechanisms have been shown to contribute or be associated to corticosteroid insensitivity in COPD patients and corticosteroid-resistant asthmatics. Of these, impaired HDAC2 function is the best studied. Another postulated mechanism is impaired GR nuclear translocation, which is essential for corticosteroid activity. The mechanisms that regulate GR nucleocytoplasmic shuttling are mostly unknown.

Recent evidence in an *in vitro* setting, suggests nuclear importin receptors 7, 8, 13 and β may facilitate GR nuclear translocation. Amongst these, importin-7 is one of the least characterised in its role in inflammatory cells and under oxidative stress. Additionally, another member of the nuclear importin receptor family, Importin- β mediated transport has been shown to be impaired under oxidative stress.

I propose oxidative stress associated with heightened inflammation may cause corticosteroid insensitivity and this may be partly due to impaired GR nuclear translocation. Furthermore, I believe importin-7 may contribute to GR nuclear translocation and under conditions of oxidative stress it may be impaired leading to corticosteroid insensitivity. Two potential targets I propose that may be degraded under oxidative stress and lead to corticosteroid insensitivity are 1) cytoplasmic importin-7 and 2) nuclear RanGTP levels, both essential for nuclear import of cargo protein (Fig 1.12).

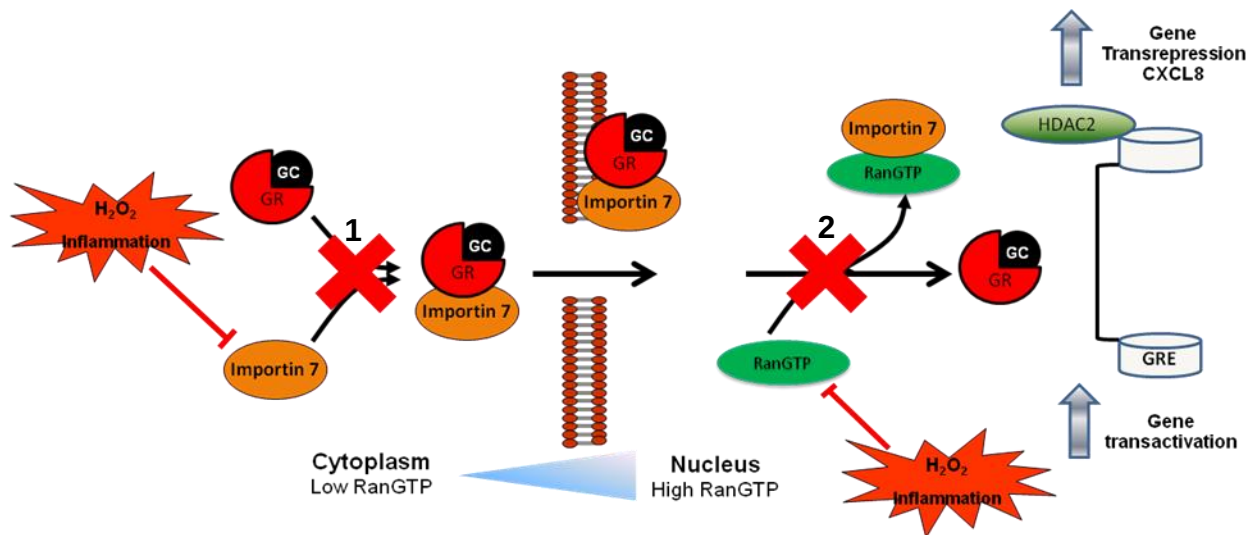


Figure 1.12 Potential mechanisms of reduced glucocorticoid receptor (GR) nuclear translocation. Corticosteroid-induced GR nuclear translocation is essential for corticosteroid-mediated suppression of pro-inflammatory mediators. Importin-7 binds to and mediates nuclear translocation of the glucocorticoid receptor (GR). Once in the nucleus, nuclear RanGTP binds to importin-7 resulting in the dissociation of the importin-7 and GR complex. Consequently, the GR is able to move through the nucleus and either binds to glucocorticoid response elements on corticosteroid-inducible genes or recruits histone deacetylase (HDAC)-2 to prevent the gene transcription of pro-inflammatory mediators. Activated GR may also prevent gene transcription by binding as a monomeric unit to other transcription factors. Under conditions of oxidative stress, importin-7 or nuclear RanGTP levels may be reduced leading to reduced GR nuclear translocation and consequently corticosteroid insensitivity.

CHAPTER 2

MATERIALS AND METHODS

2.1 U937 Cell culture

The U937 cell line (American Tissue Culture Collection, Rockville, MD) was established from malignant cell lines obtained from the pleural effusion of a 37 year old Caucasian male with diffuse histiocytic lymphoma. U937 cells express many of the monocytic-like characteristics exhibited by cells of histocystic origin and exhibit macrophage-like characteristics.

U937 cells were grown in full RPMI-1640 media (containing 1% L-glutamine and 10% foetal calf serum (FCS, Invitrogen Ltd, Paisley, UK)) and split every 2-3 days. Cells were serum starved in minimal RPMI-1640 media (1% L-glutamine and 0.5% FCS) to reduce endogenous steroid content and drive cells towards cell cycle synchronisation before experiments.

2.2 Compounds

Fluticasone furoate (FF), salmeterol (SLM) and vilanterol trifenate (VI) were obtained from GlaxoSmithkline (Greenford, UK). Fluticasone propionate (FP) and budesonide (BUD) were purchased from Sigma-Aldrich (Sigma-Aldrich, Poole, UK). Stock concentrations of BUD, FP, FF & VI were made up in dimethylsulfoxide (DMSO; Sigma, UK) and SLM in ethanol at a concentration of 10^{-2} M. Subsequent dilutions were made in medium to yield the final chosen concentrations. Final concentrations of DMSO used were 0.25%. All chemicals/reagents were purchased from Sigma-Aldrich unless otherwise stated.

2.3 (MTT) assay

The MTT assay was performed on U937 cells (200µl/well of 0.5×10^6 cells/ml) seeded in 96-well plates. 125µl of supernatant was removed and 10µl of MTT stock solution (5mg/ml; Sigma) was added to the remaining solution, before incubation for 2hr at 37°C. Thereafter, DMSO (150µl) was added to each well for 15min at room temperature, before the optical density was determined at 550nm.

2.4 MitoSOX Red fluorescence (Mitochondrial ROS)

U937 monocytes were seeded in low serum media at 2ml of 0.5×10^6 cells/ml per well in 6-well plates (Sigma-Aldrich). Following 3h serum starvation, cells were treated with 200µM of H_2O_2 for 90min followed by 1ng/ml of IL-1β for 30mins. Thereafter, cells were washed and resuspended in 5µM MitoSOX Red (M360008, Invitrogen) diluted in hanks buffered salt solution (HBSS, Invitrogen), containing calcium (Ca^{2+}) and magnesium (Mg^{2+}), and incubated in the dark at 37°C for 30mins. Cells were then centrifuged at 1200rpm for 3mins, washed and then resuspended in HBSS (containing Ca^{2+} and Mg^{2+}) in a sterile flow cytometry (FACS) tube at a concentration of 0.5×10^6 cells/ml. Antimycin A (AntaA) was used as a positive control for superoxide production and cells were incubated for 30min at room temperature (RT) in the dark. Mitochondrial superoxide levels were measured by FACS with a 510/580 excitation emission. At least 5,000 events in each sample were collected. Cell debris with low forward scatter (FSC) and side scatter (SSC) were excluded from analyses. Histogram plots of mean fluorescence intensity for each sample were generated.

2.5 DCF assay

U937 monocytes were seeded at 100µl of 1×10^6 cells/ml in a 96-well collagen-coated plate 1 day before treatment. The next day, cells were centrifuged (1200rpm at 4°C

for 3mins) and washed with KRH buffer (118mM NaCl, 4.8mM KCl, 1.2mM MgSO₄, 2.5mM CaCl₂, 10nM HEPES, pH 7.4). Cells were then incubated with 100μM 2, 7-dichlorofluorescein diacetate (DCFH-DA) in low serum media at 5% CO₂/95% air at 37°C for 30mins. After 30min DCFH-DA incubation, cells were washed and incubated with KRH buffer with stimulants, H₂O₂ and IL-1β, for indicated time points. Fluorescence was measured using a plate reader (Synergy HT, Biotek, Winooski, VT USA), with an excitation filter set at 485nm and the emission filter set at 530nm.

2.6 Western blotting

2.6.1 Cytoplasmic and nuclear extraction

Nuclear and cytoplasmic fractions were extracted using a Nuclear Extraction kit (Active Motif, Carlsbad, CA). U937 Cells (9ml of 0.5 x10⁶/ml) were washed 2x with ice-cold HBSS and collected by pipetting up and down. Cells were pelleted by centrifugation (1500rpm, 5min, 4°C), resuspended in hypotonic buffer (200μl), vortexed and incubated on ice for 15min. Detergent (8μl) was added, the suspension vortexed and centrifuged (14000rpm, 30sec, 4°C), and the supernatant (cytoplasmic fraction) collected. The remaining nuclear pellets were resuspended via scraping on a metal tube rack, in complete lysis buffer (25μl) containing the protease inhibitors and 10mM DTT, and incubated on ice for 30min. The suspension was centrifuged (14000rpm, 10min, 4°C) and the supernatant (nuclear fraction) was collected.

2.6.2 Bradford assay

Bradford's reagent (BioRad Laboratories, Hemel Hempstead, Hertfordshire, UK) was used to determine cytoplasmic and nuclear protein concentrations. A set of 6 different final protein concentrations of BSA standard solution (1, 2, 4, 6, 8 & 10μg) were added to a 96-well plate in duplicate. Unknown concentrations of nuclear and

cytoplasmic (2µl) protein fractions were also added in duplicate. To each well, Biorad buffer (200µl) was added. After 5min, absorbance at 595nm was measured using a plate reader. A standard curve of absorbance vs. concentration was plotted, and used to determine concentrations of nuclear and cytoplasmic fractions.

2.6.3 Polyacrylamide gel electrophoresis

NuPAGE 4-12% Bis-Tris Pre-Cast gels (Invitrogen) were used under reducing conditions to separate proteins of different molecular weights. Gels were firstly rinsed with deionised water before being assembled into the gel tank. NuPAGE MOPS Running Buffer (Invitrogen) was added into the central compartment, before the remaining external compartments were filled, to ensure no leakage. All ten wells of the gel were washed with running buffer before adding nuclear or cytoplasmic fractions and rainbow marker (Amersham Biosciences, Little Chalfont, UK). Corresponding volumes of NuPAGE LDS sample buffer (4X) were added to each cytoplasmic and nuclear fraction. These were heated (5min, 95°C) to denature proteins, followed by brief centrifugation. Finally, cytoplasmic and nuclear fractions were loaded onto the gels and electrophoresis was performed at 200V and 90A for 35min.

2.6.4 iBlot dry Western transfer

After electrophoresis, the polyacrylamide gel was removed from the tank and opened. The gel was kept wet at all times and cut with a clean scalpel, removing wells and the bottom segment of the gel. The gel was then placed on top of the nitrocellulose membrane in the iBlot Western detection bottom stack. Pre-wetted filter paper was placed on top of the gel and any bubbles were removed using the blotting roller. The Western detection top stack was placed over the filter paper with

the electrode side facing up. Finally, the iBlot disposable sponge was positioned so that the metal contact was at the upper right corner of the lid, and electro-transfer using the iBlot device was performed for 7mins.

2.6.5 Protein immunodetection

Following the dry Western transfer, the nitrocellulose membrane was removed and blocked in 5% non-fat milk TBS-Tween 20 (Sigma) at room temperature for 20min. This was removed and 10ml of primary antibody (GR H-300, 1:500, Santa Cruz Biotechnology, Santa Cruz, CA) diluted in 5% blocking solution was added and incubated overnight at 4°C on a rocking platform. Thereafter the nitrocellulose membrane was washed with TBS-Tween (3x for 5min).

Subsequently, the membranes were incubated with 10ml horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-rabbit, 1:2000, Dako, Cambridge, UK) diluted in the blocking solution for 1h on a rocking platform (room temperature). Three Washes at 5min with TBS-Tween 20 were repeated, before the antigen-antibody reactions were detected with the chemiluminescence detection kit (Amersham Biosciences). Protein loading control was determined with the use of TATA binding protein (TATA BP) and β -actin antibodies (Dako).

2.6.6 Band quantification

The GelDoc Imaging System (UVP, Upland, CA, USA) was used to measure band density on the exposed X-ray film. This was standardised against the loading controls and compared to untreated cells. Nuclear levels of protein were standardised against TATA binding protein (TATA BP; TATA is a conserved DNA sequence).

2.6.7 Primary and secondary antibodies

Primary antibody	Company	Secondary antibody
GR (H-300)	Santa Cruz	Goat anti-rabbit
GR (E-20)	Santa Cruz	Goat anti-rabbit
Importin-7 (H-78)	Santa Cruz	Goat anti-rabbit
Importin-7 (C-12)	Santa Cruz	Rabbit anti-goat
β -actin HRP-conjugated	Santa Cruz	N/A
TATA BP	Abcam	Goat anti-mouse
Anti-Ran	Cell Biolabs	Goat anti-mouse

Table 2.1 Primary and secondary antibodies.

2.7 Enzyme-linked immunosorbent assay (ELISA)

Extracellular concentrations of CXCL8 and IL-6 were measured with human CXCL8 and IL-6 DuoSet ELISA kits (R&D Systems Europe, Abingdon, UK) according to the manufacturer’s instructions (see below).

2.7.1 CXCL8 and IL-6

96 well plates were pre-coated with capture antibody (mouse anti-human CXCL8, 4ug/ml) overnight at room temperature. Each well was aspirated and washed three times with wash buffer after incubation with each solution (3X200μl, 0.05% Tween 20 in PBS). After washing, cells were incubated for 1h with 300μl of blocking buffer per

well (1% BSA in PBS). Diluted supernatants (1:2, 100µl, in 0.1% BSA, 0.05% Tween 20 and Tris-buffered saline (20mM Trizma base, 150mM NaCl)) and standards were added to corresponding wells for 2h at room temperature. After the 2h incubation, detection antibody (biotinylated goat anti-human, 100µl, 20ng/ml in 0.1% BSA, 0.05% Tween 20 and Tris-buffered saline) was added and incubated for a further 2h at room temperature. This was followed by another wash with washing buffer, before incubating for 20min with streptavidin-HRP (1:200 dilution, 100µl) at room temperature. The remaining steps were conducted in the dark. This was followed by the last wash and addition of substrate solution (1:1 mixture of Color Reagent A (H₂O₂) and Color Reagent B (Tetramethylbenzidine)) for 20min. Finally, 50µl of Stop Solution (2N H₂SO₄) was added to each well before determining optical density using a microplate reader set to 450nm (wavelength correction set at 540nm).

2.8 DNA promoter binding assays

2.8.1 GR-GRE Assay

The TransAM GR kit (Active Motif) was used to determine GR binding to DNA. 96-well plates consisting of 8-well strips coated with streptavidin were immobilised with biotinylated DNA oligonucleotides (GRE sequence, GGTACAnnnTGTCT). Nuclear and cytoplasmic extracts (15µg in 20µl complete lysis buffer) were incubated with complete binding buffer (30µl) for 1h at room temperature on a gently rocking platform. Thereafter, the wells were washed with wash buffer. Followed by incubation with an anti-GR antibody (100µl, 1:1000) and an HRP-conjugated anti-rabbit antibody (100µl, 1:1000), for 1h each, at room temperature. After washing, developing solution (100µl) was added to each well in the dark for 10min, before terminating the reaction with stop solution (100µl). Finally, the colour change was

determined using a microplate reader set at 450nm (wavelength correction set at 655nm).

2.8.2 Nrf2 DNA binding assay

The TransAM Nrf2 kit (Active Motif) was used to determine Nrf2 binding to DNA. 96-well plates consisting of 8-well strips coated with streptavidin were immobilised with biotinylated DNA oligonucleotides (ARE sequence, GTCACAGTGA CT CAGCAGAATCTG). Nuclear and cytoplasmic extracts (10µg in 10µl complete lysis buffer) were incubated with complete binding buffer (40µl) for 1h at room temperature on a gently rocking platform. Thereafter, the wells were washed with wash buffer. Followed by incubation with an anti-Nrf2 antibody (100µl, 1:1000) and an HRP-conjugated anti-rabbit antibody (100µl, 1:1000), for 1h each, at room temperature. After washing, developing solution (100µl) was added to each well in the dark for 10min, before terminating the reaction with stop solution (100µl). Finally, the colour change was determined using a microplate reader set at 450nm (wavelength correction set at 655nm).

2.8.3 p65 (NF-κB) DNA binding Assay

The TransAM p65 (NF-κB) kit (Active Motif) was used to determine p65 (NF-κB) binding to DNA. 96-well plates consisting of 8-well strips coated with streptavidin were immobilised with biotinylated DNA oligonucleotides (sequence, GGGACTTTCC). Nuclear extracts (5µg in 20µl complete lysis buffer) were incubated with complete binding buffer (30µl) for 1h at room temperature on a gently rocking platform. Thereafter, the wells were washed with wash buffer. Followed by incubation with an anti-p65 antibody (100µl, 1:1000) and an HRP-conjugated anti-rabbit antibody (100µl, 1:1000), for 1h each, at room temperature. After washing,

developing solution (100µl) was added to each well in the dark for 5min, before terminating the reaction with stop solution (100µl). Finally, the colour change was determined using a microplate reader set at 450nm (wavelength correction set at 655nm).

2.9 Quantitative real time reverse transcription polymerase chain reaction (RT-qPCR)

2.9.1 mRNA extraction

mRNA extraction was performed using the RNeasy kit (Qiagen, Crawley, East Sussex, UK). Briefly, cells were centrifuged at 4500 rpm at 4°C for 5mins and resuspended in 350µl RLT buffer, cell lysis buffer. Ensure all medium is removed before resuspending in RLT buffer to prevent incomplete cell lysis. Cells were then homogenized in RLT buffer using a QIAshredder spin column by centrifuging for 2min at 8,000xg. An equal volume of 70% ethanol was mixed with the homogenized lysate. 700µl of homogenized lysate in ethanol was transferred to an RNeasy spin column and centrifuged for 15sec at 8,000xg. Flow-through was discarded after the centrifugation and 700µl of buffer RW1 was added to the RNeasy spin column. Once again, the RNeasy spin column was centrifuged for 15sec at 8,000xg to wash the spin column and the flow-through was discarded. Next, 500µl of buffer RPE was added to the RNeasy spin column and centrifuged for 15sec at 8,000xg. This was repeated again; however this time the spin column was centrifuged for 2min to ensure no ethanol is carried over during RNA elution. Flow through was discarded and RNeasy spin column was placed in a new 1.5ml collection tube. Finally, 30µl of RNase-free water was added to the RNeasey spin column and centrifuged for 1min at 8,000xg to elute the RNA. RNA was stored at -80°C for future use. The

concentration and purity of RNA was evaluated by spectrophotometry using a Nanodrop (NanoDrop Technologies, Wilmington, Delaware, USA).

2.9.2 Reverse transcription

Reverse transcriptase was performed using the Qiagen Quantitect kit (Qiagen). mRNA samples were thawed on ice. 20µl eppendorfs were pre-labelled and 1µg of corresponding mRNA samples and 2µl of gDNA Wipeout buffer were added. The remaining volume was made up to 14µl with RNase-free water (see Table 2.2). Eppendorfs were then incubated for 2min at 42°C and immediately placed on ice thereafter.

Component	Amount
gDNA Wipeout Buffer, 7X	2 µl
Template mRNA	1µg
RNase-free water	Variable
Total volume	14 µl

Table 2.2 Genomic DNA elimination components

During the 2 min incubation period, the reverse-transcription master mix was prepared on ice for cDNA synthesis (see Table 2.3).

Component	Amount
Reverse Transcriptase	1µl
RT buffer, 5X	4µl
RT Primer Mix	1µl
Total	6µl

Table 2.3 Reverse-transcription master mix components

6µl of reverse-transcription master mix was added to 14µl of template mRNA and then incubated for 15min at 42°C. Finally, samples were incubated for a further 3min at 95°C to inactivate the reverse transcriptase.

2.9.3 Quantitative RT-PCR

mRNA levels of *DUSP-1* and *18S* were quantified by real-time qPCR using TaqMan Universal master mix (Applied Biosystems, Foster City, CA, USA). The TaqMan probes are based on the 5-3 exonuclease activity of the Taq DNA polymerase which leads to cleavage of fluprescent dye-labelled probes during PCR. Each transcript was analysed by delta (Δ) CT method and corrected to the CT values of the *18S* reference gene. Cycle parameters were 95°C for 15min to activate HotStarTaq DNA Polymerase, followed by annealing and extension for 50 cycles at 94°C for 15sec, followed by 60°C for 25sec and finally 72°C for 25sec. mRNA expression levels of *DUSP-1* was normalised to reference gene *18S*.

<i>Gene name</i>	<i>Assay ID</i>
<i>DUSP-1</i>	Hs00610256_g1
<i>18S</i>	Hs99999901_s1

Table 2.4 List of assay probes used

2.10 Transfection (importin-7 knockdown)

Transfection of U937 monocytes was performed in 6-well plates and 96-well plates for analysis of intracellular protein and extracellular pro-inflammatory mediators, respectively. Cells were washed with hanks buffer and carefully resuspended in 100µl Nucleofector solution per sample, at a concentration of 1×10^6 cells/100µl. Immediately, 200nM of human importin-7 ON-TARGET_{plus} SMARTpool small-interfering RNA (siRNA) or negative siRNA control, ON-TARGET_{plus} Non-Targeting pool (Thermo Fisher Scientific, Epsom, UK) was added to cells. Cells were transferred immediately into certified cuvettes, avoiding bubbles. The cuvette was immediately inserted into the Nucleofector Cuvette holder and an electrical current was passed through the cells. Post-transfection, 500µl of the pre-equilibrated complete RPMI-1640 media (1% L-glutamine and 10% FCS) was added immediately to the cells to neutralise the effects of the Nucleofector solution. The cells were diluted further with complete RPMI-1640 media in a final volume of 3ml/well in a 6-well plate. Subsequently, cells were incubated for 36h at 37°C and 5% CO₂, followed by serum starvation for 3h in minimal media before treatment.

2.11 Ran activation assay

Intracellular levels of RanGTP were measured with Ran activation assay kit (Cell Biolabs, San Diego, CA, USA). The procedure is detailed below.

2.11.1 Cell lysates

Serum starved U937 monocytes were seeded at a density of 0.5×10^6 cell/ml in large flasks. Cells were treated with H₂O₂ for 90min, followed by IL-1β for a further 30min.

Cells were washed with ice-cold PBS and resuspended in ice-cold 1X Assay/Lysis Buffer. Lysates were centrifuged for 10min at 14,000xg at 4 °C.

2.11.2 GTP γ S/GDP (Positive and Negative Controls)

0.5-1ml of each cell lysate was made up to 1ml with 1X Assay Lysis Buffer, followed by, the addition of 20 μ l of 0.5M EDTA. 10 μ l of 100X GTP γ S was added to the positive control tube and 10 μ l of 100X GDP was added to the negative control tube. Positive and negative controls were incubated for 30min at 30 °C with agitation. Then 65 μ l of 1M MgCL₂ was added to stop the reaction. Tubes were placed on ice.

2.11.3 Ran Pull-Down Assay

In separate eppendorfs, 40 μ l of thoroughly resuspended RanBP1 PBD Agarose beads were added to 1ml of cell lysate made up with 1X Assay Lysis Buffer. Cell Lysates were incubated with gentle agitation for 1h at 4 °C followed by 10sec centrifugation at 14,000xg. Supernatant was carefully discarded and bead-pellet conjugate was washed 3 times with 0.5ml of 1X Assay Buffer. Thereafter, the bead pellet was resuspended in 40 μ l of 2X reducing SDS-PAGE sample buffer and boiled for 5min, finally followed by centrifugation at 14,000xg for 10sec. Positive and negative control samples (see section 2.11.2) were treated in a similar manner before separation by SDS-PAGE.

2.11.4 Western blotting

See sub-sections 2.6.4-2.6.6.

Antibody	Dilution
Primary Mouse Anti-Ran antibody	1:1000
Secondary Goat Anti-Mouse IgG	1:2000

Table 2.5 Details of anti-Ran antibody

2.12 Blood monocyte extraction

80ml of whole blood was collected from each patient. 15ml of Ficoll-Paque solution (GE Healthcare, Chalfont St Giles, UK) was added to 4x 50ml conical flasks. 20ml of blood was carefully pipetted over the Ficoll-Paque solution, followed by centrifugation for 30min at 400xg at room temperature with the brake off. The top layer, plasma, was carefully discarded from the 50ml falcon tubes. The cloudy layer, containing all the PBMCs, was gently collected using a Pasteur pipette and transferred into a newly labeled 50ml falcon tube. The remaining 50ml volume was made up with PBS and centrifuged at 200xg for 10min with the brake on. PBMCs were seeded at a density of 2×10^6 cells/ml in complete media for 2h at 37°C and 5% CO₂. The supernatant was carefully aspirated off and adherent cells, monocytes, were washed and resuspended in minimal media with relevant stimulants.

2.13 Replicate experiments and statistical analysis

At least three independent experiments were performed, with each sample reported as the mean of duplicate or triplicate analysis. For most experiments, due to the numbers we assumed a non-Gaussian distribution and analysed data using non-parametric tests. For comparison of one group to a hypothetical value or comparison of two paired groups we used the Wilcoxon test. Comparison of two unpaired groups

was determined by the Mann-Whitney test, whereas analysis of three or more unmatched groups was initially analysed by Kruskal-Wallis ANOVA prior to specific non-parametric comparisons. Results were considered significant if $p < 0.05$.

CHAPTER 3

COMPARISON OF THREE CORTICOSTEROIDS ON GLUCOCORTICOID RECEPTOR FUNCTION AND ACTIVITY

3.1 Introduction

Corticosteroids are widely used in the treatment of asthma as monotherapy and, in both asthma and chronic obstructive pulmonary disease (COPD) in combination with bronchodilators. As previously discussed, corticosteroids exert their effects by binding to the glucocorticoid receptor (GR), causing GR nuclear translocation, where activated GR either upregulates transcription of anti-inflammatory genes or downregulates the transcription of pro-inflammatory genes. Activated GR may bind directly to the promoter regions of genes that encode pro-inflammatory proteins via GREs or negative GREs. GR binding to the palindromic promotor induces the transcriptional induction of anti-inflammatory genes such as *DUSP1* and *secretory leukocyte protease inhibitor (SLPI)*. In contrast, activated GR recruits HDAC2 to NF- κ B complexes, which inhibits a range of pro-inflammatory mediators. By this process of transrepression, corticosteroids suppress pro-inflammatory mediators such as TNF- α and CXCL8 in mild-to-moderate asthmatic patients whereas they are far less effective in COPD patients (Barnes, 2006b, Barnes, 2011).

Better understanding of the structure activity relationships of inhaled corticosteroids has led to the development of new corticosteroids with higher therapeutic ratios and lower systemic bioavailabilities. Fluticasone propionate (FP) has been shown to have high affinity and selectivity for GR and low systemic absorption (Phillipps, 1990). GR affinity of FP and mometasone furoate (MF) is estimated to be about 12 and 5 times of dexamethasone and BUD, respectively (Crim et al., 2001). Recently, fluticasone furoate (FF), a novel new longer-acting ICS, under development as once-daily treatment in combination with the LABA, vilanterol trifenate (VI), for asthma and COPD, has been shown to have a greater affinity for GR than FP. Valotis and Hogger have shown, FF has a very fast association and a slow dissociation from human GR binding kinetics, with a relative receptor affinity (RRA; relative to dexamethasone (RRA: 100)) of 2,988, in comparison with other corticosteroids, which have a much lower receptor affinity: MF, 2,244; FP, 1,775; and BUD, 855 (Valotis and Hogger, 2007). This may be partly due to better H-bonding and van der Waals interaction of the 17- α ester group of FF with the ligand-binding site of GR, giving rise to a prolonged duration of action as seen in dose ranging studies in asthmatics, where FF had at least 23 hours duration of efficacy (Woodcock et al., 2011, Oliver et al., 2012).

FP and FF are structurally related; however their chemical and pharmacological properties are different. These differences in physiochemical properties, may contribute to a favourable efficacy profile for FF. FF was identified after chemical changes on the 17- α ester group of FP. Based on these pharmacodynamic and pharmacokinetic differences between these corticosteroids it would be important to

determine whether they have an impact on corticosteroid-induced GR nuclear translocation and subsequent downstream events, such as transactivation and suppression of pro-inflammatory mediators.

The combination of corticosteroids and LABA has been shown to improve clinical effectiveness and anti-inflammatory properties in asthma (Juniper et al., 1999, Woolcock et al., 1996). The addition of LABA to low dose corticosteroids has been shown to be more clinically beneficial in asthma than the use of high dose inhaled corticosteroids, allowing a reduction in the dose of corticosteroid and minimising adverse side effects related to corticosteroid therapy. Corticosteroids such as BUD, beclomethasone dipropionate (BDP) and FP have been used in combination with LABA such as FORM and SLM. These combination treatments are established in International Guidelines for treating patients with asthma (GINA, 2011) and also, COPD (Rabe et al., 2007). Evidence suggests that LABA enhance GR function *in vitro*. In an asthmatic study, the combination of SLM and FP was as effective as high dose FP on GR activation, gene transactivation and transrepression (Usmani et al., 2005b). The LABA SLM and FORM and recently indacaterol are the only licensed LABA for clinical use as monotherapy in COPD and in combination with corticosteroid for both asthma and COPD. FORM is used in combination with BUD, or in combination with BDP, whereas salmeterol is used in combination with FP. LABA may interact and bind with the β_2 -adrenergic receptor (β_2 -AR) in different ways, and may elicit different responses (Coleman et al., 1996). For example, in bronchial tissue preparations FORM acts as a nearly full β_2 -AR agonist, whereas SLM acts as a partial β_2 -AR agonist (Kallstrom et al., 1994, Scott et al., 1999).

Increased potency may lead to greater phosphorylation of GR via increased MAPK activation (Adcock et al., 2002, Miller et al., 2005). Phosphorylation of GR may alter its nuclear translocation (Weigel and Moore, 2007). Based on this evidence, it would be important to investigate the effects of LABA in combination with their clinically co-administered corticosteroids in comparison to the newer and longer-acting combination of FF and VI, which are currently under development.

Differences in the chemical structure and physiochemical properties of corticosteroids, as well as their affinity for GR may determine their duration of action (Valotis and Hogger, 2007, Crim et al., 2001). Together, these factors will control and affect the clearance of inhaled drug from the lungs. Processes that govern drug clearance in the lungs are mostly absent in the *in vitro* setting. In order to mimic lung clearance and thereby to better model the *in vivo* environment, I have incorporated a 20h washout step, following 4h corticosteroid incubation in my experiments.

The duration of action of a corticosteroid will affect its efficacy. Increasing the duration of corticosteroid-induced GR nuclear translocation may improve the corticosteroid's duration of action. GR nuclear translocation is a dynamic process governed by nuclear import and export receptors. A nuclear export signal (NES) located on the cargo protein is recognised by exportins. Recognition of the NES of a protein results in the formation of an exportin-cargo protein complex and subsequent nuclear export of the complex. Several studies have suggested that the nuclear exportin pathway CRM-1 may govern GR nuclear export. In an *in vitro* study, leptomycin B (LMB), a specific inhibitor of CRM-1 mediated nuclear export, inhibited

GR nuclear export, which led to enhanced unliganded and hormone-treated GR nuclear localisation (Savory et al., 1999). It would be important to characterise the role of CRM-1 mediated transport on GR nuclear export in inflammatory cells important in asthma and COPD. Unravelling the mechanisms that govern GR nuclear export would allow one to potentially increase the pharmacokinetic profile of a corticosteroid, as well as more importantly, the duration of the anti-inflammatory effects of the corticosteroid.

The alveolar macrophage is predominantly found in the alveolar spaces of the lung and in the small conducting airways and has been linked to the pathogenesis of asthma and COPD (Peters-Golden, 2004). As there are no human macrophage cell lines available, we used a well established model of monocytic U937 cells, which has similar phenotypes to macrophages (Harris and Ralph, 1985).

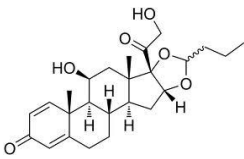
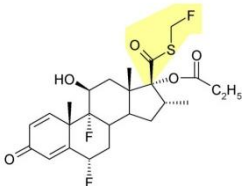
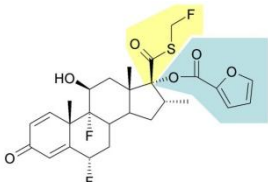
	Budesonide (BUD)	Fluticasone propionate (FP)	Fluticasone furoate (FF)
Structure <i>(Valotis, 2007)</i>			
GR affinity (Relative receptor affinity; DEX:100) <i>(Valotis, 2007)</i>	855	1775	2989
IC₅₀ (Suppression of IL-1β -induced CXCL8 (Fig. 3.3.2.1))	5.4±3.0×10 ⁻¹⁰ M	1.2±3.0×10 ⁻¹⁰ M	2.3±3.1×10 ⁻¹¹ M

Fig. 3.1 Comparison of the structures and GR affinities of budesonide, fluticasone propionate and fluticasone furoate.

3.2 Experimental protocols

3.2.1 GR nuclear translocation

Following 3h serum-starvation, U937 cells (20ml of 0.5×10^6 cells/ml) were incubated with different corticosteroids for different time points. Cells were immediately washed with HBSS and extraction of cytoplasmic and nuclear fractions was performed (section 2.6.1). A pilot *in vitro* study was conducted to determine the optimal concentration of each corticosteroid to be used (The final concentration of DMSO (0.25%) used to dissolve the corticosteroid had no effect on GR subcellular localisation (Fig. 3.2.1.1). As seen in Figure 3.2.1.2, FF-induced GR nuclear localisation in a concentration-dependent manner with a sub-maximal effect being seen with 10^{-9} M FF. This sub-maximal concentration was used to compare the activity and function of each corticosteroid for all future experiments.

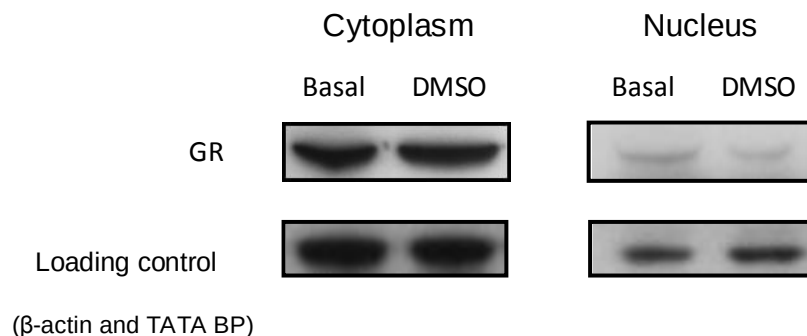


Fig. 3.2.1.1 Effect of DMSO (0.25%) on GR nuclear translocation. Serum-starved U937 monocytes were incubated with DMSO (0.25%).

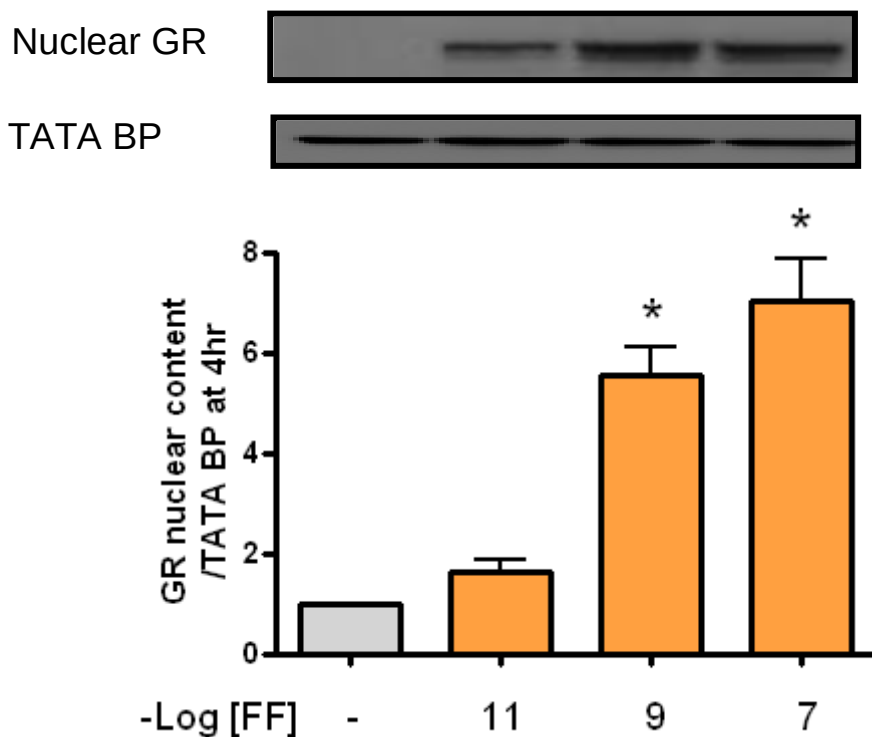


Fig. 3.2.1.2 Effect of varying concentrations of fluticasone propionate (FF, 10^{-11} - 10^{-7} M) on GR nuclear localisation. Serum-starved U937 monocytes were incubated with FF for 4h. Nuclear fractions were immediately isolated followed by Western blot analysis of GR. GR levels were normalised to levels of TATA binding protein (TATA BP). Results represent the mean $\pm$ SEM of 3 independent experiments. * p <0.05 vs. basal.

3.2.2 CXCL8 ELISA

Serum-starved U937 cells (200 μ l/well, 0.5×10^6 cells/ml) were seeded in 96-well plates and treated with a range of IL-1 β concentrations (0.1-100ng/ml) in order to determine the sub-maximal concentration of IL-1 β required to stimulate CXCL8 release from these cells after 16h. As shown in Figure 3.2.2, IL-1 β increased extracellular CXCL8 secretion in a concentration-dependent manner. IL-1 β at 1, 10 and 100ng/ml increased CXCL8 secretion by 290 ± 28 , 350 ± 27 and 480 ± 23 ng/ml respectively, with no significant difference seen between 1 and 10ng/ml. All future experiments were conducted with the sub maximal concentration of IL-1 β (1ng/ml).

In subsequent experiments, serum-starved U937 cells (200µl/well, 0.5×10^6 cells/ml) seeded in 96-well plates were treated with corticosteroid (BUD, FP or FF) and LABA (FORM, SLM or VI), alone and in combination, following 30min stimulation with IL-1 β (1ng/ml). 16 h post IL-1 β stimulation, the cell supernatant was collected to determine CXCL8 secretion levels.

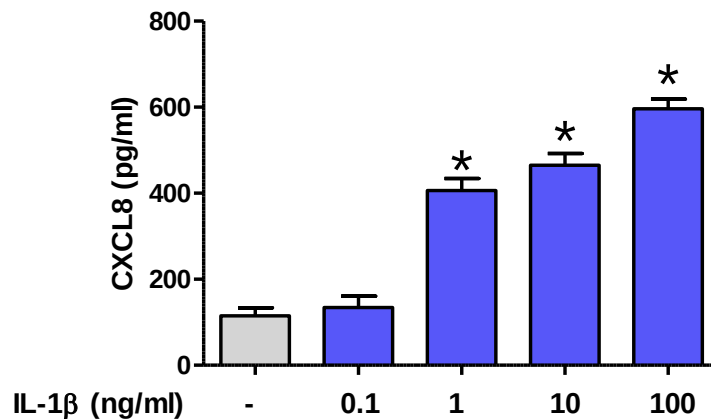


Fig. 3.2.2 Effect of IL-1 β on CXCL8 secretion in U937 monocytes. Serum-starved U937 monocytes were stimulated with varying concentrations of IL-1 β for 16 h. Results represent the mean $\pm$ SEM of 3 independent experiments. * $p < 0.05$ vs. basal

3.2.3 *DUSP1* mRNA expression

Serum-starved U937 cells seeded in 24-well plates, as 0.5×10^6 cells/ml were treated with corticosteroids for 3h. *DUSP1* mRNA expression was quantified by RT-qPCR (see section 2.9).

3.2.4 Corticosteroid washout

Serum-starved U937 cells were seeded at 20ml of 0.5×10^6 cells/ml in flasks and 200µl of 0.5×10^6 cells/ml in 96-well plates for GR nuclear localisation and CXCL8 ELISA, respectively. Cells were treated with corticosteroid for 4h followed by 20h

washout. In addition, U937 cells seeded in 96-well plates were also pre-treated with IL-1 β (1ng/ml).

3.3 Results

3.3.1 Comparison of BUD-, FP- and FF-induced GR nuclear translocation

GR nuclear translocation is essential for corticosteroid action. We explored whether differences in the physiochemical properties and binding affinities of corticosteroids would impact on the level of GR nuclear translocation. GR nuclear translocation post corticosteroid incubation was examined at 2h and 24h. At 2h continual exposure, BUD, FP and FF (all at 10^{-9} M) significantly induced GR nuclear localisation by 2.96 ± 0.74 -, 6.06 ± 0.95 - and 7.3 ± 1.48 -fold, respectively, relative to basal levels (* $p<0.05$ vs. basal). FF-induced GR nuclear localisation was significantly greater than BUD ($^{\#}p<0.05$ vs. BUD 10^{-9} M), but similar to that for FP. There was a corresponding decrease in cytosolic GR levels of $43.1\pm6.43\%$ (BUD), $70.9\pm5.0\%$ (FP) and $80.5\pm6.14\%$ (FF), relative to basal levels (* $p<0.05$ vs. basal) (Fig. 3.3.1a). Additionally, levels of GR nuclear translocation 2h post BUD, FP and FF challenge were maintained with 24h continual exposure. Continual exposure for 24h to BUD, FP and FF at 10^{-9} M significantly induced GR nuclear localisation by 3.0 ± 0.61 , 5.6 ± 0.88 and 6.3 ± 0.84 -fold, respectively (* $p<0.05$ compared to unstimulated controls). Similarly, FF-induced GR nuclear localisation was significantly greater than BUD, but comparable to that achieved with FP, as was observed during the 2h continual exposure experiments. Once again, there were corresponding decreases in cytosolic GR post corticosteroid treatment (Fig. 3.3.1b).

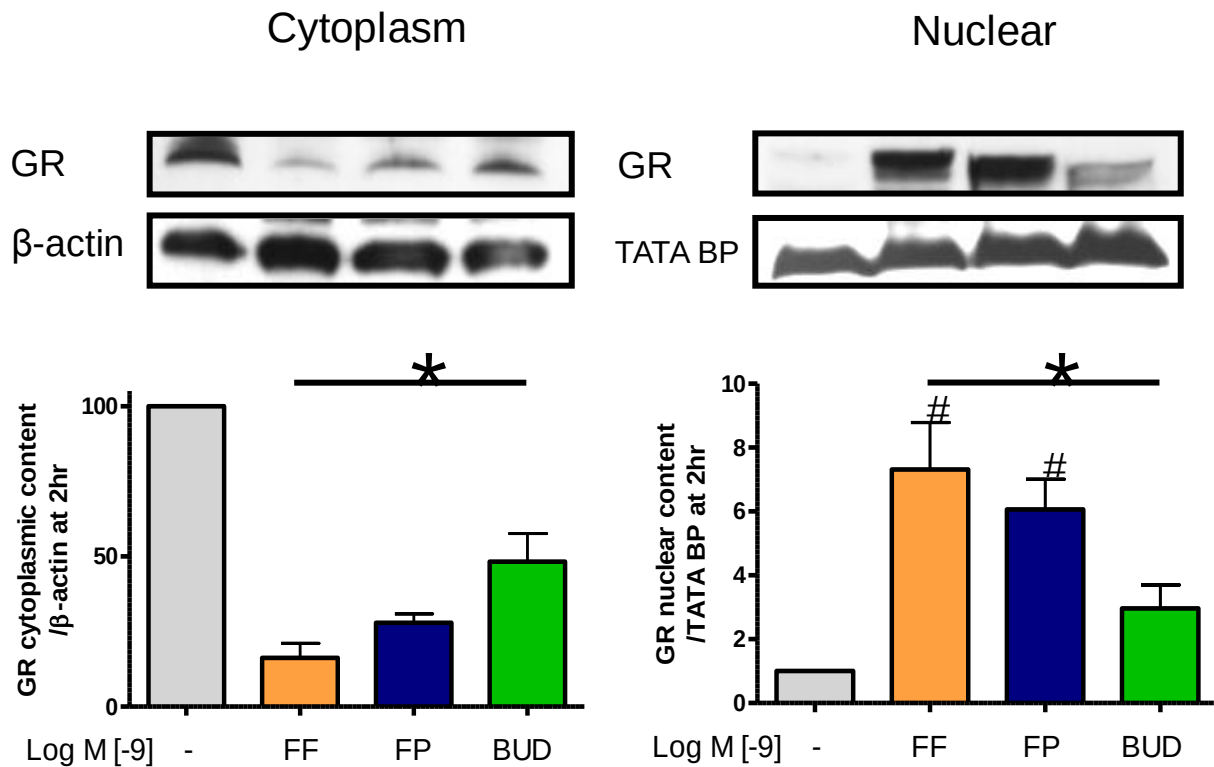


Figure 3.3.1a Effect of budesonide (BUD), fluticasone propionate (FP) and fluticasone furoate (FF), all at 10^{-9} M, on GR nuclear translocation. U937 monocytes were incubated in low-serum media to reduce endogenous steroid content and to drive cells towards synchrony. U937 monocytes were then incubated with BUD, FP and FF for 2h. Results represent the mean $\pm$ SEM of 3 independent experiments. * $p < 0.05$ vs. basal. # $p < 0.05$ vs. BUD 10^{-9} M.

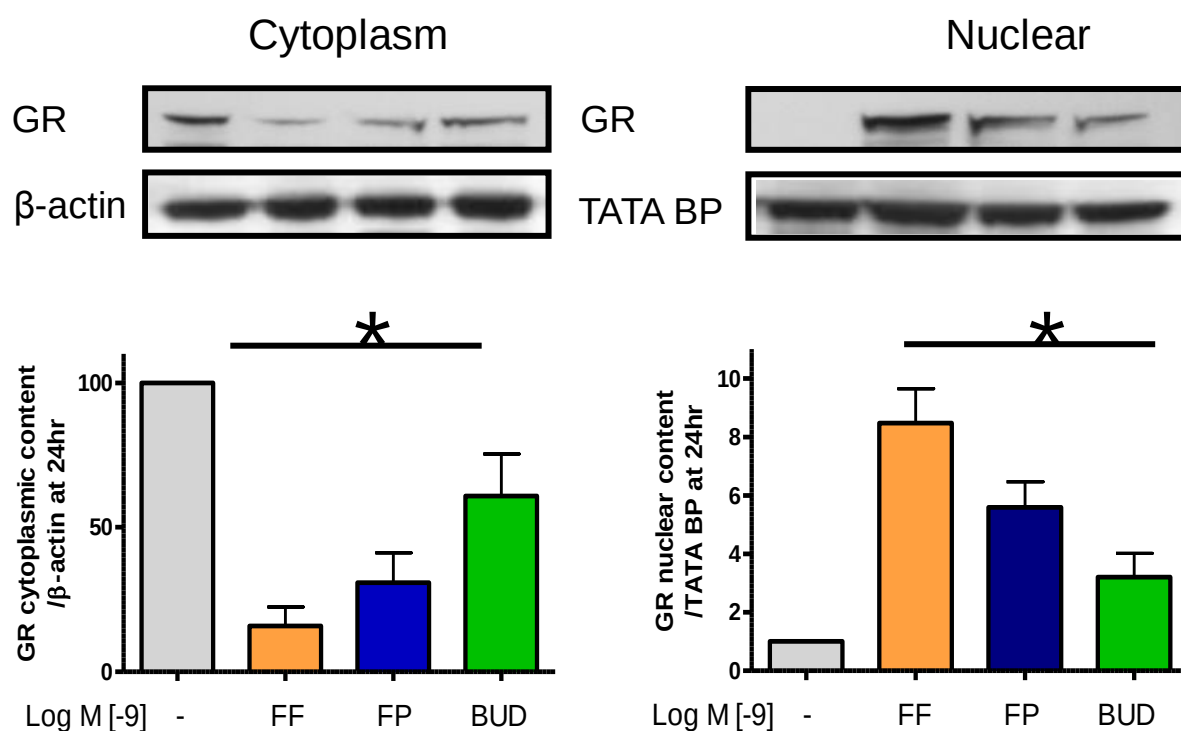


Fig 3.3.1b Effect of budesonide (BUD), fluticasone propionate (FP) and fluticasone furoate (FF), all at 10^{-9} M, on GR nuclear translocation. U937 monocytes were incubated in low-serum media to reduce endogenous steroid content and to drive cells towards synchrony. U937 monocytes were then incubated with BUD, FP and FF for 24h. Results represent the mean $\pm$ SEM of 3 independent experiments. * $p < 0.05$ vs. basal.

3.3.2 Cytokine secretion

3.3.2.1 Corticosteroids suppress CXCL8 secretion with varying potencies

Next we undertook to determine whether the differences seen in BUD-, FP- and FF-induced GR nuclear translocation would result in different levels of IL-1 β -induced CXCL8 suppression. Briefly, U937 cells were stimulated with the sub-maximal concentration of IL-1 β (1ng/ml) for 30min, followed by incubation with BUD, FP and FF. 16h post IL-1 β stimulation, supernatant was collected and CXCL8 levels were measured. BUD, FP and FF suppressed CXCL8 in a concentration-dependent

manner, with IC_{50} values of $5.4 \pm 3.0 \times 10^{-10} M$ (BUD), $1.2 \pm 3.0 \times 10^{-10} M$ (FP) and $2.3 \pm 3.1 \times 10^{-11} M$ (FF) (Fig. 3.3.2.1). There was more than a 10-fold increase in the concentration required to give 50% inhibition between FF and BUD, which reflects the differences seen in levels of GR nuclear translocation. Additionally, the maximal inhibition of CXCL8 achieved by BUD, FP and FF were $69.1 \pm 19.8\%$ (BUD), $85.1 \pm 12.2\%$ (FP) and $81.7 \pm 5.3\%$ (FF) with no significant difference seen between all three corticosteroids. At $10^{-11} M$, BUD only inhibited CXCL8 secretion by $19.3 \pm 2.3\%$, whereas the corresponding concentrations of the more potent FP and FF suppressed CXCL8 secretion by significantly greater levels ($41.6 \pm 7.1\%$ and $54.2 \pm 7.4\%$ respectively, $p < 0.05$ vs. BUD $10^{-9} M$).

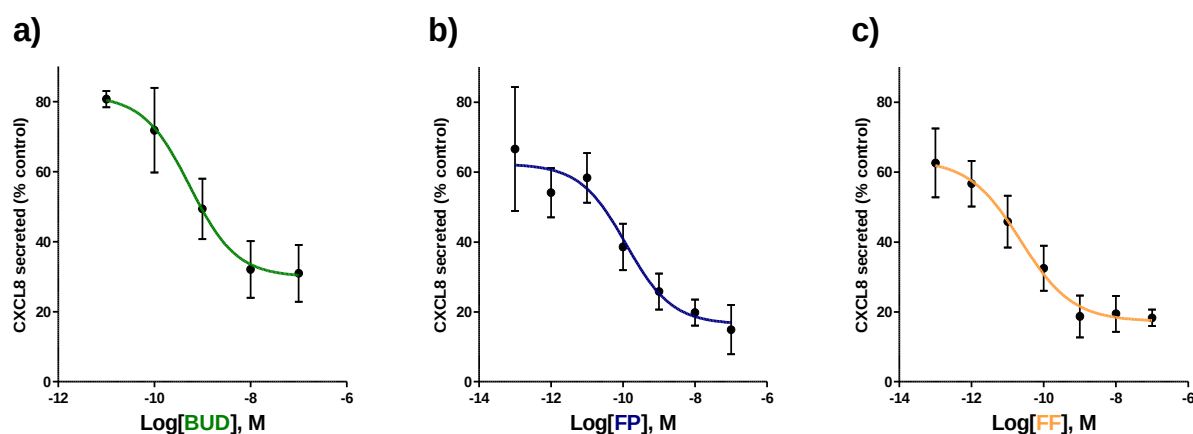


Fig. 3.3.2.1 Effect of budesonide (BUD), fluticasone propionate (FP) and fluticasone furoate (FF) on IL-1 β -induced CXCL8. Serum-starved U937 monocytes were incubated with IL-1 β for 30min, followed by incubation with (a) BUD, (b) FP or (c) FF. 16h post IL-1 β stimulation, supernatant was collected for CXCL8 analysis. Results represent the mean $\pm$ SEM of a minimum of 3 independent experiments.

3.3.2.2 LABA enhance corticosteroid suppression of CXCL8

Next we explored the effects of different concentrations of corticosteroid (10^{-11} and $10^{-9} M$) alone, and in combination with LABA ($10^{-9} M$) on IL-1 β -induced CXCL8

secretion. BUD, FP and FF at 10^{-11} M significantly suppressed IL-1 β -induced CXCL8 by $25.0\pm5.5\%$, $34.0\pm4.9\%$ and $35.0\pm5.2\%$, respectively. The suppression seen with both FP and FF being significantly greater than that seen for BUD. In contrast, none of the LABA tested (FORM, SLM and VI, all at 10^{-9} M) had any effect on IL-1 β -induced CXCL8. However, the addition of LABA (FORM, SLM and VI all at 10^{-9} M) to their respective corticosteroids (BUD, FP and FF all at 10^{-11} M) resulted in greater suppression of IL-1 β -induced CXCL8 release to $46.8\pm11.1\%$, 43.0 ± 7.0 and $52.9\pm7.86\%$ of control levels respectively (Fig. 3.3.2.2). However, although the combination of FP (10^{-11} M) with SLM (10^{-9} M) further enhanced FP-induced suppression of CXCL8 by $9.29\pm4.88\%$ this was not statistically significant. In contrast, FORM and VI (both at 10^{-9} M) significantly enhanced BUD and FF (both at 10^{-11} M)-induced suppression of CXCL8 secretion by 22.0 ± 11.0 and $18.0\pm7.9\%$ respectively, relative to BUD (10^{-11} M) and FF (10^{-11} M) alone (* $p<0.05$). Furthermore, BUD (10^{-11} M) in combination with FORM (10^{-9} M) was as effective as the higher concentration of BUD (10^{-9} M). In contrast, FF (10^{-11} M) in combination with VI (10^{-9} M) was not as effective as the higher concentration of FF (10^{-9} M).

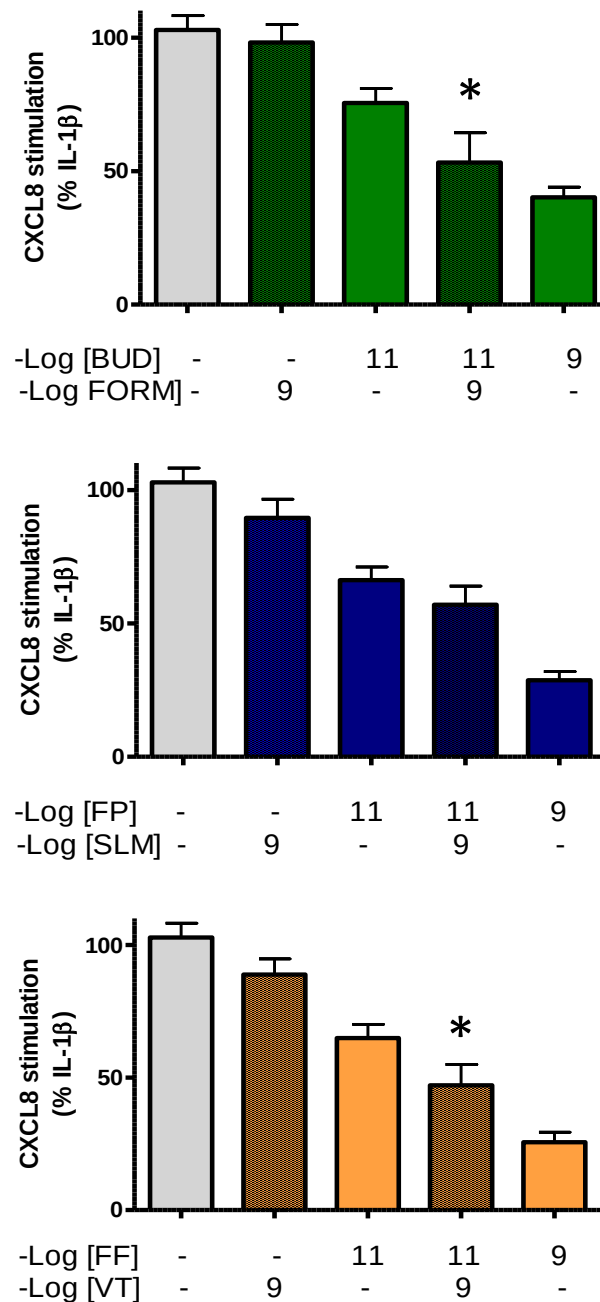


Fig. 3.3.2.2 Effect of budesonide (BUD), fluticasone propionate (FP) and fluticasone furoate (FF), alone and in combination with their respective LABAs on IL-1 β -induced CXCL8 release. Serum-starved U937 monocytes were incubated with IL-1 β for 30min, followed by incubation with corticosteroids (BUD, FP and FF all at 10^{-11} M) and LABAs (formoterol; FORM, salmeterol; SLM and vilanterol trifenate; VI all at 10^{-9} M) alone and in combination. 16h post IL-1 β stimulation, supernatant was collected for CXCL8 analysis. Results represent the mean $\pm$ SEM of a minimum of 3 independent experiments. * $p < 0.05$ vs. corticosteroid (10^{-11} M) alone.

3.3.3 *DUSP1* expression

So far we have established that FF-induced GR nuclear translocation and suppression of IL-1 β is significantly greater than that of BUD, but similar to that observed with FP, when corticosteroids are used at 10⁻⁹M. We next wanted to determine whether this was also true for corticosteroid-induced gene transactivation. We investigated the effects of BUD, FP and FF (all at 10⁻⁹M) on *DUSP1* mRNA expression (Fig. 3.3.3). BUD-, FP- and FF-induced *DUSP1* mRNA expression by 6.7 $\pm$ 1.3-, 4.0 $\pm$ 1.7- and 4.9 $\pm$ 1.7-fold respectively relative to basal levels (*p<0.05 vs. basal). Interestingly, unlike GR nuclear translocation and suppression of IL-1 β -induced CXCL8, FF was not superior to BUD. In fact, BUD caused a greater increase in *DUSP1* mRNA expression, compared to both FP and FF, although this did not reach statistical significance.

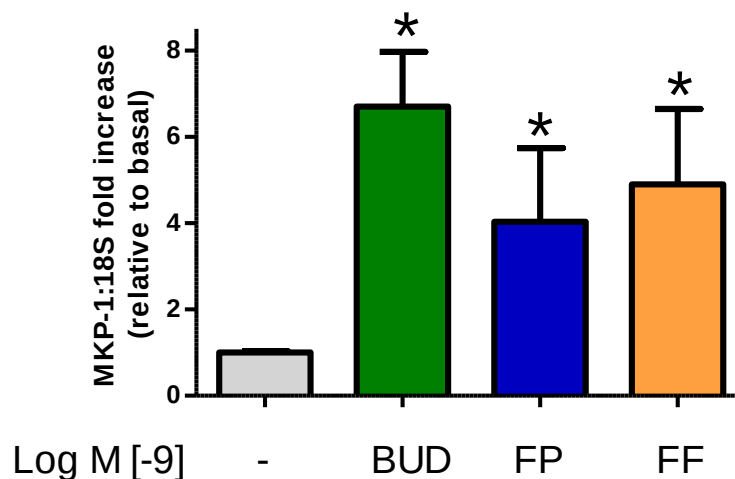


Fig. 3.3.3 Effect of budesonide (BUD), fluticasone propionate (FP) and fluticasone furoate (FF) on *DUSP-1* mRNA induction. Serum-starved U937 monocytes were incubated with BUD, FP and FF for 3h. mRNA extraction was performed immediately. Results represent the mean $\pm$ SEM of 3 independent experiments. (*p<0.05 vs. basal).

3.3.4 Corticosteroid washout

3.3.4.1 FF maintains GR nuclear localization following 20h washout

BUD, FP and FF all induced sustained GR nuclear translocation over a 24h period in a constant drug contact setting. However, in the lungs, ICS are exposed to drug clearance; that is, as inhaled drug reaches the airways it becomes exposed to a variety of mechanisms that clear the drug from the airways. In order to determine whether the differences in the physiochemical properties between these three corticosteroids and their binding affinities for GR affect corticosteroid duration of action, I introduced a 20h corticosteroid washout model following a 4h pre-incubation. In the 20h washout experiments, there was a similar level of GR nuclear translocation seen with FF (10^{-9} M) treatment at 24h (4+20h) as seen with continual FF exposure (5.4 ± 1.0 - versus 6.3 ± 0.84 -fold increase, $p=ns$). However, FP- and BUD-induced GR nuclear translocation was dramatically reduced back to basal levels in the 20h washout model (Fig. 3.3.4.1).

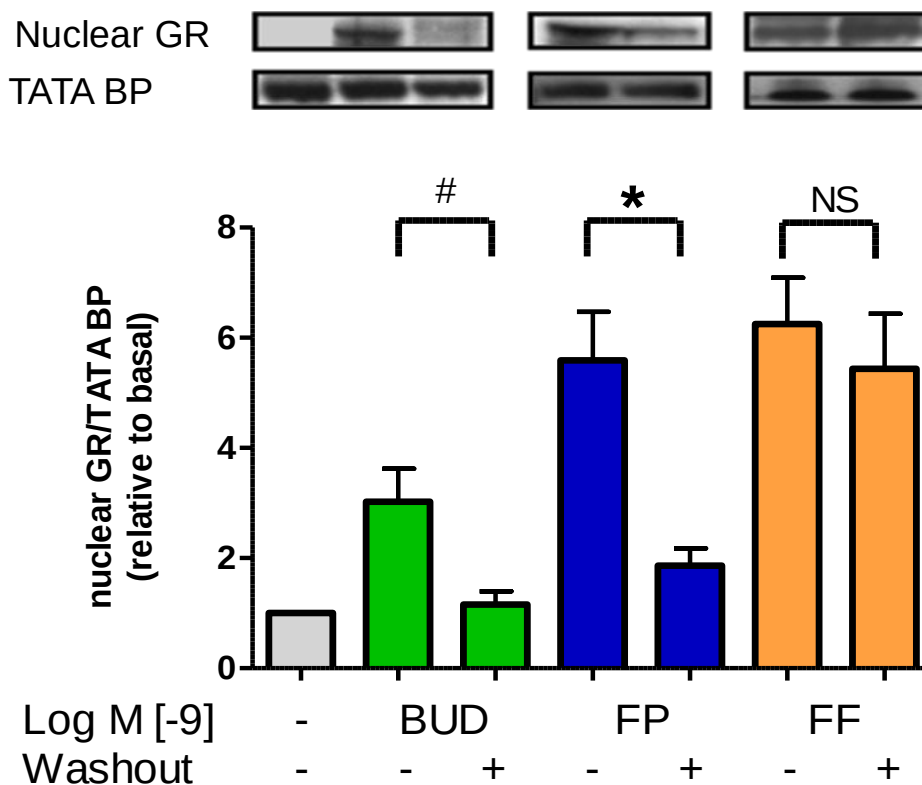


Fig. 3.3.4.1 Effects of budesonide (BUD), fluticasone propionate (FP) and fluticasone furoate (FF) on GR nuclear localisation. Serum-starved U937 monocytes were incubated with BUD, FP and FF for 4h, followed by a 20h washout. Nuclear extraction was conducted immediately and nuclear levels of GR were determined by Western blot. Results represent the mean $\pm$ SEM of a minimum of 3 independent experiments. # p <0.05 vs. 24h BUD 10^{-9} M contact. * p <0.05 vs. 24h FP 10^{-9} M contact.

3.3.4.2 FF maintains IL-1 β -induced CXCL8 suppression during 20h washout

Similarly, FF (52 $\pm$ 6.4 vs. 69 $\pm$ 6.6%, p =ns)-mediated suppression of IL-1 β -induced CXCL8 was not affected in the 20h washout model. In contrast, BUD (2.2 $\pm$ 2.2 vs. 45.9 $\pm$ 7.7%, # p <0.05) and FP (38.2 $\pm$ 9.0 vs. 69.0 $\pm$ 5.1%, * p <0.05)-mediated suppression of IL-1 β -induced CXCL8 was significantly reduced.

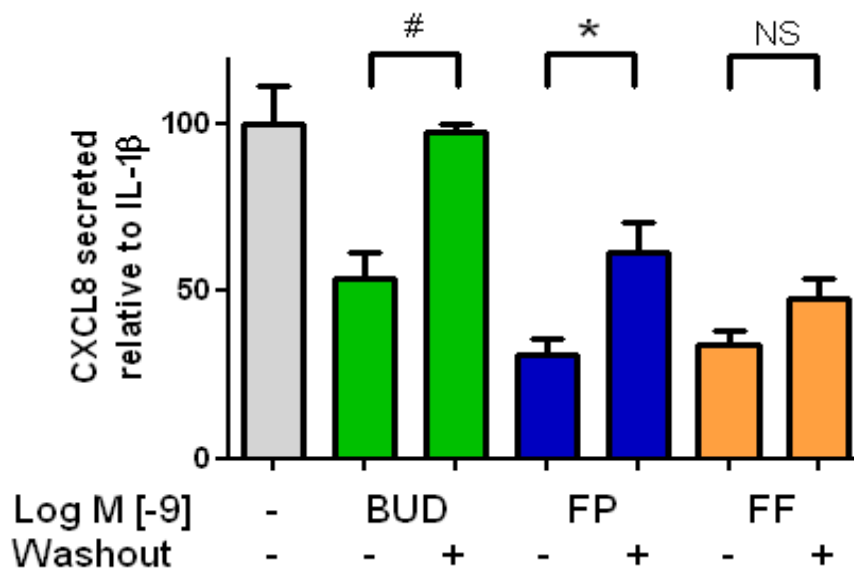


Fig 3.3.4.2 Effects of budesonide (BUD), fluticasone propionate (FP) and fluticasone furoate (FF) on IL-1 β -induced CXCL8. Serum-starved U937 monocytes were firstly stimulated with IL-1 β for 30min followed by 4h BUD, FP or FF incubation. After 4h, corticosteroid was washed out, IL-1 β was replaced and cells further incubated for another 20h. Results represent the mean $\pm$ SEM of a minimum of 3 independent experiments. # p <0.05 vs. 24h BUD 10⁻⁹M contact. * p <0.05 vs. 24h FP 10⁻⁹M contact.

3.3.5 LMB enhances FF-induced GR nuclear localisation

So far, we have shown that a 20 h washout leads to reduced FP- and BUD-induced GR nuclear import. GR nucleocytoplasmic shuttling is a dynamic process, a balance between GR nuclear import and export. Inhibiting GR nuclear export may shift the balance towards GR nuclear import and therefore lead to increased retention of corticosteroid-induced GR nuclear localisation. We therefore explored the effects of inhibition of exportin-1 on corticosteroid-induced GR nuclear localisation. Nuclear export inhibitor, LMB (10nM) alone, had no effect on GR subcellular localisation (Fig. 3.3.5.1). I pre-treated cells with LMB (10nM) for 30 min, followed by incubation with FF (10⁻⁷M) for 2h. I observed that FF-induced GR nuclear localisation in a

concentration dependent manner with a 4.7 ± 0.54 - and 6.2 ± 0.65 -fold increase seen at 10^{-9} and 10^{-7} M respectively (both $*p < 0.05$ vs. basal levels) (Fig. 3.3.5.2). More importantly, pre-treatment with LMB (10^{-8} M) significantly enhanced FF (10^{-7} M)-induced GR nuclear localisation at 2h by a further 1.4-fold (8.8 ± 0.90 vs. 6.2 ± 0.65 ; $^{\#}p < 0.05$).

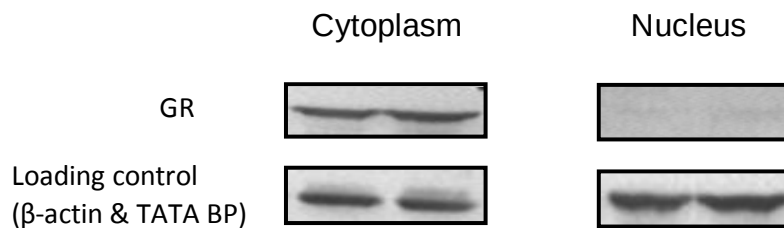


Fig. 3.3.5.1 Effect of leptomycin B (LMB) on glucocorticoid receptor (GR) subcellular localisation. Serum-starved U937 cells were incubated with LMB (10nM) for 4 h.

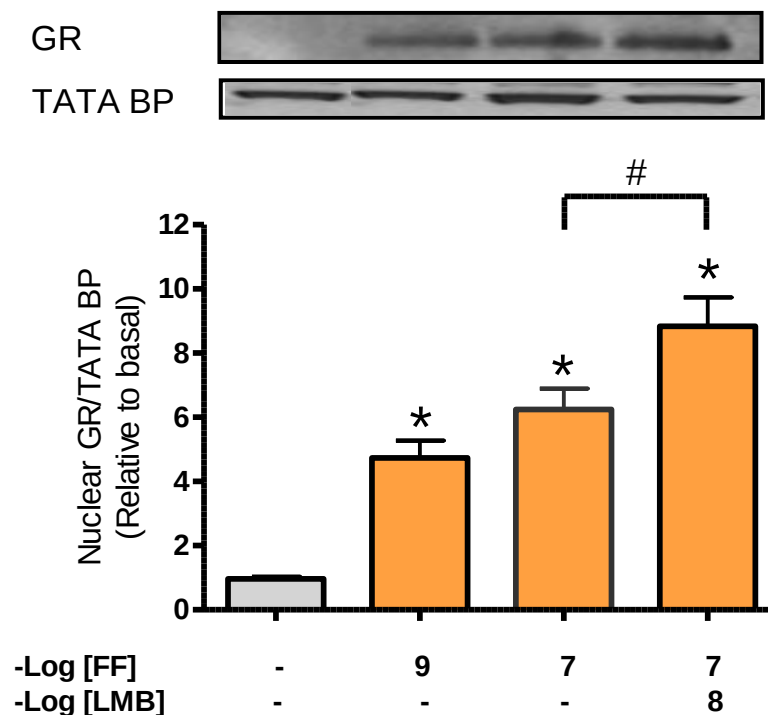


Fig. 3.3.5.2 Effect of leptomycin B (LMB) on fluticasone fuorate (FF)-induced GR nuclear localisation. Serum-starved U937 cells were stimulated with LMB (10nM) for 30min followed by FF for 4h. Results represent the mean $\pm$ SEM of 3 independent experiments. $*p < 0.05$ vs. basal levels. $^{\#}p < 0.05$ vs. FF (10^{-7} M).

3.3.6 LMB enhances corticosteroid induced GR-GRE binding

GR nuclear translocation is essential for activated GR to bind to GRE on corticosteroid-inducible genes. FF significantly induced GR-GRE binding in a concentration-dependent manner with the highest concentration of FF (10^{-7} M) increasing GR-GRE binding by more than 4-fold in comparison to basal nuclear levels (3.2 ± 0.43 vs. 0.84 ± 0.30 , $^*p < 0.05$) (Fig. 3.3.6). Furthermore, pre-treatment with LMB for 30min further enhanced FF (10^{-7} M)-induced GR-GRE binding (4.7 ± 0.38 - vs. 3.2 ± 0.43 -fold increase, $^{\#}p < 0.05$).

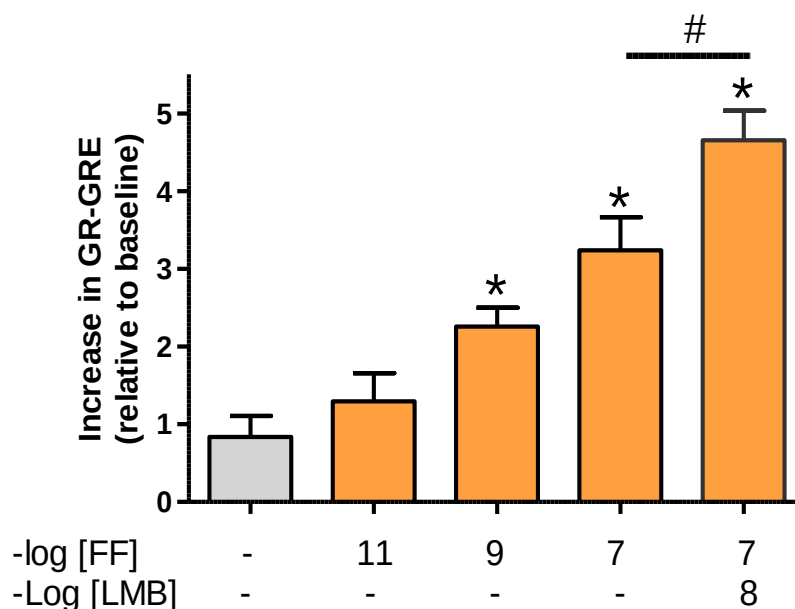


Fig. 3.3.6 Effect of leptomycin B (LMB) on fluticasone fuorate (FF)-induced GR-GRE binding. Serum-starved U937 cells were stimulated with LMB (10nM) for 30min followed by FF for 2h. Results represent the mean $\pm$ SEM of 3 independent experiments. $^*p < 0.05$ vs. basal levels. $^{\#}p < 0.05$ vs. FF (10^{-7} M).

3.3.7 LMB enhances BUD-induced GR nuclear localisation

3.3.7.1 Continual drug contact

So far, we have shown LMB (10nM) enhances FF-induced GR nuclear localisation and GR-GRE binding. In Fig. 3.3.4.1, we showed that BUD- and FP-induced GR nuclear localisation were significantly reduced post 20h corticosteroid washout, whereas FF-induced GR nuclear localisation was not affected. We next investigated whether LMB could reduce the loss in nuclear GR post 20h BUD washout. We first examined the effects of LMB on 4h continual BUD incubation. As seen with FF, pre-treatment with LMB (10nM) for 30min significantly enhanced BUD (10^{-9} M)-induced GR nuclear localisation in comparison to BUD (10^{-9} M) alone (6.4 ± 1.4 - vs. 3.0 ± 0.43 -fold increase, $^{\#}p < 0.05$) (Fig. 3.3.7.1).

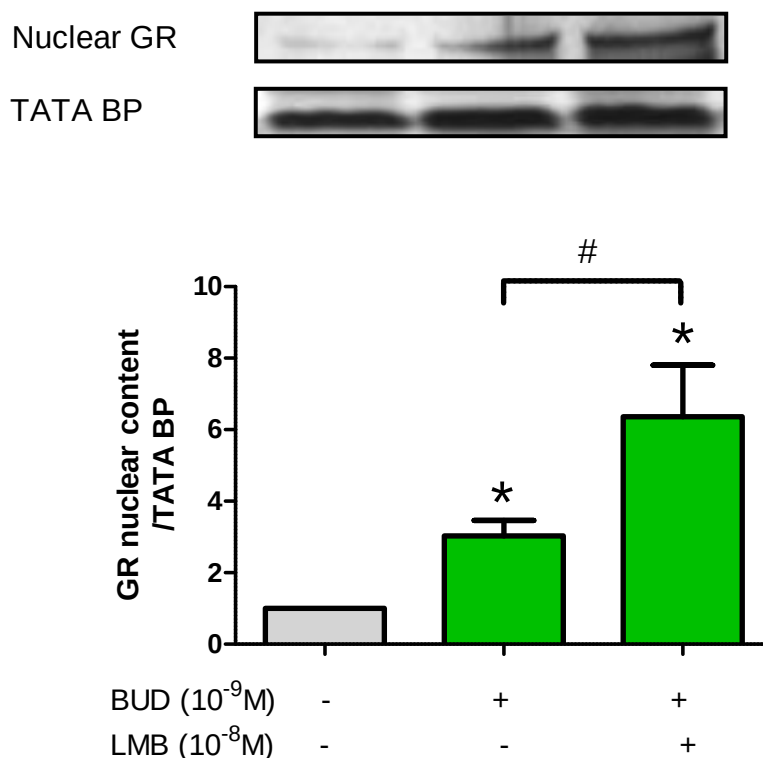


Fig. 3.3.7.1 Effect of leptomycin B (LMB) on budesonide (BUD)-induced GR nuclear localisation. Serum-starved U937 cells were stimulated with LMB (10nM) for 30min followed by incubation with BUD (10^{-9} M) for 4h. Results represent the mean $\pm$ SEM of 3 independent experiments. * $p < 0.05$ vs. basal levels. $^{\#}p < 0.05$ vs. BUD (10^{-9} M).

3.3.7.2 Drug washout

We next investigated whether we could restore BUD-induced GR nuclear import following 20 h BUD washout. Cells were treated with either continual 24h BUD treatment, 4h BUD followed by 20h washout, or 30 min LMB followed by 4h BUD treatment followed by 20h BUD washout. As seen previously, BUD (10^{-9} M)-induced GR nuclear localisation was reduced back to basal levels (3.0 ± 0.61 - vs. 1.2 ± 0.25 - fold increase, $\#p < 0.05$) after 20h BUD washout (Fig. 3.3.7.2). In contrast to continual BUD incubation, LMB (10^{-9} M) did not reduce the loss of BUD-induced GR nuclear localisation post 20h BUD washout (1.2 ± 0.37 vs. 1.2 ± 0.25 , $p = \text{ns}$).

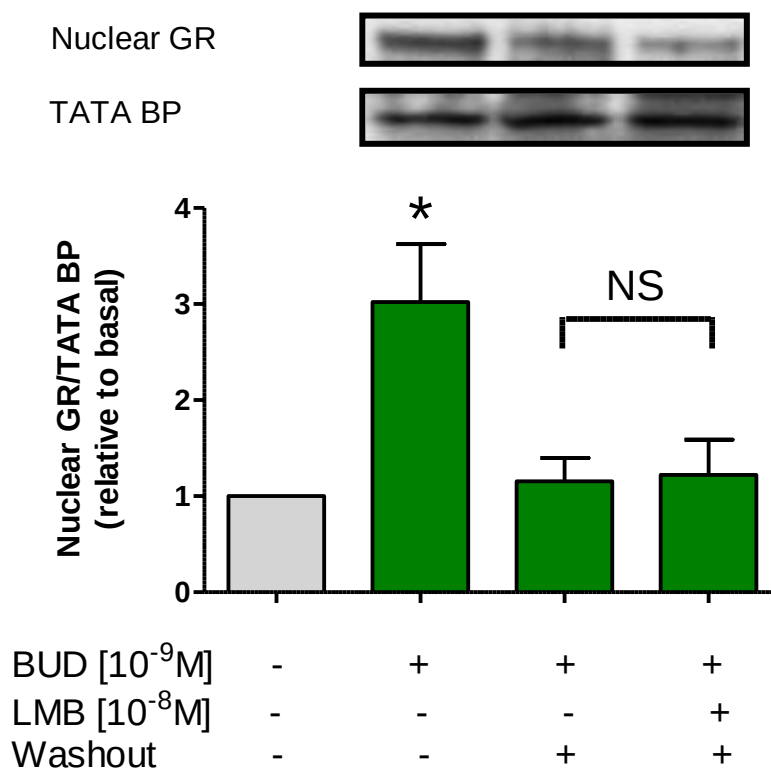


Fig. 3.3.7.2 Effect of leptomycin B (LMB) on budesonide (BUD)-induced GR nuclear localisation post 20h washout. Serum-starved U937 cells were stimulated with LMB (10nM) for 30 min, followed by incubation with BUD (10^{-9} M) for 4h. Following 4h incubation, cells were washed and incubated for a further 20h in the presence of LMB. Results represent the mean $\pm$ SEM of 4 independent experiments. * $p < 0.05$ vs. basal levels. $p = \text{ns}$.

3.4 Discussion

Corticosteroids are recommended in the treatment of patients with asthma and COPD. Since their use in the early 20th Century in respiratory diseases, drug development has been focused on developing corticosteroids with better pharmacodynamic and pharmacokinetic profiles to improve patient compliance and minimise adverse effects. Recently, FF, a new longer-acting corticosteroid under development for asthma and COPD, has been shown to have superior affinity and a slower rate of dissociation from GR than FP (Salter et al., 2007). Furthermore, X-ray crystallography has revealed the superior affinity of FF maybe due to the furoate group of FF in combination with other functional groups on the corticosteroid backbone, allowing better hydrogen-bonding and generation of van der Waal's forces with the amino acids within the GR ligand binding site (Valotis and Hogger, 2007).

We demonstrated that FF at 2h and 24h induced GR nuclear translocation to a greater extent than BUD (section 3.3.1). Additionally, FF suppressed IL-1 β -induced CXCL8 to a greater extent than BUD (Fig. 3.3.2.1). In addition, both BUD and FP, maintained similar levels of GR nuclear translocation at 2h and 24h (section 3.3.1). Determining the duration of action of corticosteroids in the *in vitro* setting is very difficult due to the absence of parameters that dictate ICS clearance from the lungs. However, the introduction of a 20h corticosteroid washout period in our experiments allowed us to better model the *in vivo* environment, as well as better represent the differences in the PK profiles of BUD, FP and FF that have been observed in several studies (Woodcock et al., 2011, Valotis and Hogger, 2007). In the 20h drug washout

experiments, following 4h corticosteroid incubation, FF not only maintained its level of GR nuclear localisation and suppression of IL-1 β -induced CXCL8, it was also far superior to both BUD and FP. Whereas, in contrast, BUD- and FP-induced GR nuclear translocation were significantly reduced to basal levels following a 20h washout. In addition, BUD-mediated suppression of IL-1 β -induced CXCL8 was completely abolished and FP-mediated suppression of IL-1 β -induced CXCL8 was reduced significantly. The longer duration of action of FF in the drug washout experiment can be explained by its enhanced affinity for GR and slower rate of dissociation. In freshly isolated human lung tissue, FF was retained for longer than FP (Valotis and Hogger, 2007). Further studies using higher concentrations of Bud and FP will determine whether the effect of FF can be replicated with other corticosteroids when used at equipotent concentrations.

Inhibiting GR nuclear export may shift the balance towards GR nuclear import and therefore lead to increased retention of corticosteroid-induced GR nuclear localisation. We found, in the continual drug contact setting, pre-treatment with LMB (10nM) for 30 min considerably enhanced BUD- and FF-induced GR nuclear localisation at 2h. However, LMB failed to prevent loss of BUD-induced GR nuclear localisation post 20h BUD washout. Our data is consistent with the findings of Savory *et al*, who showed in an *in vitro* study in Cos7 cells, 200nM of LMB for 1h promoted the movement of wild-type (WT) GR from the cytoplasm into the nucleus (Savory *et al.*, 1999). Additionally, they showed LMB failed to promote GR nuclear translocation in Cos7 cells expressing GR lacking the NL1 fragment, suggesting that the effects of LMB on WT GR were NLS dependent (Savory *et al.*, 1999). In contrast,

Holaska and colleagues showed that GR nuclear export is insensitive to LMB. In their study they demonstrated that GR nuclear export was governed by the nuclear export receptor calreticulin (CRT) (Holaska et al., 2001). They demonstrated that CRT mediated GR nuclear export using cell permeabilisation, microinjection and transfection assays.

The reported differences in the pathways that may regulate GR nuclear export clearly seem contradictory. Several groups have demonstrated multiple sites on GR may be implicated in GR nuclear import and export (Galigniana et al., 2010, Freedman and Yamamoto, 2004, Tao et al., 2006). This suggests that GR nucleocytoplasmic shuttling maybe governed by more than one pathway, which may explain why GR nuclear export maybe sensitive in some cases but not others to LMB. This may also partially explain why LMB failed to prevent loss of nuclear GR post 20h BUD washout, despite enhancing BUD-induced GR nuclear localisation in the drug contact setting at 2h. It is likely that different mechanisms regulate GR nuclear shuttling in the presence and absence of ligand, and these mechanisms may be time-dependent. For example, Savory *et al* showed, rapid nuclear import of GR (4 to 6min) was mediated through the NL1 fragment of GR, whereas slow GR nuclear import (45 to 60min) was NL2 mediated (Savory et al., 1999).

We also compared the effects of combination treatment of corticosteroid and LABA compared to corticosteroid alone. We found FORM and VI respectively, enhanced BUD- and FF-mediated suppression of IL-1 β -induced CXCL8. However, SLM had no statistically significant effect on FP-mediated suppression of IL-1 β -induced CXCL8.

Furthermore, FORM enhanced BUD mediated suppression of IL-1 β -induced CXCL8 by an additional 22 $\pm$ 11%, which was not different to the additional suppression achieved with VI in combination with FF, 18 $\pm$ 8%. This is interesting, as FORM is a full β_2 -agonist, whereas VI, similar to SLM, is a partial β_2 -agonist. This suggests that VI, a partial agonist, is as effective as the full agonist, FORM, in relation to suppression of the pro-inflammatory mediator, CXCL8, in combination with a corticosteroid. A full concentration-response curve for each LABA in combination with their respective corticosteroid used in these experiments may provide additional information. A full β_2 -agonist will induce greater receptor activation and down-stream signal transduction than a partial β_2 -agonist (Lotvall, 2001). One would assume, therefore a full β_2 -agonist, like FORM, would have a greater effect on corticosteroid-mediated suppression of pro-inflammatory mediators than a partial β_2 -agonist, like VI.

There is growing evidence for the molecular rationale of the complementary interactions and effects between corticosteroids and LABA both *in vitro* (Mak et al., 1995, Eickelberg et al., 1999, Papakonstantinou et al., 2012) and *in vivo* (Usmani et al., 2005b, Orsida et al., 2001, Li et al., 1999), where studies have demonstrated that LABA increase GR nuclear translocation and corticosteroids up-regulate β_2 -receptors. In primary human lung fibroblasts and vascular smooth muscle cells, Eickelberg and colleagues observed that both SLM- and salbutamol-induced GR nuclear translocation in the absence of the corticosteroid. This induction of GR nuclear translocation was shown to be protein kinase A (PKA) dependent (Eickelberg et al., 1999). The biological and therapeutic effects of all β_2 -agonists are

exerted via the cell surface β_2 -adrenoceptors (β_2 -A), a member of the G-protein-coupled receptor family. Upon β_2 -agonist binding, the associated α -component dissociates and activates adenylate cyclase, which then leads to the enzymatic production of cyclic adenosine monophosphate (cAMP). Subsequently, PKA is activated, which then phosphorylates intracellular proteins (Johnson, 2004). An investigation in human embryonic kidney cells demonstrated that stimulation of the β_2 -receptor by SLM resulted in the activation of the mitogen-activated protein kinase (MAPK) pathway (Daaka et al., 1997, McDonnell et al., 1998). Activation of MAPK may lead to GR phosphorylation at a number of serine residues (Adcock et al., 2002). This molecular basis may explain the complementary effects of β_2 -agonists on corticosteroids in improving lung function, symptoms, disease exacerbations in both asthma and COPD (Woolcock et al., 1996, Greening et al., 1994, Pauwels et al., 1997, Juniper et al., 1999, Calverley et al., 2007). Comparisons between VI and FORM effects on down-stream signalling pathways such as PKA induction and MAPK activation for example across a range of concentrations will provide further evidence for the mechanism of ICS-LABA cross-talk in these cells.

Finally, we also compared BUD-, FP- and FF-driven induction of *DUSP1* mRNA expression. As expected BUD-, FP- and FF-induced *DUSP1* mRNA expression, with no difference in levels of induction between all three corticosteroids. This is in clear contrast to GR nuclear translocation and suppression of CXCL8 release, where FF was superior to BUD. This may be explained by the use of a concentration of corticosteroid that resulted in saturated levels of *DUSP1* mRNA expression, as the regulation of *DUSP1* mRNA expression maybe very sensitive to corticosteroids.

Therefore, it would be important to perform a full concentration response of corticosteroids on *DUSP1* mRNA expression. In addition, experiments to detail the effects of LABA on GR-induced *DUSP1* mRNA would prove interesting in delineating the mechanisms for GR-LABA cross-talk. Oxidative stress, as detailed in the Introduction, can affect inflammatory gene expression and corticosteroid function. The next chapter therefore examines the effect of oxidative stress on corticosteroid function in these cells.

CHAPTER 4

OXIDATIVE STRESS-INDUCED CORTICOSTEROID INSENSITIVITY

4.1 Introduction

In COPD, a key pathophysiological trait central to the disease pathogenesis is a heightened inflammatory response to oxidative stress (Lee and Yang, 2012, Rahman, 2005). Hydrogen peroxide (H_2O_2) and other biomarkers of oxidative stress, such as 8-isoprostane, are found in elevated levels in exhaled breath condensate, sputum and in the systemic circulation of COPD patients (Dekhuijzen et al., 1996, Montuschi et al., 2000, MacNee, 2005). Oxidative stress is due to the generation of excessive reactive oxygen species (ROS). Under conditions of cellular stress, nuclear factor erythroid 2-related factor (Nrf2) is activated to induce a multitude of antioxidant genes that protect cells from the oxidative stress (Singh et al., 2009).

The lungs are exposed to environmental- and cellular-derived ROS (MacNee, 2005). The major sources of environmental-derived ROS in COPD includes outdoor pollution and cigarette smoke (Rahman, 2005). Inhaled cigarette smoke and other noxious particles induce acute pulmonary inflammation through activation of pro-inflammatory redox-sensitive transcription factors, such as NF- κ B and AP-1 and signalling pathways, such as p38 MAPK and PI3K (Rahman, 2005). The bronchial epithelium of COPD patients and smokers has been shown to have increased expression of p65, a major subunit of NF- κ B, which is negatively correlated to airflow

limitation (Di Stefano et al., 2002). NF- κ B upregulates the transcription of many inflammatory genes which are highly expressed in COPD patients including CXCL8, TNF- α and IL-6 (Keatings et al., 1996). In an *in vitro* setting, cigarette smoke exposure to alveolar macrophages resulted in increased oxidative metabolism and impaired phagocytosis (Drath et al., 1979).

The two main sources of intracellular ROS are the mitochondria and NADPH oxidase (Lee and Yang, 2012, Rahman, 2005). Mitochondria play a crucial role in the pathological generation of ROS in disease (Pacher et al., 2007, Kirkham and Rahman, 2006). The recognition of the important role of mitochondria in oxidative-stress related diseases has resulted in the development of experimental techniques to analyse mitochondrial $O_2^{\cdot -}$ production (Kirkham and Rahman, 2006). Traditionally, hydroethidine (HE) which is converted into the fluorescent product 2-hydroxyethidium, upon oxidation by $O_2^{\cdot -}$, has been used to detect intracellular levels of ROS. More recently, a novel derivative of HE, MitoSOX Red was developed to specifically detect $O_2^{\cdot -}$ in the mitochondria of live cells. MitoSOX Red accumulates in the mitochondria as a function of mitochondrial membrane potential. Several confocal fluorescent microscopy studies in cell lines have validated the use of MitoSOX Red for selective detection of mitochondrial $O_2^{\cdot -}$ production. In human coronary artery endothelial cells (HCAECs), confocal microscopy showed oxidised MitoSOX Red co-localised with GFP targeted to the mitochondria. Furthermore, the specific detection of the $O_2^{\cdot -}$ by MitoSOX Red was demonstrated with pre-treatment with a cell-permeable superoxide dismutase that scavenges superoxide (SOD-PEG) or a nitric oxide donor that promptly reacts with superoxide (DETANONOate). Both

SOD-PEG and DETANONOate markedly reduced the intensity of MitoSOX (Mukhopadhyay et al., 2007).

There is growing evidence that suggests oxidative stress may contribute to corticosteroid insensitivity in patients with COPD (Barnes, 2010a). In peripheral blood mononuclear cells (PBMCs) of COPD patients, reversal of the effects of oxidative stress on the signalling molecule PI3K- δ resulted in restoration of corticosteroid sensitivity (To et al., 2010). The purpose of the next series of experiments in this Chapter was to determine whether oxidative stress in the *in vitro* setting could lead to corticosteroid insensitivity. We explored the effects of H₂O₂ on intracellular and mitochondrial ROS. Followed by, its effects on IL-1 β -induced-CXCL8 secretion and, NF- κ B transactivation. Finally, we assessed whether H₂O₂ in combination with IL-1 β leads to corticosteroid insensitivity.

4.2 Experimental protocols

4.2.1 Intracellular ROS

Starved cells were cultured in RPMI supplemented with CS-FCS (0.5%) and L-glutamine (1%) in 96 well plates overnight. Cells were washed with KRH buffer, followed by incubation with 100 μ M 2', 7'-dichlorofluorescein (DCFH-DA) for 30mins. Thereafter, cells were again washed with KRH buffer, before treatment with H₂O₂ (50-200 μ M) for 2h. The maximal concentration of H₂O₂ used was 200 μ M as this concentration had no effect on cell viability at 18h (Fig. 4.2.1 & Fig. 4.2.2). Fluorescence with an excitation filter set at 485nm (and emission filter set at 530nm) was read immediately. The acetate ester of DCFH-DA is a membrane permanent molecule that passes across the cell membrane. Once inside the cell, intracellular esterases convert DCFH-DA into the non-fluorescent ionic moiety H₂DCF-DA, causing it to be trapped inside the cell. Upon oxidation by ROS, H₂DCF-DA is converted into 2, 7-dichlorodihydrofluorescein (DCF), which is highly fluorescent.

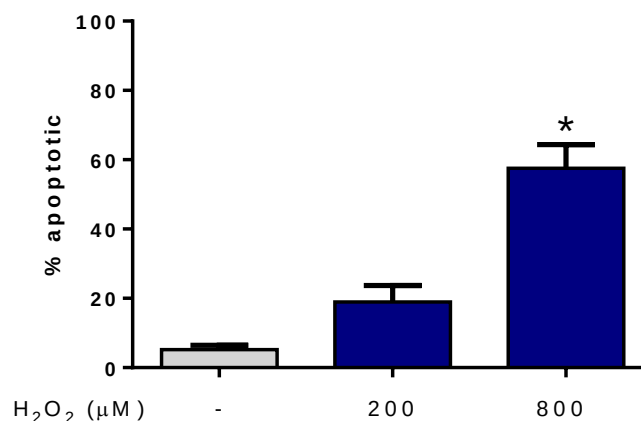


Fig. 4.2.1 Effect of hydrogen peroxide (H₂O₂) on cell viability. Serum-starved U937 monocytes were stimulated with H₂O₂ at 200 and 800 μ M for 18h, followed by 15min incubation with phycoerythrin (PE), a fluorogenic dye that detects early apoptosis. *p<0.05 vs. basal.

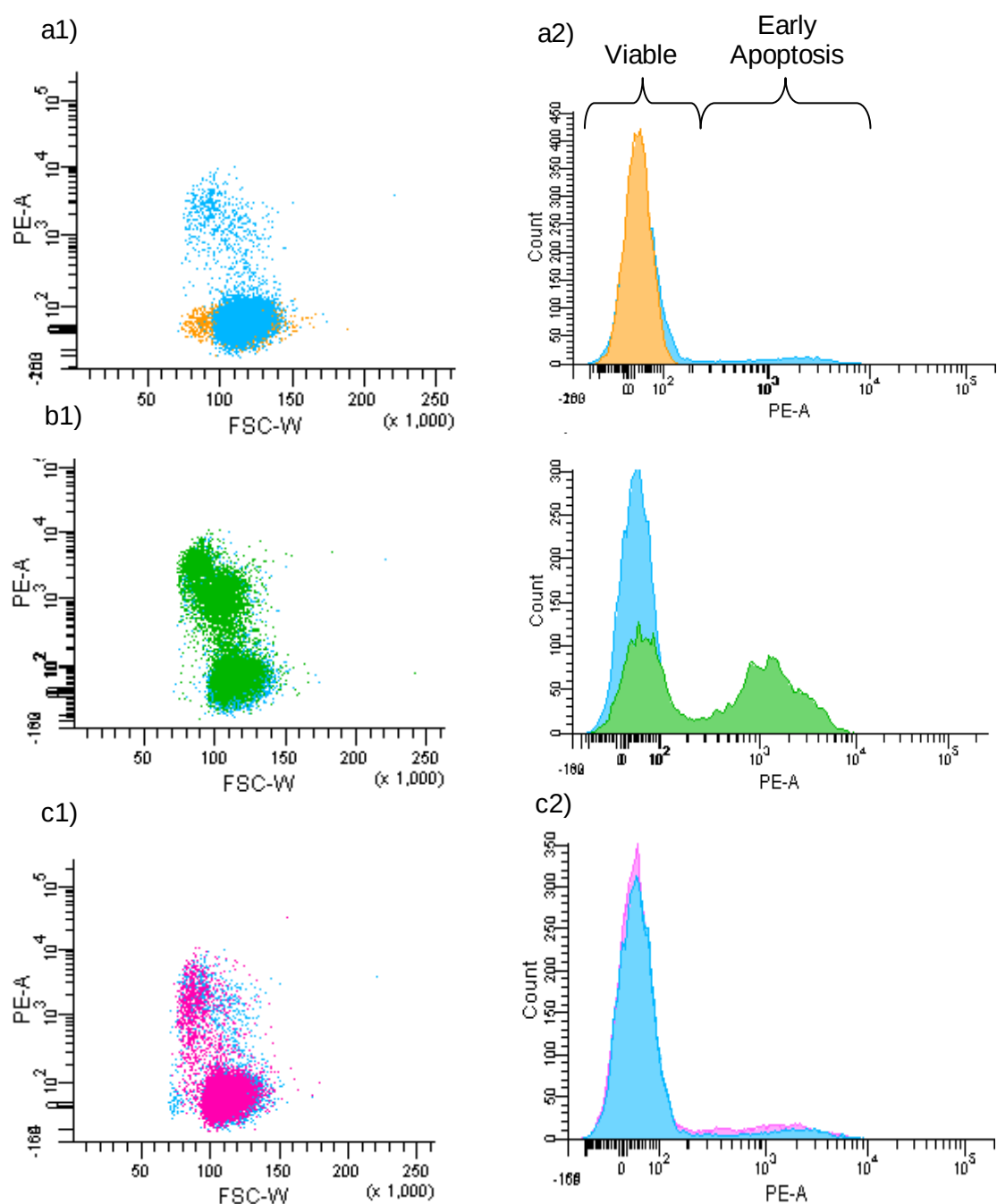


Fig 4.2.2 Effect of hydrogen peroxide (H_2O_2) on cell viability. Serum-starved U937 monocytes were stimulated with H_2O_2 at 200 and $800\mu M$ for 18h, followed by 15min incubation with phycoerythrin (PE), a specific flurogenic dye for detection of early apoptosis, in the dark. Levels of PE fluorescence were detected by flow cytometry at the PE channel. Dot-plots represent basal (a), $800\mu M$ H_2O_2 (b) and $200\mu M$ H_2O_2 (c). Dots-plots are accompanied with their corresponding histogram plots showing level of alive and dead cells. These are representative of three independent experiments.

4.2.2 Mitochondrial ROS

Starved cells were cultured in 6 well plates at a seeding density of 0.5×10^6 cell/ml. Cells were treated with H_2O_2 (50-200 μ M) for a period of 2h, followed by MitoSOX Red (5 μ M) incubation for 30min at 37°C in the dark. Mitochondrial superoxide levels were measured using a novel highly selective fluorogenic dye, MitoSOX Red superoxide detector. MitoSOX Red is oxidised by superoxide which leads to fluorescence of the dye. Levels of MitoSOX Red fluorescence were detected by flow cytometry, using the 488nm argon laser and emission through the PE filter (575nm).

4.2.3 Involvement of oxidative stress in cytokine synthesis and secretion

Starved cells were cultured in 96-well plates at a seeding density of 0.5×10^6 cell/ml. Cells were treated with H_2O_2 (200 μ M) for 90min, followed by IL-1 β (1ng/ml) for 30min. Cells were then stimulated with varying concentrations of BUD, FP and FF. 16h post IL-1 β stimulation, supernatant was collected and stored at -20°C for future cytokine/chemokine analyses.

4.2.4 NF- κ B DNA-binding

Starved cells were cultured in flasks at a seeding density of 0.5×10^6 cell/ml. Cells were treated with H_2O_2 (200 μ M) for 90min, followed by IL-1 β (1ng/ml) for 30min. Post IL-1 β stimulation, nuclear extraction was conducted immediately and p65 (NF- κ B) activation was measured.

4.2.5 Oxidative stress on corticosteroid activity and function

Starved cells were either cultured in flasks or 96-well plates at a seeding density of 0.5×10^6 cell/ml for intracellular GR detection and pro-inflammatory mediator secretion respectively. Cells were treated with H_2O_2 (200 μ M) for 90mins followed by IL-1 β (1ng/ml) for 30min. Cells were then challenged with BUD, FP and FF 2 and 16h post IL-1 β stimulation cells and supernatant were collected for immediate detection of intracellular GR and pro-inflammatory mediator secretion. In the absence of corticosteroid, H_2O_2 (200 μ M) and IL-1 β (1ng/mL) alone had no effect on GR subcellular localisation (Fig. 4.2.5).

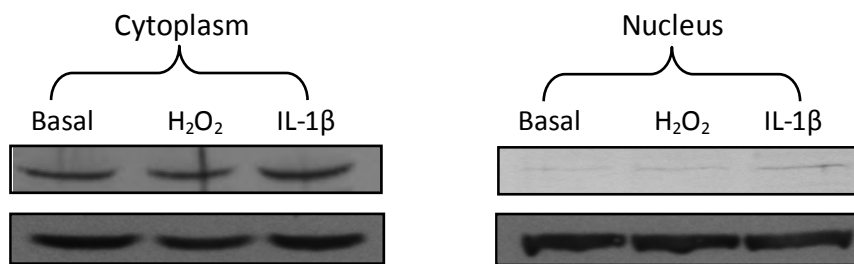


Fig 4.2.5 Effect of hydrogen peroxide (H_2O_2) and IL-1 β on glucocorticoid receptor (GR) subcellular localisation in resting cells. Serum-starved U937 monocytes were stimulated with either H_2O_2 (200 μ M) or IL-1 β (1ng/ml) to determine effects on GR subcellular localisation.

4.3 Results

4.3.1 H₂O₂ causes oxidative stress

4.3.1.1 H₂O₂ induces Intracellular ROS

In order to establish an *in vitro* model that characterises the oxidative stress and heightened inflammation observed in the *in vivo* setting of COPD patients, we first explored the effects of H₂O₂, an oxidant that is elevated in COPD patients, on intracellular ROS, a marker of oxidative stress. H₂O₂ (50-200μM) induced intracellular ROS in a concentration-dependent manner at 2h. Specifically, H₂O₂ at 100 and 200μM increased intracellular ROS by 2.4±0.34- and 2.3±0.1-fold respectively relative to basal levels (*p<0.05) (Fig. 4.3.1.1).

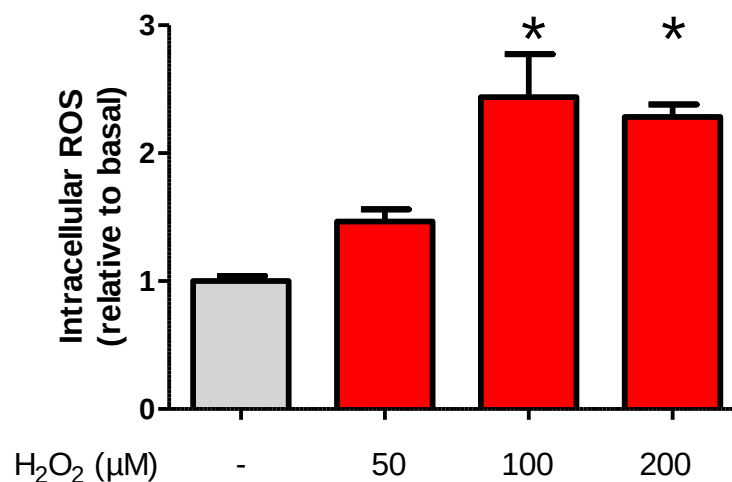


Fig 4.3.1.1 Effect of hydrogen peroxide (H₂O₂) on intracellular ROS. Serum-starved U937 monocytes were treated with 100μM of 2', 7'-dichlorofluorescein, followed by stimulation with H₂O₂ (50-200μM) for 2h. Fluorescence with an excitation filter set at 485nm was read immediately. Results represent the mean±SEM of 3 independent experiments. *p<0.05 vs. basal.

4.3.1.2 H₂O₂ induces mitochondrial oxidative stress

Next we investigated the effects of H₂O₂ on mitochondrial superoxide levels using a flurogenic dye, MoSOX Red. An increase in mean fluorescence intensity, a shift in the histogram towards the right represents an increase in mitochondrial superoxide levels. H₂O₂ at 100μM (blue histogram) caused a slight increase in mean fluorescence intensity; however this was not significant, relative to basal levels (red histogram) (Fig. 4.3.1.2). However, H₂O₂ at 200μM (red histogram) caused a 2.5-fold shift towards the right in mean fluorescence intensity in comparison to basal levels (p<0.05). Based upon the effects of a range of concentrations of H₂O₂ on intracellular and mitochondrial ROS, we decided to use the concentration of 200μM for all future experiments.

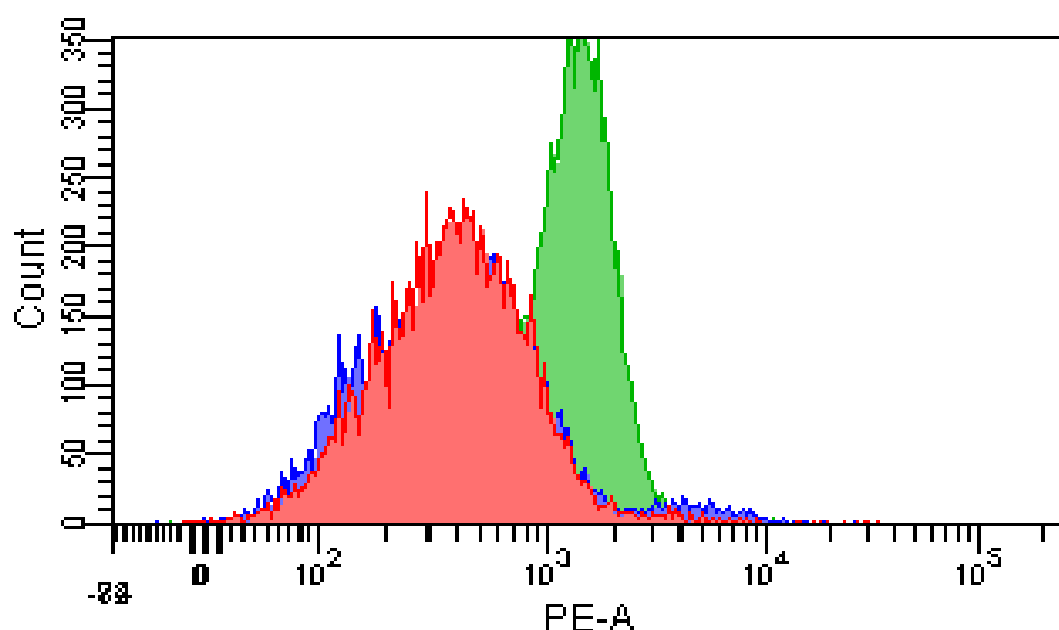


Fig 4.3.1.2 Effect of H₂O₂ on mitochondrial superoxide levels. Serum-starved U937 monocytes were stimulated with H₂O₂ for 2h. After 2h, cells were incubated with MitoSOX Red, a specific fluoregenic dye for detection of superoxide levels in live mitochondrial cells, for 30min. Levels of MitoSOX Red fluorescence were detected by flow cytometry at the PE channel. Red, blue and green histograms represent basal, 100 and 200μM H₂O₂ induced superoxide levels. Results represent the mean±SEM of 3 independent experiments.

4.3.2 Nrf2 activation

In the presence of cellular stress, Nrf2, a transcription factor, upregulates the transcription of antioxidant genes. We next explored the effects of H₂O₂ on Nrf2 transactivation, measured as binding of Nrf2 to anti-oxidant response elements (AREs). H₂O₂ (200μM induced a significant increase in Nrf2 transactivation compared with basal levels at both 1h (1.9±0.40-fold increase, *p<0.05 vs. basal) and 2h (1.6±0.12-fold increase, *p<0.05 vs. basal).

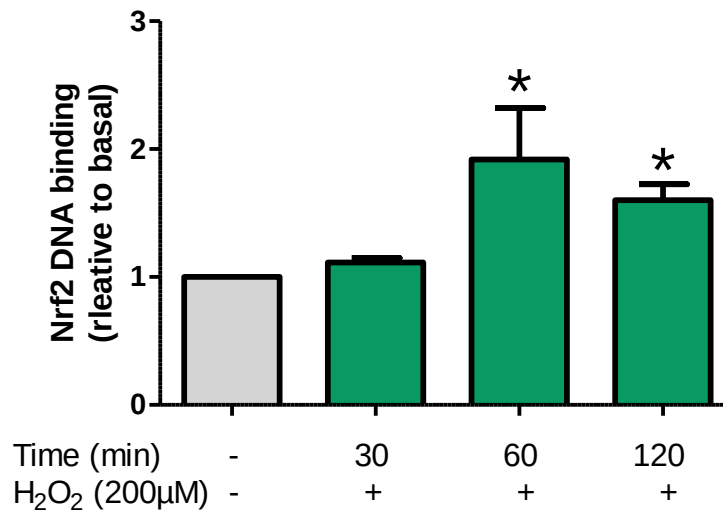


Fig 4.3.2 Effect of H₂O₂ on Nrf2 transactivation. Serum-starved U937 monocytes were treated with H₂O₂ (200μM) for 2 h. After the various time points indicated nuclear extraction was performed. Results represent the mean±SEM of 4 independent experiments. *p<0.05 vs. basal.

4.3.3 H₂O₂ enhances markers of inflammation

4.3.3.1 H₂O₂ enhances-IL-1β induced CXCL8

So far, we have established an *in vitro* model of oxidative stress, where H₂O₂ induces intracellular and mitochondrial oxidative stress. Next we explored whether H₂O₂ would enhance the inflammatory response in U937 monocyte cells. We used IL-1β to stimulate CXCL8 secretion. As seen previously (Fig. 3.2.2), IL-1β (1-100ng/ml) significantly increased CXCL8 levels in a concentration-dependent manner with a submaximal concentration of 1ng/ml. The combination of H₂O₂ (25-200μM) with IL-1β (1ng/ml) enhanced IL-1β-induced CXCL8 levels in a concentration-dependent manner, with the highest concentration of H₂O₂ (200μM) increasing CXCL8 secretion 10 fold, (2900±110 vs. 290±28 pg/ml, #p<0.05) (Fig. 4.3.3.1). H₂O₂ (25-200μM) alone had no effect on IL-1β-induced CXCL8 levels.

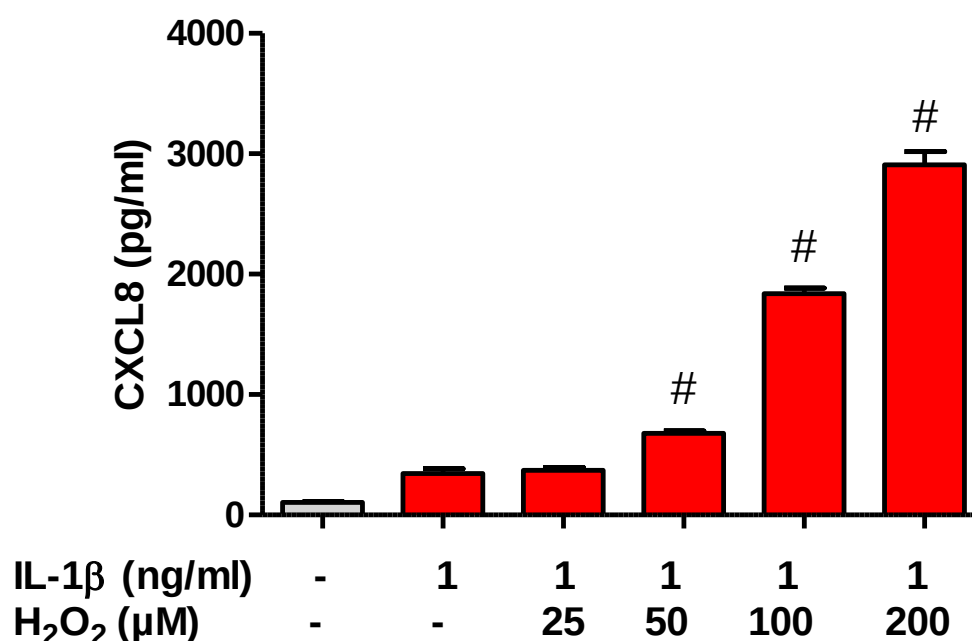


Fig. 4.3.3.1 Effect of H₂O₂ on IL-1β-induced CXCL8. Serum-starved U937 monocytes were firstly stimulated with varying concentrations of H₂O₂ for 90 min followed by IL-1β (1ng/ml) for a further 16h. Thereafter supernatant was collected and CXCL8 levels were measured. Results represent the mean±SEM of 3 independent experiments. #p<0.05 vs. IL-1β alone.

4.3.3.2 H₂O₂ enhances-IL-1β induced NF-κB activation

IL-1β-induced secretion of extracellular CXCL8 is mediated by NF-κB (Patel et al., 2002). We therefore examined whether H₂O₂ could enhance IL-1β-induced p65 transactivation. As expected IL-1β (1ng/ml) significantly increased p65 transactivation by 1.9±0.12-fold (*p<0.05). This was significantly enhanced when cells were pre-treated with H₂O₂ (200μM) (2.6±0.10 vs. 1.9±0.12, #p<0.05). In contrast, H₂O₂ alone (200μM) alone had no effect on p65 transactivation.

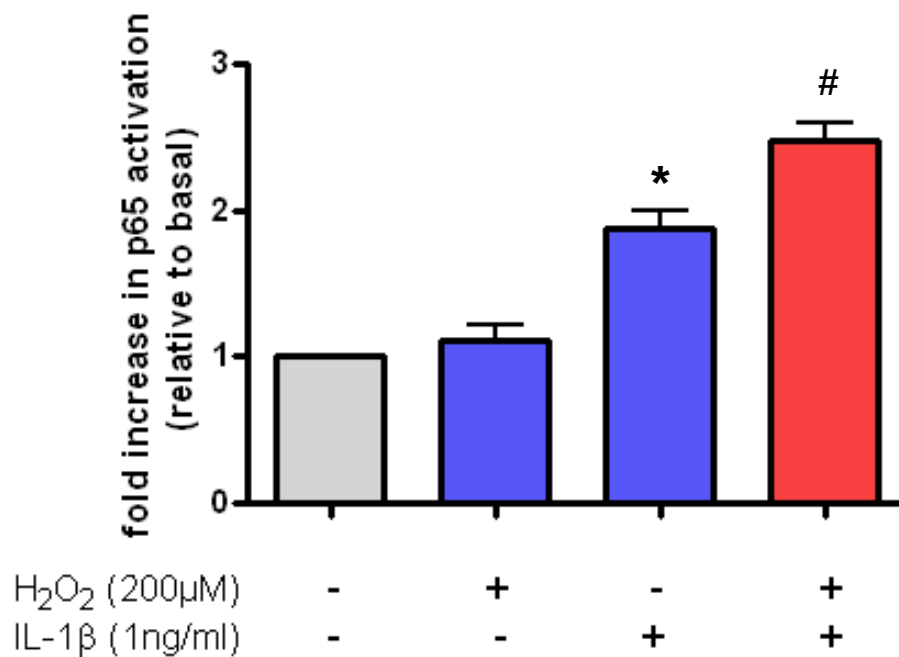


Fig 4.3.3.2 Effect of H₂O₂ on IL-1β-induced NF-κB transactivation. Serum-starved U937 monocytes were firstly stimulated with H₂O₂ (200μM) for 90min followed by IL-1β (1ng/ml) for a further 30min. Thereafter nuclear extractions were performed and NF-κB binding to DNA was assessed. Results represent the mean±SEM of 3 independent experiments. *p<0.05 vs. basal. #p<0.05 vs. IL-1β alone.

4.3.4 Oxidative stress impairs BUD- and FP-driven suppression of IL-1β-induced CXCL8

So far, we have shown that oxidative stress leads to a heightened inflammatory response in U937 monocytes. Next we wanted to determine whether this would lead to corticosteroid insensitivity, as observed in COPD. We therefore explored the effects of our *in vitro* oxidative stress model on corticosteroid-driven suppression of IL-β-induced CXCL8. As shown in Fig. 3.3.2.1, in the absence of H₂O₂, BUD, FP and FF all suppressed IL-β-induced CXCL8 secretion, with FF and FP being more potent than BUD. However, in the presence of H₂O₂ (200μM), BUD- and FP-mediated suppression of IL-1β-induced CXCL8 was significantly impaired particularly at higher

concentrations (Fig. 4.3.5.2). Pre-treatment with H₂O₂ significantly impaired FP-mediated suppression of IL-1 β -induced CXCL8 by 11 $\pm$ 1.5% (10⁻⁹M) and 23 $\pm$ 2.3% (10⁻⁷M), respectively. The effect of H₂O₂ on suppression of CXCL8 release by BUD was similar with a 16 $\pm$ 8.7% and 24 $\pm$ 7.7% attenuation of BUD effects at 10⁻⁹ and 10⁻⁷M respectively. Although there was an effect on the maximal inhibition seen, there were no differences in IC₅₀ values for either BUD or FP in the presence and absence of H₂O₂ (Fig. 4.3.4.1). Suppression of IL-1 β -induced CXCL8 at lower concentrations of both BUD and FP (10⁻¹²-10⁻¹⁰M), were not affected by pre-treatment with H₂O₂.

In contrast, H₂O₂ had no effect on FF-mediated suppression of IL-1 β -induced CXCL8 at any concentration tested. These set of results clearly demonstrate our *in vitro* model of oxidative stress associated with heightened inflammatory response causes relative corticosteroid insensitivity, at least for two out of three corticosteroids tested.

	Corticosteroid-mediated suppression of IL-1 β -induced CXCL8 (IC ₅₀)	
	Without H ₂ O ₂	With H ₂ O ₂
BUD	3.3 $\pm$ 4.7 x10 ⁻¹⁰	5.4 $\pm$ 3.0 x10 ⁻¹⁰
FP	7.2 $\pm$ 1.9 x10 ⁻¹¹	1.2 $\pm$ 3.0 x10 ⁻¹⁰
FF	1.8 $\pm$ 4.2 x10 ⁻¹¹	2.3 $\pm$ 3.1 x10 ⁻¹¹

Fig. 4.3.4.1 Effect of H₂O₂ (200 μ M) on IC₅₀ values for corticosteroid-driven suppression of IL-1 β -induced CXCL8.

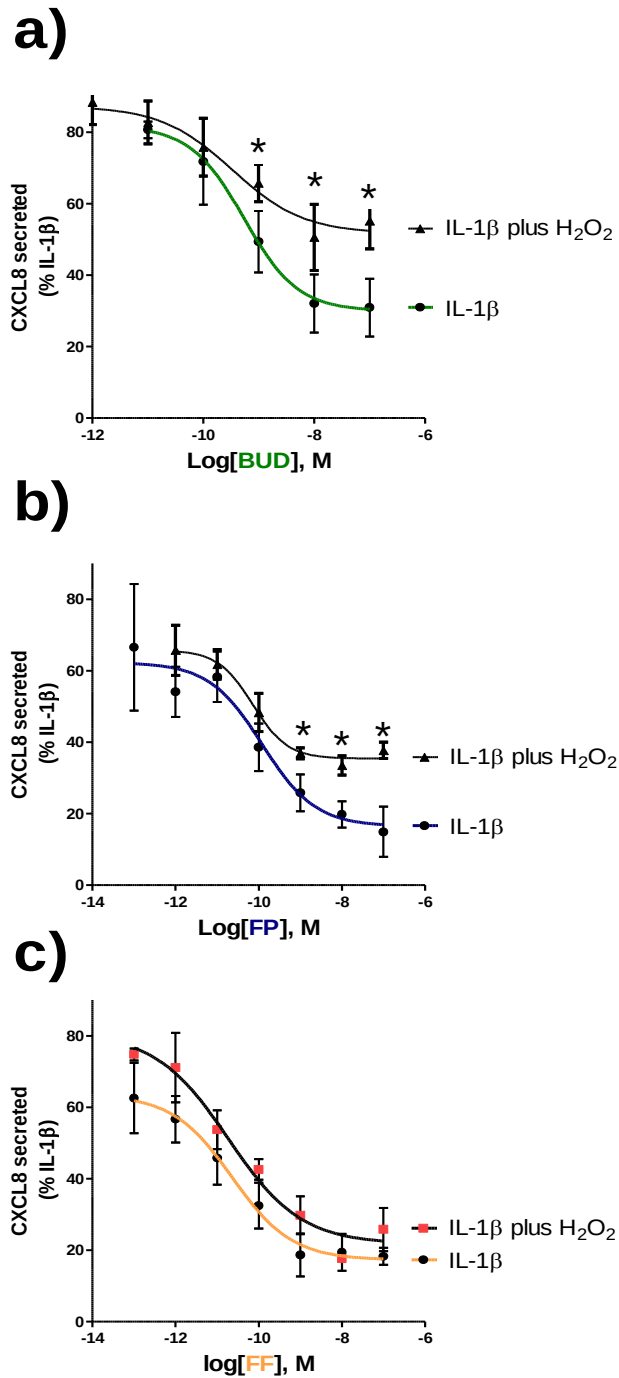


Fig. 4.3.4.2 Effect of H₂O₂ on corticosteroid suppression of IL-1β-induced CXCL8. Serum-starved U937 monocytes were stimulated with H₂O₂ (200μM) for 90min followed by IL-1β (1ng/ml) for a further 30min. Cells were then incubated with varying concentrations of (a) BUD, (b) FP and (c) FF. Supernatant was collected 16h post IL-1β stimulation. Results represent the mean±SEM of a minimum of 3 independent experiments. *p<0.05 vs. without H₂O₂.

4.3.5 Oxidative stress impairs BUD and FP induced GR nuclear localisation

In our *in vitro* model, oxidative stress was associated with a heightened inflammatory response and resulted in corticosteroid insensitivity, with reduced suppression of IL-1 β -induced CXCL8 by both BUD and FP. We next assessed whether this corticosteroid insensitivity could be due to impaired GR nuclear localisation. Cells were incubated with BUD, FP or FF (all at 10⁻⁹M) for 2h following H₂O₂ and IL-1 β pre-treatment. Both BUD- and FP-induced GR nuclear localisation was significantly reduced in the presence of H₂O₂ and IL-1 β (Fig. 4.3.5.2). In the absence of H₂O₂ and IL-1 β , BUD- and FP-induced GR nuclear localisation by 3.0 $\pm$ 0.43- and 5.7 $\pm$ 0.85-fold respectively (both *p<0.05). In the presence of H₂O₂ and IL-1 β , BUD- and FP-induced GR nuclear localisation was attenuated with a reduced 1.7 $\pm$ 0.15-fold and 3.4 $\pm$ 0.51-fold increase seen respectively (both #p<0.05 compared to no H₂O₂ and IL-1 β). Pre-treatment with H₂O₂ alone or IL-1 β alone had no effect on FP- and FF-induced GR nuclear import (Fig. 4.3.5.1).

In contrast, FF-induced GR nuclear localisation was not affected by pre-treatment with H₂O₂ and IL-1 β (6.6 $\pm$ 1.5 fold increase vs. 7.3 $\pm$ 1.5 fold increase, p = ns). This corresponded with the observed failure of H₂O₂ to modify FF suppression of IL-1 β -induced CXCL8 release.

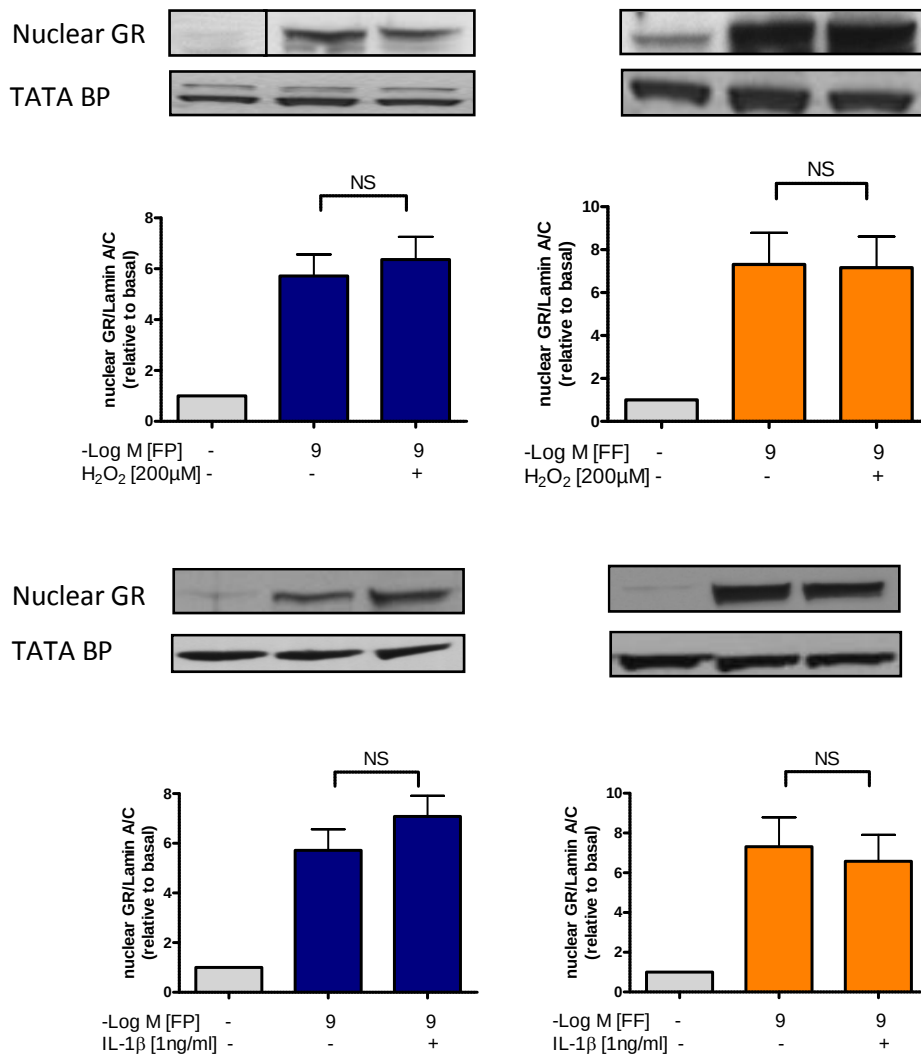


Fig. 4.3.5.1 Effect of H₂O₂ or IL-1β alone on corticosteroid-induced GR nuclear localisation. Serum-starved U937 monocytes were stimulated with either H₂O₂ (200μM) for 2 h or IL-1β (1ng/ml) for 30min. Cells were then incubated with FP or FF (all at 10⁻⁹M) for 2h. Nuclear extraction and determination of GR levels by Western blotting was performed immediately. Results represent the mean±SEM of 3 independent experiments. p=ns.

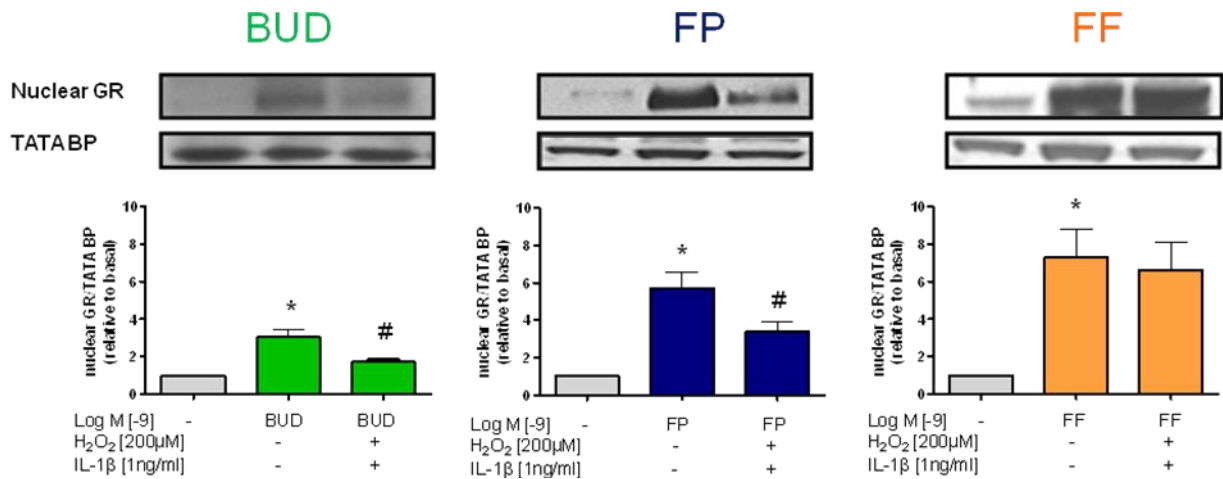


Fig. 4.3.5.2 Effect of H_2O_2 in combination with $IL-1\beta$ on corticosteroid-induced GR nuclear localisation. Serum-starved U937 monocytes were stimulated with H_2O_2 (200 μ M) for 90min followed by $IL-1\beta$ (1ng/ml) for a further 30min. Cells were then incubated with BUD, FP and FF (all at 10^{-9} M) for 2h. Nuclear extraction and determination of GR levels by Western blotting was performed immediately. Results represent the mean $\pm$ SEM of 3 independent experiments. * p <0.05 vs. basal. # p >0.05 vs. corticosteroid without H_2O_2 and $IL-1\beta$.

4.3.6 Oxidative stress impairs FP-induced GR-GRE binding

GR nuclear translocation is essential for activated GR to bind to promoter regions of corticosteroid-inducible genes. We explored whether a reduction in FP-induced GR nuclear translocation would result in reduced GR-GRE binding. Corticosteroid FP (10^{-9} M) enhanced GR-GRE binding by 1.6 ± 0.05 -fold (* p <0.05). The combination of H_2O_2 and $IL-1\beta$, in the absence of FP, had no effect on GR-GRE binding, relative to basal levels. But, in the presence of H_2O_2 and $IL-1\beta$, FP-induced GR-GRE binding was significantly attenuated resulting in a 1.3 ± 0.06 -fold increase only (# p <0.05 vs. FP-induced GR-GRE binding without H_2O_2 and $IL-1\beta$).

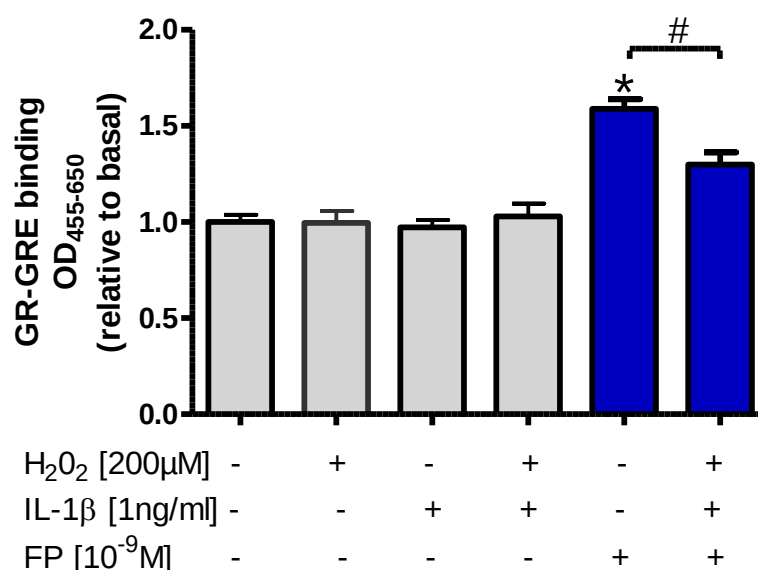


Fig. 4.3.6 Effect of H₂O₂ and IL-1β on FP-induced GR-GRE binding. Serum-starved U937 monocytes were stimulated with H₂O₂ (200μM) for 90min followed by IL-1β (1ng/ml) for a further 30min. Then cells were incubated with FP (10⁻⁹M) for 2h. Nuclear extraction was performed immediately. Results represent the mean±SEM of 3 independent experiments. *p<0.05 vs. basal. #p>0.05 vs. FP (10⁻⁹M) alone.

4.3.7 Oxidative stress on BUD- and FP-induced *DUSP1* expression

Finally, we determined the effect of H₂O₂ on BUD- and FP-induced *DUSP1* mRNA expression. In the absence of H₂O₂ and IL-1β, BUD and FP significantly induced *DUSP1* mRNA expression (Fig. 3.3.3). Interestingly, we found that H₂O₂ in combination with IL-1β also induced *DUSP1* mRNA expression by 7.2±1.8-fold in the absence of corticosteroid (Fig 4.3.7). This was similar to levels seen with BUD, FP and FF alone. However, in the presence of H₂O₂ and IL-1β, BUD and FP did not cause a further increase in *DUSP1* mRNA expression compared to H₂O₂ and IL-1β (Fig. 4.3.7).

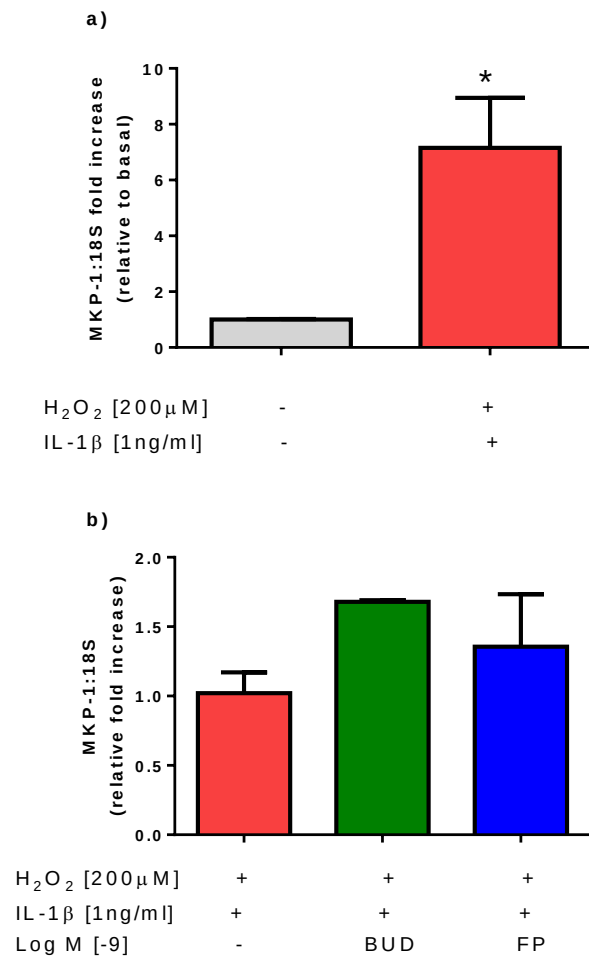


Fig. 4.3.7 Effect of H₂O₂ and IL-1β on corticosteroid-induced *DUSP1* mRNA expression. Serum-starved U937 monocytes were stimulated with H₂O₂ (200µM) for 90min followed by IL-1β (1ng/ml) for a further 30min. Then cells were incubated with BUD or FP (both at 10⁻⁹M) for 3h PCR. Results represent the mean±SEM of 3 independent experiments. *p<0.05 vs. basal.

4.4 Discussion

In this Chapter we explored the effects of oxidative stress on the inflammatory response, on corticosteroid-mediated suppression of CXCL8 and, on corticosteroid-induced GR nuclear localisation. We found H_2O_2 in a concentration-dependent manner induced intracellular and mitochondrial ROS. This demonstrated the importance of H_2O_2 , which is elevated in COPD patients (Dekhuijzen et al., 1996), in further propagating ROS generation by targeting key intracellular sources of ROS. In addition, pre-treatment with H_2O_2 also significantly enhanced IL-1 β -induced CXCL8 extracellular secretion and NF- κ B transactivation, pro-inflammatory markers that are elevated in COPD (Keatings et al., 1996, Di Stefano et al., 2002). This is consistent with findings that suggest oxidative stress drives the inflammatory response in COPD (Dekhuijzen et al., 1999, Rahman et al., 2005, Lee et al., 2012). Therefore, further investigations of the signalling pathways in our *in vitro* model of oxidative stress may reveal novel therapeutic targets for COPD.

This increase in oxidative stress was associated with an increase in Nrf2 transactivation, an important transcription factor that upregulates the expression of antioxidant genes under cellular stress. Gene expression of several Nrf2-regulated antioxidant genes was markedly reduced in COPD lungs compared to non-COPD lungs (Malhotra et al., 2008). This depletion in Nrf2-regulated antioxidant genes correlated to disease severity. Measurement of the temporal response to H_2O_2 on Nrf2 activation in our model may identify whether Nrf2 levels fall in our model. The importance of Nrf2 in corticosteroid insensitivity was recently demonstrated in alveolar macrophages from patients with COPD (Malhotra et al., 2011).

Sulphoraphane, an activator of Nrf2, was able to reverse nitrosylation of histone deacetylase (HDAC)-2, which resulted in restoration of dexamethasone sensitivity in alveolar macrophages. Acetylation of Nrf2 also reduces its ability to switch on anti-oxidant gene expression (Mercado et al., 2012). The effect of H₂O₂ on Nrf2 acetylation status over time may also prove useful.

Under oxidative stress conditions, both BUD (10⁻⁹M)- and FP (10⁻⁹M)-induced GR nuclear localisation and suppression of IL-1 β -induced CXCL8 were severely reduced. A full concentration-response curve of BUD- and FP-induced GR nuclear localisation would identify the extent of corticosteroid insensitivity. This reduction in corticosteroid sensitivity in our *in vitro* model is consistent with findings reported in sputum macrophages from COPD patients whereby BUD was unable to suppress pro-inflammatory mediators (Keatings et al, 1997). This loss in GR nuclear translocation observed in our model mimics the reduced GR ligand-binding affinity in the nucleus observed in peripheral blood mononuclear cells (PBMCs) of severe asthmatics (Irusen et al., 2002). These findings support the use of our model as an *in vitro* representation of COPD.

Irusen and colleagues also demonstrated that this loss in nuclear GR ligand-binding affinity was blocked by SB203580, a p38 mitogen-activated kinase (p38 MAPK) inhibitor. Under separate conditions, activation of p38 MAPK was demonstrated to phosphorylate GR, which was associated with reduced dexamethasone suppression of granulocyte-macrophage colony-stimulating factor (GM-CSF) release (Irusen et al., 2002). Recent data from Kent and colleagues has shown that p38 MAPK

inhibitors can restore corticosteroid responsiveness in BAL macrophages from patients with COPD (Kent et al., 2009). It has also been demonstrated that oxidative stress directly inhibits GR nuclear translocation (Okamoto et al., 1999). In this study in human GR-expressing CHO_{MTGR} cells, H₂O₂ inhibited GR nuclear translocation, in addition to partially inhibiting ligand-dependent dissociation of Hsp90 from GR. GR nuclear translocation was reversed with *N*-acetyl-L-cysteine.

This reduction in GR nuclear translocation may also be due to impaired nuclear receptors that govern GR nucleocytoplasmic shuttling (Kodiha et al., 2004). In HeLa cells, several components of the classical nuclear import pathway, importin- β , were affected by extremely high concentrations of H₂O₂. Importin- β belongs to the same family of receptors as importin-7 which has been reported to bind and facilitate nuclear import of the GR (Freedman and Yamamoto, 2004). Treatment with H₂O₂ also resulted in the redistribution and degradation of nuclear RanGTP, essential for nuclear import of cargo proteins, and relocalisation of importin- β (Kodiha et al., 2004). Additionally, in growing yeast *Saccharomyces cerevisiae* cells, heat, shock and H₂O₂ significantly impaired classical nuclear import of NLS fused to green fluorescent protein (NLS-GFP). This was associated with a redistribution of the small GTPase, Gsp1p, from the nucleus into the cytoplasm (Stochaj et al., 2000).

In contrast to BUD and FP, FF maintained its level of GR nuclear translocation and suppression of IL-1 β -induced CXCL8 under oxidative stress. This resistance to oxidative stress may be partly explained by its enhanced affinity for GR (Valotis and Hogger, 2007, Valotis et al., 2004). We also found FP-induced GR-GRE binding was

significantly impaired under oxidative stress conditions. Corticosteroid-inducible genes include *DUSP1* and *SLP1*, which are essential for counteracting the pro-inflammatory signalling pathways that are heightened in COPD (Shipp et al., 2010). *DUSP1* codes for mitogen-activated protein kinase phosphatase (MKP)-1, which inhibits p38 and JNK MAPK activity and can indirectly modulate NF- κ B function by altering p65 phosphorylation status (Perkins, 2007). We found, H₂O₂ in combination with IL-1 β induced *DUSP1* mRNA expression levels similar to what was seen with corticosteroids alone (Fig. 3.3.3). This is consistent with data reported in HeLa cells, where IL-1 β induced *DUSP1* mRNA expression in a p38 MAPK-dependent manner (Lasa et al., 2002). In order to verify whether H₂O₂ and IL-1 β impair corticosteroid-induced GR-GRE binding on corticosteroid-inducible genes we would need to undertake these experiments on genes that are not upregulated by H₂O₂ and IL-1 β .

Taken together, these findings support that our *in vitro* model of oxidative stress-induced corticosteroid insensitivity represents some aspects of the disease pathology in COPD. As COPD is characterised by a multitude of inflammatory cells and mediators, it would be crucial to determine the effects of H₂O₂ alone and in combination with a cocktail of inflammatory stimuli on inflammatory marker secretion to further validate our model. Stimulation of our cells with more than one inflammatory stimulus may better represent the multiple signalling pathways that contribute to inflammation in COPD (Lee et al., 2009, Wang et al., 2008). Furthermore, H₂O₂ is amongst a number of oxidants that are found elevated in COPD (Kirkham and Rahman, 2006, Lee and Yang, 2012). The role of other ROS on corticosteroid insensitivity needs to be assessed and modelled in combination with

H₂O₂. It would be important to compare the inflammatory signalling pathways that are activated in our model to cigarette smoke-induced inflammation to determine how well H₂O₂ reflects the numerous molecular signalling pathways activated by cigarette smoke, the major cause of environmental-derived ROS in COPD. In addition, it would be important to confirm our findings in primary cells from patients with COPD. In the next Chapter we will explore the role of nuclear transport receptors in corticosteroid-induced GR nuclear localisation. Hopefully this will allow us to identify potential nuclear transport receptors that govern GR nucleocytoplasmic shuttling that may be implicated in reduced GR nuclear translocation under conditions of oxidative stress.

CHAPTER 5

IMPORTIN-7-MEDIATED GR NUCLEAR IMPORT

5.1 Introduction

In Chapter 4, we demonstrated that H₂O₂ induces oxidative stress and enhances IL-1 β -induced NF- κ B (p65 transactivation) and CXCL8 in monocytes, pathological changes that are characteristic of COPD. Additionally, in our *in vitro* model of oxidative stress, fluticasone propionate (FP)- and budesonide (BUD)-mediated suppression of IL-1 β -induced CXCL8 were significantly reduced. Further experiments showed that GR nuclear translocation, essential for corticosteroid function, was dramatically reduced. The purpose of this Chapter is to investigate whether reduced corticosteroid-induced GR nuclear translocation is due to impaired mechanisms that may regulate GR nucleocytoplasmic shuttling in our *in vitro* model of oxidative stress associated with a heightened inflammatory response.

Nucleocytoplasmic shuttling of proteins across the nuclear pore complex (NPC) is governed by several sequential steps. They are (i) retrograde movement through the cytoplasm, (ii) recognition by nuclear import proteins, (iii) docking to the nuclear pore, (iv) translocation across the nuclear compartment, (v) binding to and release from nuclear sites of action, (vi) recognition by nuclear export proteins and finally, (vii) anteretrograde cytoplasmic movement (Galigniana et al., 2010). The signalling pathways that regulate GR nucleocytoplasmic shuttling remain largely unknown.

Several *in vitro* studies have suggested importin proteins- α , - β , -7, -8 and -13 may govern GR nucleocytoplasmic shuttling.

Of these, importin-7 has been shown to bind and mediate GR nuclear translocation in an *in vitro* import nuclear assay (Freedman and Yamamoto, 2004). However, the role of importin-7 in corticosteroid-induced GR nucleocytoplasmic shuttling in inflammatory cells and under oxidative stress associated with heightened inflammatory response has not been studied. Importin-7 belongs to the karyopherin family of nuclear receptors that mediate RanGTPase-dependent transport across the NPC. RanGTPases regulate interactions between the nuclear transport receptors of the importin family and their cargo proteins. Interestingly, Importin- β , a member of the same family of nuclear receptors as importin-7, has been shown to be degraded under exceptionally high concentrations of H_2O_2 (Kodiha et al., 2004). Moreover, H_2O_2 at 500 μ M, also caused degradation of nuclear RanGTP, which is essential for the dissociation of nuclear importin receptors from their cargo proteins. We therefore investigated the role of importin-7 in corticosteroid-induced GR nuclear translocation and under conditions of oxidative stress (Fig. 5.1).

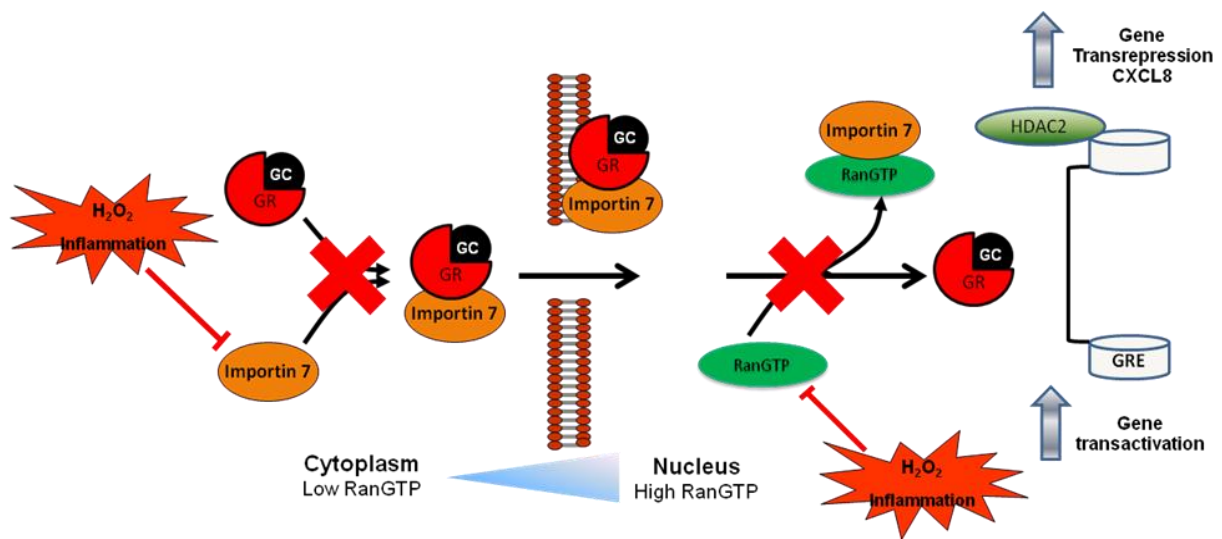


Fig. 5.1 Proposed mechanism of reduced corticosteroid-induced GR nuclear translocation. Corticosteroid-induced GR nuclear translocation is essential for corticosteroid-mediated suppression of pro-inflammatory mediators. Importin-7 binds to and mediates nuclear translocation of the glucocorticoid receptor (GR). Once in the nucleus, nuclear RanGTP binds to importin-7 resulting in the dissociation of the importin-7 and GR complex. Consequently, the GR is able to move through the nucleus and either binds to glucocorticoid response elements on corticosteroid-inducible genes or recruits histone deacetylase (HDAC)-2 to prevent the gene transcription of pro-inflammatory mediators. Activated GR may also prevent gene transcription by binding as a monomeric unit to other transcription factors. Under conditions of oxidative stress, importin-7 or nuclear RanGTP levels may be reduced leading to reduced GR nuclear translocation and consequently corticosteroid insensitivity.

5.2 Experimental protocol

5.2.1 Importin-7 knockdown

Please see section 2.10 for full details. Briefly, U937 cells in full media were transfected with either 200nM of human importin-7 ON-TARGET^{plus} SMARTpool siRNA or 200nM of ON-TARGET^{plus} Non-Targeting pool (negative control) for 36h in either 6- or 96-well plates. Fig. 5.2.1 clearly shows that transfection with importin-7 siRNA had no significant effect on cell viability compared to untransfected cells as determined by the MTT assay.

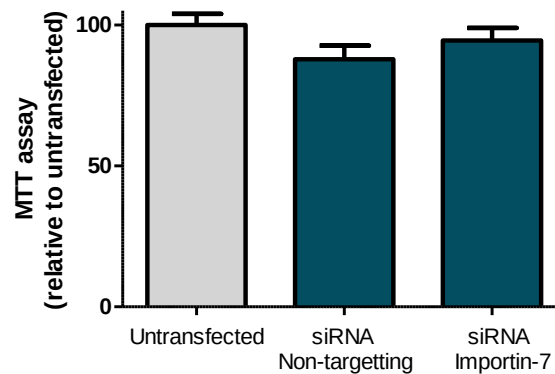


Fig. 5.2.1 Effect of electroporation with siRNA on cellular activity. U937 cells were transfected via electroporation for 36h with siRNA, followed by incubation with MTT to determine the effects of electroporation and siRNA on cellular activity. Results represent the mean $\pm$ SEM of 3 independent experiments.

5.2.2 The role of importin-7 on corticosteroid-induced GR nuclear translocation

Following 36h transfection with importin-7 siRNA, U937 cells were serum starved for 3h and then incubated with FP for 2h. Protein extracts were obtained and GR was detected by Western blotting.

5.2.3 The role of importin-7 on corticosteroid-mediated suppression of CXCL8

Following 36h transfection with importin-7 siRNA, U937 cells were serum starved for 3h and then incubated with IL- β (1ng/ml) for 30min. Cells were then stimulated with FP (10^{-9} M) and the supernatant was collected 16h after IL-1 β stimulation.

5.2.4 Importin-7 & RanGTP subcellular localisation under oxidative stress

Serum starved cells were cultured in large T175 flasks. Cells were treated with H₂O₂ (200 μ M) for 90min followed by IL- β (1ng/ml) stimulation for 30 min. Post IL-1 β stimulation, nuclear extraction was performed and protein levels of importin-7 were determined by Western blotting. RanGTP was detected by immunoprecipitation with agarose beads bound to RanBP1. In Fig. 5.2.4 we clearly demonstrated that importin-7 predominantly resides in the cytoplasm in resting U937 cells, therefore the effects of H₂O₂ and IL-1 β were determined on cytoplasmic importin-7.

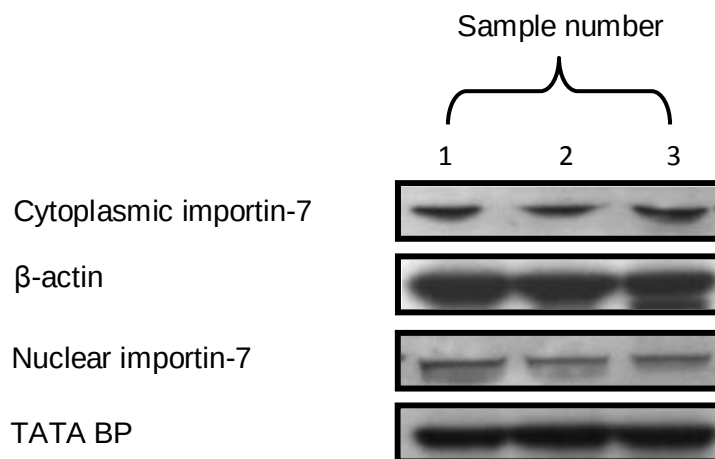


Fig. 5.2.4 Intracellular localisation of Importin-7. Cytoplasmic and nuclear extraction was carried out on serum-starved U937 monocytes. Western blot indicates importin-7 subcellular localisation from three different samples.

5.2.5 Corticosteroid-induced GR nuclear localisation under nuclear export inhibition

Serum starved cells were cultured in large flasks with leptomycin B (10nM) for 30min followed by corticosteroids for varying time points. Several *in vitro* studies have established LMB as a potent inhibitor of nuclear exportin 1 at nanomolar concentrations (Wolff et al., 1997, Menendez et al., 2003, Kudo et al., 1999). To ensure LMB had no effect on cell viability, we incubated cells with LMB for 4h followed by incubation with R-phycoerythrin (PE), a fluorogenic detector of early apoptosis. LMB had no significant effect on cell viability at 4h (Fig. 5.2.5.1 & Fig. 5.2.5.2) treatment relative to untreated cells.

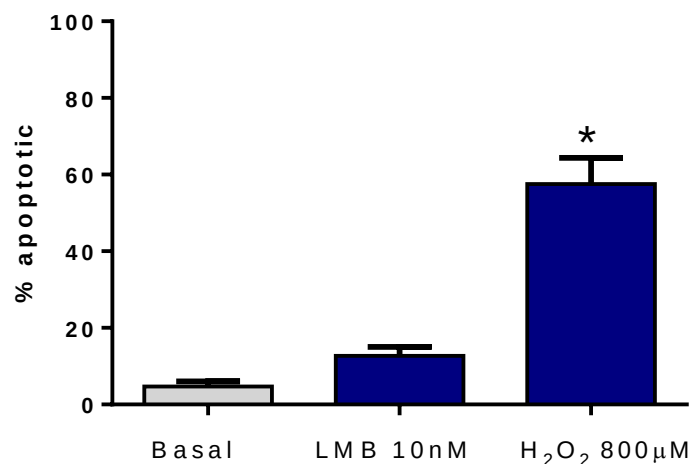


Fig. 5.2.5.1 Effects of 4 h Leptomycin B (LMB) treatment on cell viability. Serum-starved U937 monocytes were stimulated with LMB at 10nM for 30min followed by incubation with PE, a specific fluorogenic dye for detection of early apoptosis, for 15min in the dark. Levels of PE fluorescence were detected by flow cytometry using the PE channel. The effects of 800µM H₂O₂ are shown as a positive control. *p<0.05 vs. basal.

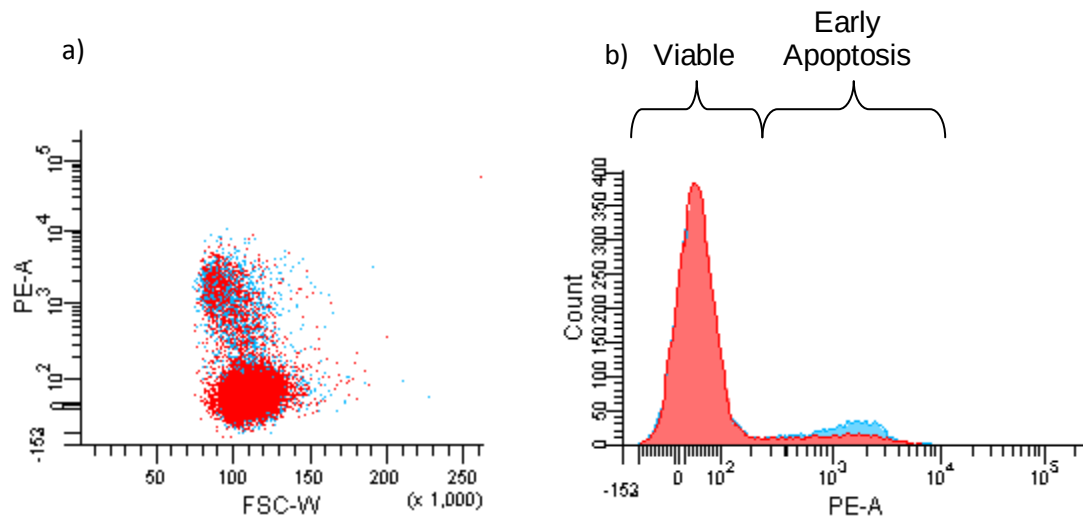


Fig. 5.2.5.2 Effect of 4 h leptomycin B (LMB) treatment on cell viability. Serum-starved U937 monocytes were stimulated with LMB (10 nM) for 4h followed by incubation with PE, a specific fluorogenic dye for detection of early apoptosis, for 15min in the dark. Levels of PE fluorescence were detected by flow cytometry at the PE channel, where a) represents a dot-plot of PE vs. forward scatter (FSC) for each cell and b) represents the distribution of those cells as either viable or apoptotic, as a histogram. Red and blue dot plots and histograms represent untreated cells and LMB at 10nM for 4h, respectively. These are representative of three experiments.

5.3 Results

5.3.1 Importin-7 siRNA reduces both cytosolic and nuclear levels of Importin-7

In order to determine the role of importin-7 on corticosteroid-induced GR nuclear import in U937 monocytes, we first assessed the effects of electroporation of importin-7 siRNA on importin-7 protein levels. We used ON-TARGET SMARTpool siRNA against human importin-7 (L-012255-00-001, Dharmacon, Lafayette, CO, USA). The siRNA pool contained four ON-TARGET*plus* SMARTpool siRNAs: CAAUUGCAGCUUUGUAUUA, UAAGCAGGCUGGUGAAGAU, GAAGAUCGCCAUUGUAUUC and CAACACUGCUCCAGAUUAC. The non-silencing negative siRNA control used in this study was ON-TARGET*plus* non-targeting siRNA. We found that both cytosolic ($38.4 \pm 11.5\%$) and nuclear ($42.7 \pm 16.5\%$) importin-7 levels were greatly reduced to a similar extent relative to non-targeting siRNA negative control 36h after importin-7 siRNA transfection (* $p < 0.05$ vs. non-targeting siRNA negative control). For all future experiments exploring the role of importin-7 knockdown on corticosteroid-induced GR nuclear translocation, we used the above conditions.

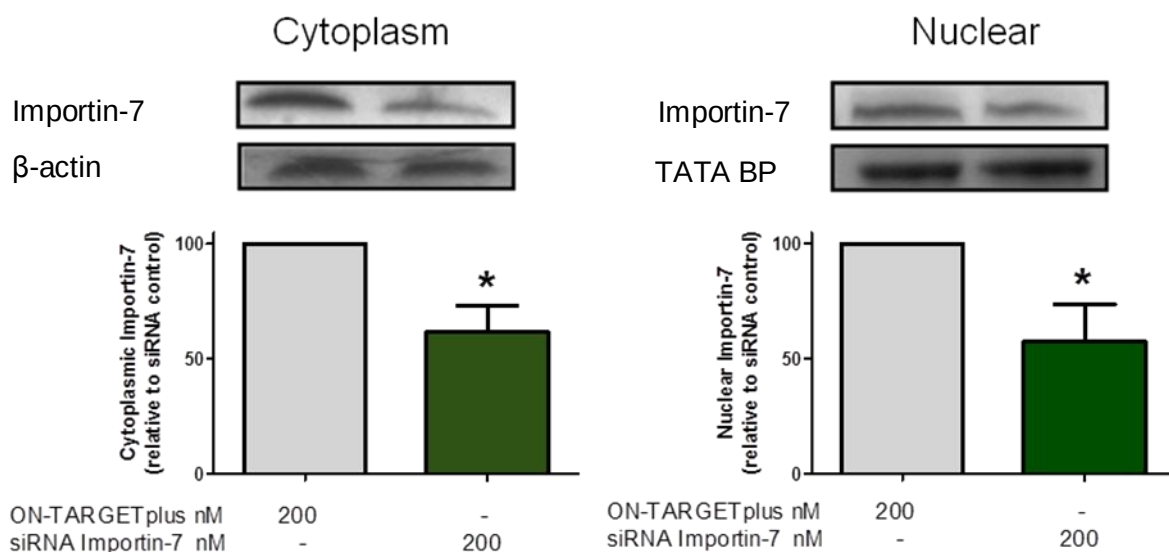


Fig 5.3.1 Effect of importin-7 siRNA transfection. Nuclear and Western blotting was carried out on serum-starved U937 monocytes 36h after electroporation with importin-7 siRNA or control. Results represent the mean $\pm$ SEM of 3 independent experiments. * $p < 0.05$ vs. non-targeting siRNA negative control.

5.3.2 Importin-7 siRNA reduces corticosteroid-induced GR nuclear localisation

After optimising *in vitro* conditions for importin-7 knockdown, we next explored the role of importin-7 on FP-induced GR nuclear localisation. FP (10^{-9} M)-induced GR nuclear localisation was markedly reduced in the importin-7 knockdown model by $39.3 \pm 8.7\%$, relative to non-targeting siRNA negative control (* $p < 0.05$). Interestingly, the percentage reduction in FP-induced GR nuclear localisation was similar to the level of importin-7 knockdown achieved in the cytoplasm ($39.3 \pm 8.71\%$ vs. 38.4 ± 11.5).

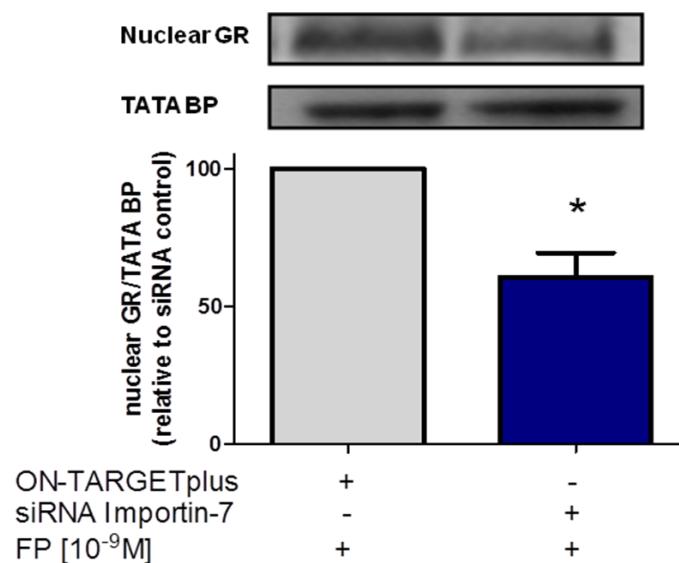


Fig. 5.3.2 Effect of importin-7 siRNA transfection on FP-induced GR nuclear localisation. 36h post importin-7 siRNA transfection, cells were serum starved for 3h, followed by incubation with FP (10⁻⁹M) for 2h. Results represent the mean±SEM of 3 independent experiments. *p<0.05 vs. non-targeting siRNA negative control.

5.3.3 Importin-7 siRNA reduces corticosteroid-induced suppression of CXCL8

We have shown that importin-7 knockdown impairs corticosteroid-induced GR nuclear translocation. Next, we investigated whether importin-7 knockdown would also have an impact on corticosteroid-mediated suppression of IL-1 β -induced CXCL8 secretion. Following 36h transfection, the non-targeting siRNA had no significant effect on FP (10⁻⁹M)-induced suppression of IL-1 β -induced CXCL8 in comparison to the non-transfected cells (76.8±2.6 vs. 68.4±3.0% stimulated levels, p=ns). Whereas, in the importin-7 knockdown model, FP was only able to suppress IL-1 β -induced CXCL8 by 40.2±16.1% which was significantly less than that seen in control untransfected cells (68.4±3.0%, *p<0.05). The percentage reduction in FP function (~40%) reflects the degree of importin-7 knockdown achieved.

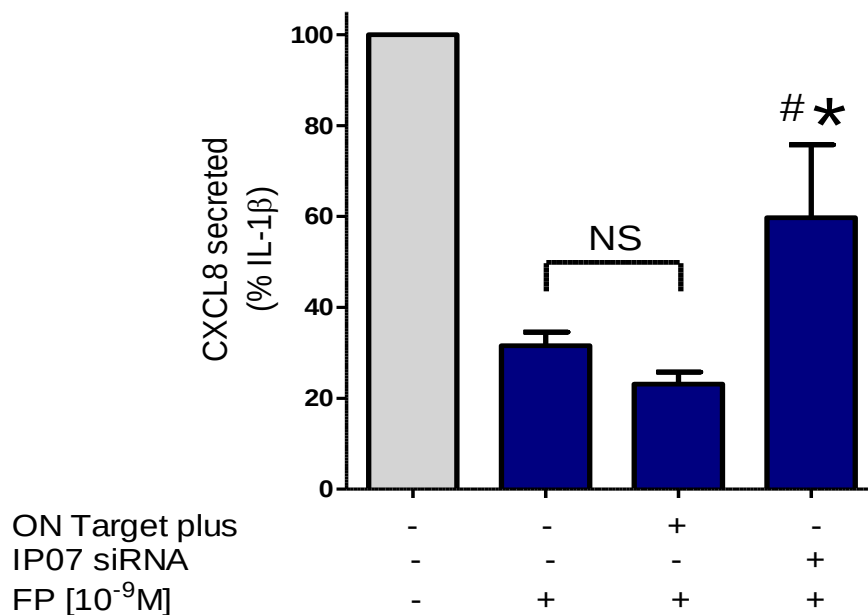


Fig. 5.3.3 Effect of importin-7 siRNA transfection on FP-mediated suppression of IL-1 β -induced CXCL8. 36 h post importin-7 siRNA transfection, cells were serum starved and stimulated with IL-1 β (1ng/ml) for 30min followed by incubation with FP (10⁻⁹M). 16h post IL-1 β stimulation the supernatant was collected and CXCL8 levels were analysed by ELISA. Results represent the mean $\pm$ SEM of 3 independent experiments. *p<0.05 vs. FP mediated suppression of IL-1 β -induced CXCL8. #p<0.05 vs. non-targeting siRNA.

5.3.4 Effects of oxidative stress on cytoplasmic importin-7

Having established that importin-7 was involved in corticosteroid-induced GR nuclear translocation, we next explored whether the impaired corticosteroid-induced GR nuclear translocation observed under conditions of oxidative stress and heightened inflammatory response (Chapter 4), was due to impaired importin-7-mediated GR nuclear transport. In our *in vitro* model of oxidative stress, pre-treatment with 200 μ M of H₂O₂ and 1ng/ml of IL-1 β had no effect on cytosolic levels of importin-7 (Fig. 5.3.4).

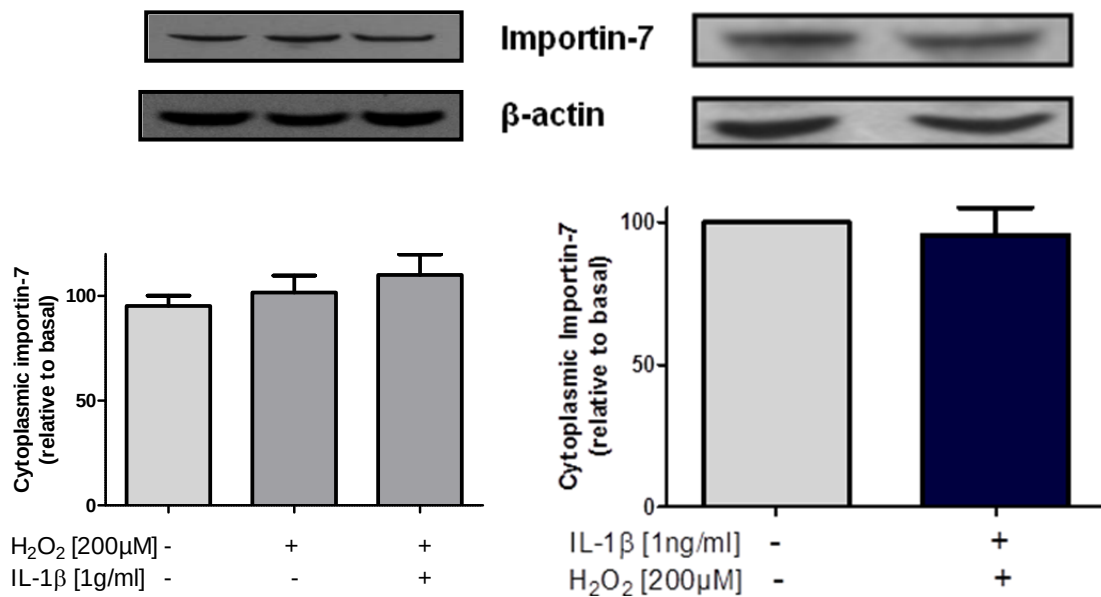


Fig. 5.3.4 No effect of H_2O_2 and IL-1 β alone or in combination on cytoplasmic importin-7 levels. Serum-starved U937 cells were stimulated with H_2O_2 (200 μ M) for 90min followed by stimulation with IL-1 β (1ng/ml) for 30min. Nuclear extraction and Western blotting were performed immediately. Results represent the mean $\pm$ SEM of 3 independent experiments.

5.3.5 Oxidative stress reduces nuclear RanGTP levels

Importin-7 mediated transport of cargo proteins is RanGTP-dependent. We next explored whether impaired corticosteroid-induced GR nuclear translocation in our *in vitro* model of oxidative stress was due to degradation of nuclear RanGTP. Levels of nuclear RanGTP were detected by pull down using RanBP1 Agarose beads. Pre-treatment with H_2O_2 (200 μ M) and IL-1 β (1ng/ml) caused a significant reduction in nuclear Ran GTP levels by 61.2 $\pm$ 12.6% relative to basal levels (*p<0.05 vs. basal).

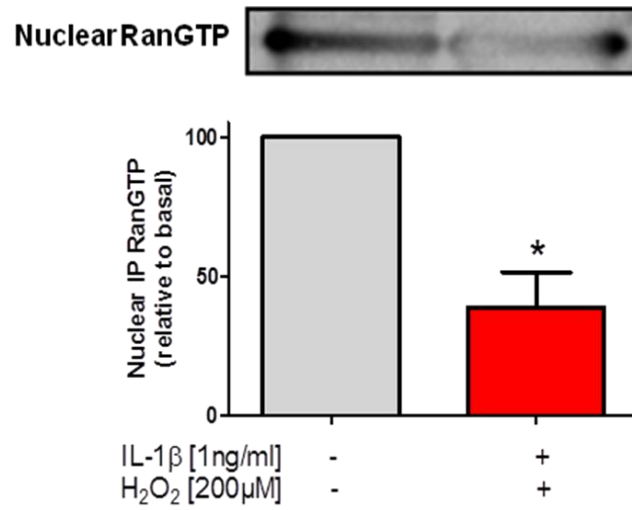


Fig. 5.3.5 Effect of H₂O₂ and IL-1β on nuclear RanGTP levels. Serum-starved U937 cells were stimulated with H₂O₂ (200μM) for 90min followed by stimulation with IL-1β (1ng/ml) for 30min. Immunoprecipitation of nuclear RanGTP was performed immediately. Results represent the mean±SEM of 3 independent experiments. *p<0.05 vs. basal nuclear levels.

5.4 Discussion

Nucleocytoplasmic shuttling of proteins across the NPC is governed by nuclear receptors. Over the last decade, several members of the karyopherin family of nuclear receptors have been shown to facilitate GR nucleocytoplasmic shuttling (Freedman and Yamamoto, 2004, Tao et al., 2006). In an *in vitro* nuclear import assay, importins- β , -7 and -8 bound to and facilitated the nuclear import of the NL1 fragment of GR, with importins-7 and -8 additionally facilitating nuclear import of the NL2 fragment of GR (Freedman and Yamamoto, 2004). We found knockdown of importin-7 by siRNA in a human monocyte cell line, considerably reduced FP-induced GR nuclear localisation and FP-mediated suppression of IL-1 β -induced CXCL8 (Figures. 5.3.1, 5.3.2 and 5.3.3). Our experiments provide evidence that importin-7 plays a vital role in corticosteroid-induced GR nuclear import and corticosteroid-mediated suppression of inflammatory markers in human monocyte cells.

After establishing the potential role of importin-7 in corticosteroid-mediated GR nuclear translocation, we next explored (in our *in vitro* model of oxidative stress associated with heightened inflammatory response) whether reduced BUD and FP function maybe partly due to impaired importin-7 mediated GR nuclear import. We found H₂O₂ (200 μ M) in combination with IL-1 β (1ng/ml) significantly reduced nuclear RanGTP levels, however cytoplasmic levels of importin-7 were not affected. As reported previously, importin-7 mediated transport of cargo proteins is RanGTP dependent (Jakel et al., 1999). The RanGTPase system is composed of a Ran gradient across the NPC, where RanGDP is predominantly found in the cytoplasm,

whereas RanGTP mainly resides in the nucleus (Poon and Jans, 2005, Wagstaff and Jans, 2009, Gorlich and Kutay, 1999). Hydrolysis of RanGTP to RanGDP releases a sufficient degree of energy to facilitate the dissociation of importin-7 and its cargo protein (for example a transcription factor) in the nucleus. This subsequently allows the transcription factor to progress through the nucleus and regulate gene transcription. Based upon our findings that nuclear RanGTP is degraded in our *in vitro* model of oxidative stress and that knockdown of importin-7 reduces corticosteroid function we suggest that BUD and FP insensitivity may be partly due to abnormal degradation of nuclear RanGTP.

Kodiha and colleagues showed importin- β , a member of the same family of nuclear transport receptors as importin-7, underwent significant degradation post H_2O_2 treatment in HeLa cells (Kodiha et al., 2004). A closer examination of the findings revealed that the group used a concentration of H_2O_2 at 10mM, which is far greater than 800 μ M, where we observed a significant level of apoptosis in U937 cells at 4h and 18h (see Fig. 5.2.5.2 and Fig. 4.2.2). Further in-depth studies by this group revealed that inhibition of caspases, a key apoptotic signalling mechanism, reduced the loss of importin- β . One therefore would assume the observed loss in importin- β reported by this group was most probably due to apoptosis induced by oxidative stress.

In this Chapter we have been able to demonstrate that importin-7 is crucial for corticosteroid-induced GR nuclear import. In addition, under conditions of oxidative stress-induced corticosteroid insensitivity, nuclear RanGTP, an essential co-factor

for importin-7, was markedly less. In the next Chapter we would like to confirm whether corticosteroid insensitivity in cells extracted from severe asthmatics and COPD patients is also associated with reduced GR nuclear localisation, as observed in our in vitro model of oxidative stress-induced corticosteroid insensitivity.

CHAPTER 6

CORTICOSTEROID SENSITIVITY IN MONOCYTES FROM SEVERE ASTHMATICS AND COPD PATIENTS

6.1 Introduction

Corticosteroids are widely used in the treatment of asthma and COPD, however in sharp contrast to their use in patients with asthma, they are less effective in controlling the underlying inflammation and reversing the decline in lung function in patients with COPD (Keatings et al., 1997, Suissa and Barnes, 2009). In Chapter 3, we compared three corticosteroids, budesonide (BUD), fluticasone propionate (FP) and fluticasone furoate (FF), which have different physiochemical and structural properties, and found FF had a greater effect on glucocorticoid receptor (GR) activity and on suppression of the pro-inflammatory mediator, CXCL8, compared to BUD. Furthermore, FF was able to sustain its effect on GR nuclear translocation and CXCL8 suppression following a 20h washout, whereas BUD was unable to achieve this. This clear difference in activity and function between BUD and FF was also explored under conditions of oxidative stress associated with heightened inflammatory response. We observed FF was resistant to the effects of oxidative stress associated with heightened inflammatory response. In contrast, BUD-induced GR nuclear import and BUD-mediated suppression of IL-1 β -induced CXCL8 were greatly reduced. Based upon these differences between BUD and FF at the *in vitro* level, the aim of this Chapter was to compare BUD and FF activity and function *ex vivo* in monocytes from healthy volunteers, severe asthmatics and COPD patients.

We also observed in Chapter 3 that the addition of long-acting β_2 -agonists (LABA), formoterol (FORM) and vilanterol trifenate (VI), to BUD and FF, respectively (combinations that are used in clinical practice) reduced CXCL8 to a greater extent than the corticosteroid treatment alone. This probably reflected the additional contribution of the anti-inflammatory effect of the corticosteroid in the combination formulations over-and-above the single-agent LABA treatment (Adcock et al., 2002). Indeed, there is growing evidence for the molecular rationale of the complementary interactions and effects between corticosteroids and LABA both *in vitro* (Mak et al., 1995, Eickelberg et al., 1999, Papakonstantinou et al., 2012) and *in vivo* (Usmani et al., 2005b, Orsida et al., 2001, Li et al., 1999), where studies have shown that corticosteroids up-regulate β_2 -receptors and LABA's increase GR nuclear translocation. This molecular basis may explain the improvement in lung function, symptoms, disease exacerbations in both asthma and COPD achieved with the combination treatment than either drug alone in large patient clinical trials (Woolcock et al., 1996, Greening et al., 1994, Pauwels et al., 1997, Juniper et al., 1999, Calverley et al., 2007). The use of corticosteroids in combination with LABA is well established in national and international guidelines for treating patients with asthma and also those with COPD. Experimental evidence suggests there is a complementary action between corticosteroids and LABA (Mak et al., 1995, Eickelberg et al., 1999, Papakonstantinou et al., 2012, Usmani et al., 2005b, Baraniuk et al., 1997). In an asthmatic study, the combination of low-dose FP and SLM was as effective as high-dose FP on GR activation, gene transactivation and gene transrepression (Usmani et al., 2005b). Based upon these previous findings, the additional aim of this Chapter was to determine whether the complementary

effect of the LABA, FORM and VI, on their respective corticosteroids used in clinical practice, BUD and FF, could have a beneficial effect in primary monocytes from severe asthmatics and COPD patients who are characterised by corticosteroid insensitivity.

6.2 Experimental protocols

6.2.1 Screening visit

Subjects attended a screening visit at the Asthma Lab of the Royal Brompton Hospital and underwent a medical history, assessment of current medications and smoking history and, if required, a medical examination. They also underwent spirometry testing to determine eligibility to enter the study. A review of the inclusion and exclusion criteria were carried out and written informed consent was obtained from each patient.

Patients were allowed to use their current short-acting β_2 agonists (SABA) and short-acting muscarinic-antagonists (SAMA), and were asked to refrain for 12h from their LABAs, inhaled corticosteroid (ICS) and long-acting muscarinic-antagonists (LAMA) prior to the study visit. Theophylline (an oral tablet bronchodilator) was required to be stopped at least 3 days prior to the study visit.

6.2.2 Study design

Six healthy volunteers, 8 mild-to-moderate COPD patients and 4 severe asthmatics participated in this study. There was only one study visit per subject that lasted approximately 30min. Clinical assessment and lung function were measured and

80ml of venous blood taken during this visit. This study had Ethical approval (Research Ethics Committee Number: 11/SC/0037).

The primary outcome of this study was to compare BUD- and FF-induced GR-GRE binding in blood monocytes from healthy volunteers, severe asthmatics and COPD patients. Secondary endpoints included the effects on inflammatory markers, CXCL8 and IL-6, alone and in combination with LABA, FORM and VI.

6.2.3 Monocytes from blood

A sample of 80ml of venous blood was taken during the patient's visit. The blood was separated by centrifugation using Ficoll-Paque (400xg for 30min at 22°C). The peripheral blood mononuclear cell (PBMC) monolayer was collected and washed with HANKS buffered solution at 300xg for 5min at 4°C. PBMCs were resuspended in RPMI-1640 complete media and seeded as 100µl of 2×10^6 cells/ml or 3ml of 2×10^6 cells/ml in 96 and 6 well-plates respectively for 90min to allow monocyte adherence. Following 90min incubation, the supernatant was removed and monocytes forming a layer on the flask were resuspended in RPMI-1640 minimal media.

6.2.4 Corticosteroid-induced GR activation

Nuclear protein was extracted from blood monocytes using a nuclear protein extraction kit (Active Motif, Carlsbad, CA, USA). GR-GRE binding was detected using a TransAM-GR kit (Active Motif). We decided to use a concentration of 10^{-9} M for each corticosteroid based on experimental data in U937 cells, as shown in Fig. 6.2.3.

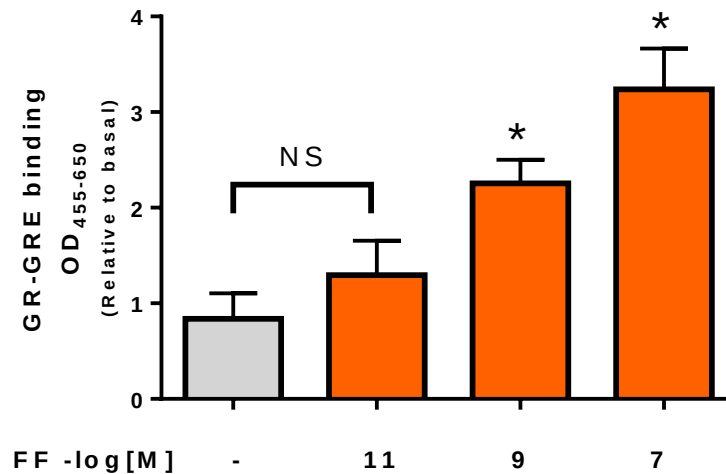


Fig. 6.2.3 Fluticasone furoate (FF)-induced GR-GRE binding. U937 mononocytes were treated with FF for 2h. Nuclear extraction was carried out immediately and activated glucocorticoid receptor (GR) binding to glucocorticoid response elements (GRE) was determined by GR-GRE assay. Results are based on 3 independent experiments. * $p < 0.05$ vs. basal.

6.2.5 Enzyme-linked immunosorbent assay (ELISA)

Extracellular concentrations of CXCL8 and IL-6 were measured with the human DuoSet ELISA kits (R&D Systems Europe, UK), according to the manufacturer's instructions.

6.2.6 Statistical methods

All statistical comparisons were conducted using a multiplicative analysis of variance (ANOVA) model. P-values less than 5% were considered statistically significant.

6.3 Results

6.3.1 Patient demographics

Age-matched patients with mild-to-moderate COPD, severe asthma and healthy patients were studied. Eight patients with mild-to-moderate COPD (GOLD 1 and 2 guidelines) and four patients with severe asthma defined according to the GINA guidelines were recruited. COPD patients had significantly lower mean FEV₁ (% pred.) compared to their healthy age-matched counterparts (*p<0.05).

	Healthy (n=6)	Severe Asthma (n=4)	COPD (n=8)
Age	55 ± 6.7	60 ± 2.5	68 ± 8.0
Gender (M/F)	3/3	2/2	4/4
FEV ₁ (% pred.)	100 ± 15	54 ± 16	67 ± 22*
FVC (% pred.)	100 ± 16	76 ± 14	90 ± 22
FEV ₁ /FVC (% pred.)	99 ± 5.5	72 ± 9.1	76 ± 18

Table 6.1 Patient demographics. 6 healthy volunteers, 4 severe asthmatics and 8 COPD patients were recruited. Age, gender and several parameters of lung function were recorded. Definition of abbreviation: FEV₁ = forced expiratory volume in 1 second; FVC = forced vital capacity.

6.3.2 Corticosteroid-induced GR activation in blood monocytes

6.3.2.1 Comparison of BUD- and FF-induced GR-GRE binding

We first compared BUD- and FF-induced GR-GRE binding in blood monocytes from healthy volunteers. As expected, BUD and FF at 10^{-9} M, significantly induced GR-GRE binding by 1.4 ± 0.18 - and 1.6 ± 0.35 -fold increase respectively relative to basal levels. No significant difference was observed between FF- and BUD-induced GR-GRE binding (Fig. 6.3.2.1).

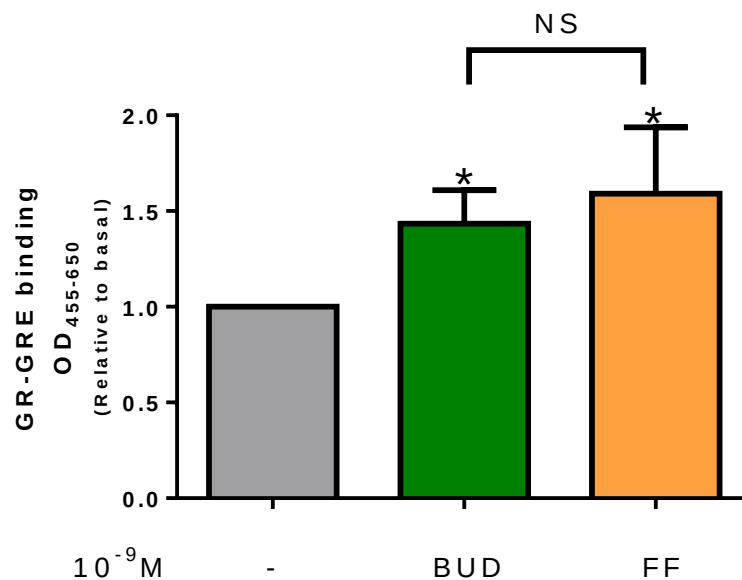


Fig. 6.3.2.1 Comparison of budesonide (BUD)- and fluticasone furoate (FF)-induced GR-GRE binding blood monocytes derived from blood of healthy volunteers. Monocytes were isolated from peripheral blood mononuclear cells from 6 healthy volunteers. Following isolation, monocytes were stimulated with BUD or FF (both at 10^{-9} M) for 2h. Immediately after 2h corticosteroid stimulation, nuclear extraction and GR-GRE binding was performed. *p<0.05 vs. basal.

6.3.2.2 BUD and FF-induced GR-GRE binding in severe asthma and COPD

It has been previously reported that corticosteroid-induced GR activation in severe asthmatics is impaired (Irusen et al., 2002, Mercado et al., 2012). As a subpopulation of patients with severe asthma and COPD patients show a degree of corticosteroid insensitivity, we compared BUD- and FF-induced GR-GRE binding in blood monocytes from healthy volunteers, severe asthmatics and COPD patients. Following a 2h stimulation, BUD (10^{-9} M) significantly induced GR-GRE binding by 1.4 ± 0.18 (healthy volunteers) and 1.9 ± 0.2 (COPD) in blood monocytes, relative to basal levels (Fig. 6.3.2.2.1). Whereas in severe asthmatics, BUD (10^{-9} M) was unable to induce GR-GRE binding relative to basal levels. There was no significant difference in BUD-induced GR-GRE binding between the treatment groups (Fig. 6.3.2.2.1). FF (10^{-9} M) also induced GR-GRE binding in COPD patients to a similar extent to that seen in healthy volunteers (2.4 ± 0.77 - vs. 1.6 ± 0.35 -fold increase, $p = \text{ns}$) (Fig. 6.3.2.2.2). In severe asthmatics, FF was also unable to cause a significant increase in GR-GRE binding compared to basal levels. There was also no significant difference in FF-induced GR-GRE binding between the treatment groups studied.

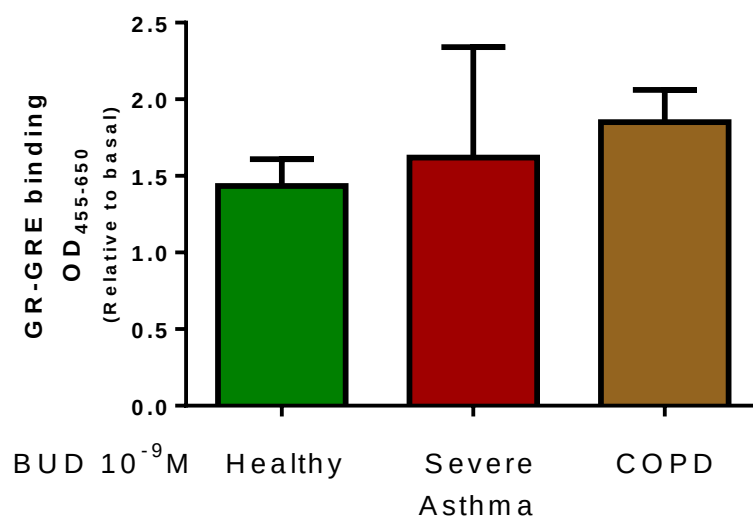


Fig. 6.3.2.2.1 Comparison of budesonide (BUD)-induced GR-GRE binding in blood monocytes from 6 healthy volunteers, 4 severe asthmatics and 8 COPD patients. Monocytes were isolated from peripheral blood mononuclear cells and stimulated with BUD at 10⁻⁹M for 2h. Immediately after this nuclear extraction and GR-GRE binding assays were performed. Results represent mean±SEM.

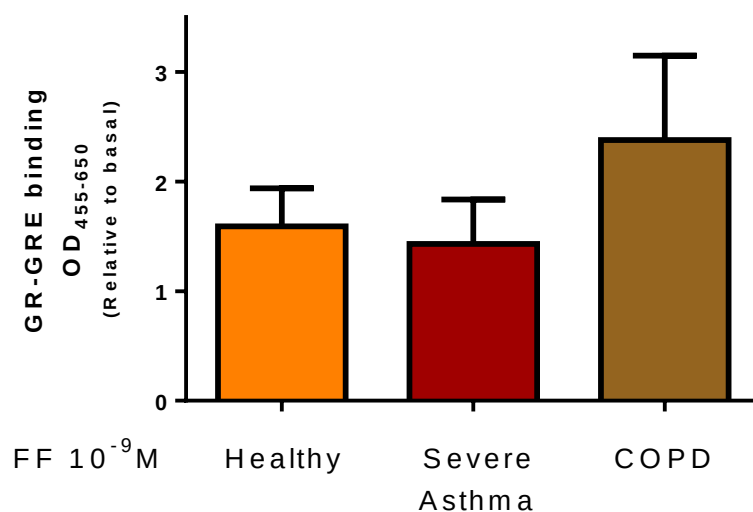


Fig. 6.3.2.2.2 Comparison of fluticasone furoate (FF)-induced GR-GRE binding in blood monocytes from 6 healthy volunteers, 4 severe asthmatics and 8 COPD patients. Monocytes were isolated from peripheral blood mononuclear cells from subjects and stimulated with FF (10⁻⁹M) for 2h and nuclear extraction and GR-GRE assays performed. Results are presented as mean±SEM.

6.3.3 Pro-inflammatory cytokine secretion in monocytes

Following corticosteroid-induced GR activation, activated nuclear GR dampens down gene transcription of pro-inflammatory genes, such as *IL-8* and *IL-6*, which respectively encode for the pro-inflammatory mediators, CXCL8 and IL-6 (Barnes, 2006b). The neutrophil chemoattractant, CXCL8, and the pro-inflammatory mediator, IL-6, are found in elevated levels in COPD and asthmatic patients (Keatings et al., 1996, Barnes, 2008). We compared basal levels and IL-1 β -induced CXCL8 and IL-6 release from blood monocytes from 6 healthy volunteers, 4 severe asthmatics and 8 COPD patients. We observed that IL-1 β -induced a significant increase in CXCL8 and IL-6 levels in blood monocytes derived from all three subject groups (Fig. 6.3.3.1 & Fig. 6.3.3.2). We also noted basal levels of CXCL8 from COPD patients were significantly higher than in healthy volunteers (1940 ± 277 vs. 808 ± 191 pg/ml, $*p < 0.05$). Although, severe asthmatics had similar basal levels of CXCL8 compared to COPD patients, this did not quite reach statistical significance when compared to healthy volunteers (2304 ± 810 vs. 808 ± 191 pg/ml, $p = 0.17$) (Fig. 6.3.3.1a). Furthermore, after monocytes were stimulated for 16h with IL-1 β (1 ng/ml), CXCL8 was markedly higher in monocytes from both severe asthmatics (10900 ± 2600 vs. 4670 ± 667 pg/ml, $*p < 0.05$) and COPD patients (11500 ± 1630 vs. 4670 ± 667 pg/ml, $*p < 0.05$), compared to healthy volunteers. There was no significant difference in CXCL8 secretion between severe asthmatics and COPD patients.

We also observed that basal levels of IL-6 were significantly higher in severe asthmatics compared to (i) COPD patients (2300 ± 811 vs. 216 ± 125 pg/ml, $^{\#}p < 0.05$) and (ii) healthy volunteers (2300 ± 811 vs. 12.3 ± 5.90 pg/ml, $*p < 0.05$). Although there

was a substantial difference in IL-6 basal levels between COPD and healthy volunteers, this did not reach statistical significance (216 ± 125 vs. 12.3 ± 5.90 pg/ml, $p=0.9$).

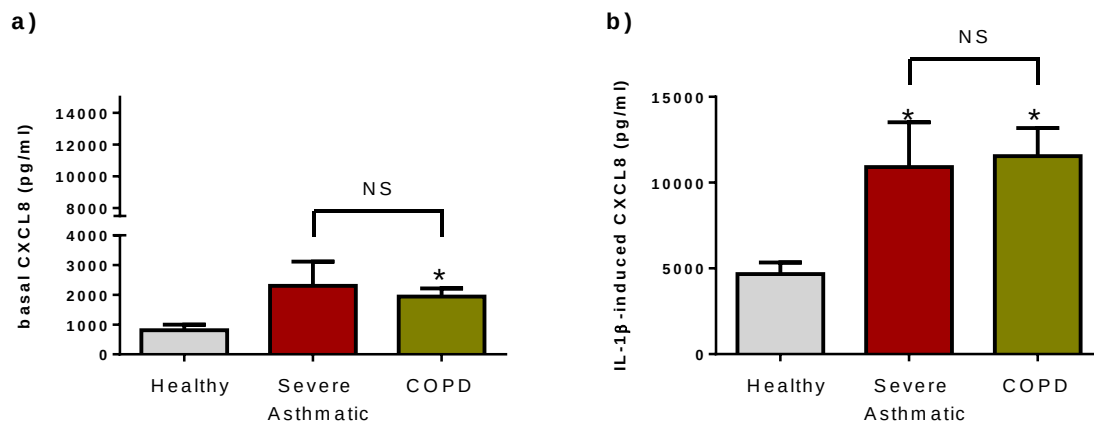


Fig. 6.3.3.1 Basal and IL-1 β -induced CXCL8 secretion from monocytes. Monocytes were isolated from peripheral blood mononuclear cells of 6 healthy, 4 severe asthmatic and 8 COPD patients. Following isolation, monocytes were stimulated with IL-1 β (1ng/ml) for 16h and CXCL8 levels were determined by ELISA. * $p < 0.05$ vs. Healthy.

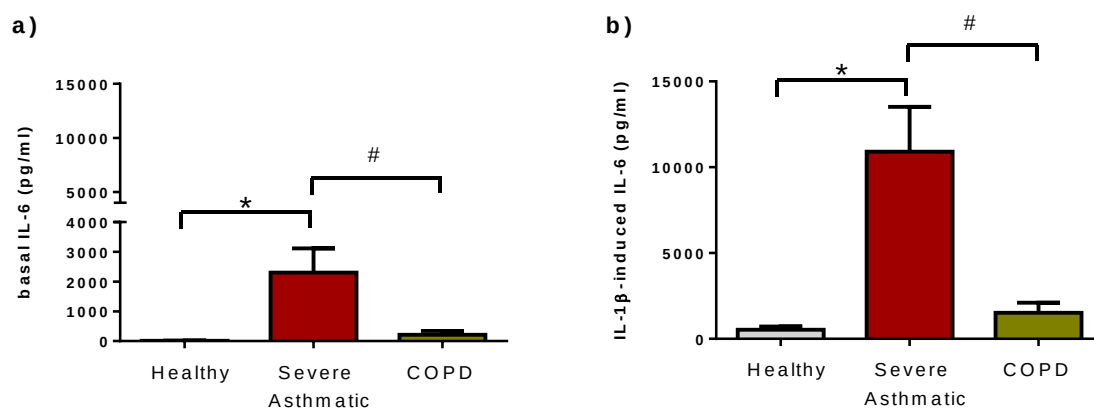


Fig. 6.3.3.2 Basal and IL-1 β -induced IL-6 secretion from monocytes. Monocytes were isolated from peripheral blood mononuclear cells of 6 healthy, 4 severe asthmatic and 8 COPD patients. Following isolation, monocytes were stimulated with IL-1 β for 16 h and IL-6 levels were determined by ELISA. * $p < 0.05$ vs. Healthy. # $p < 0.05$ vs. COPD.

Similarly, IL-1 β -induced IL-6 was substantially higher in blood monocytes from severe asthmatics compared to (i) COPD patients (10900 $\pm$ 2610 vs. 1520 $\pm$ 592pg/ml, [#]p<0.05) and (ii) healthy volunteers (10900 $\pm$ 2610 vs. 536 $\pm$ 180pg/ml, *p<0.05). IL-1 β -induced IL-6 levels were not statistically different between COPD and healthy volunteers (1520 $\pm$ 592 vs. 536 $\pm$ 179pg/ml, p=0.29) even though mean levels were almost 3 times higher in COPD patients.

6.3.4 Corticosteroid-mediated suppression of CXCL8

So far, we have shown that IL-1 β induces CXCL8 and IL-6 secretion in blood monocytes from severe asthmatics and COPD patients, compared with that of healthy volunteers. We next explored the suppressive effects of BUD and FF, alone and in combination with FORM and VI, respectively, on these pro-inflammatory mediators.

6.3.4.1 Comparison of BUD and FF in monocytes from healthy volunteers

Both BUD and FF reduced IL-1 β -induced CXCL8 release in blood monocytes from healthy volunteers in a concentration-dependent manner with IC₅₀ values of 7.7 $\pm$ 2.5 $\times 10^{-9}$ and 2.4 $\pm$ 2.2 $\times 10^{-9}$ M respectively. At the highest concentration (10⁻⁷M), BUD and FF suppressed CXCL8 by 59.1 $\pm$ 5.1 and 64.0 $\pm$ 7.4% respectively. Thus, there was no difference in the concentration-response curves for BUD- and FF-mediated suppression of IL-1 β -induced CXCL8 (Fig. 6.3.4.1.1).

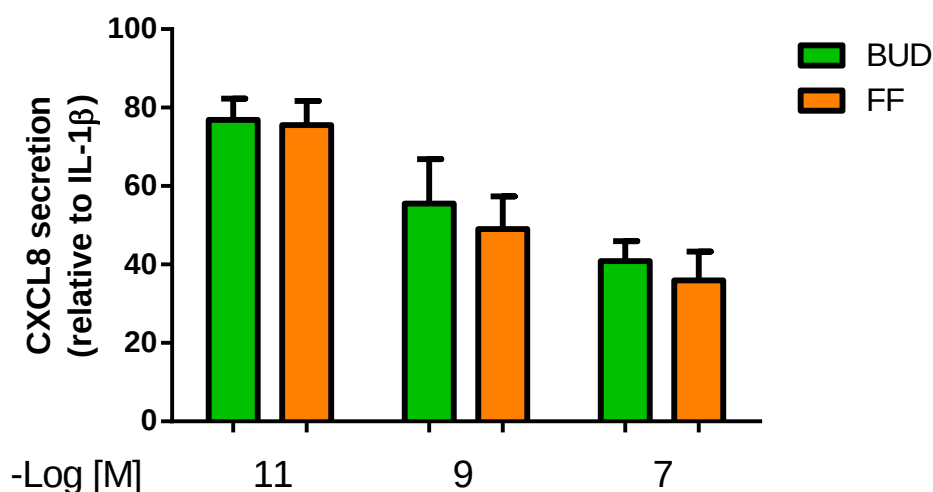


Fig. 6.3.4.1.1 Comparison of budesonide (BUD)- and fluticasone furoate (FF)-mediated suppression of IL-1 β -induced CXCL8 secretion from monocytes. Monocytes were isolated from peripheral blood mononuclear cells from 6 healthy subjects and stimulated with IL-1 β (1ng/ml) for 16h in the presence and absence of BUD or FF. CXCL8 levels were determined and results shown as means $\pm$ SEM of percent suppression of IL-1 β -stimulated levels.

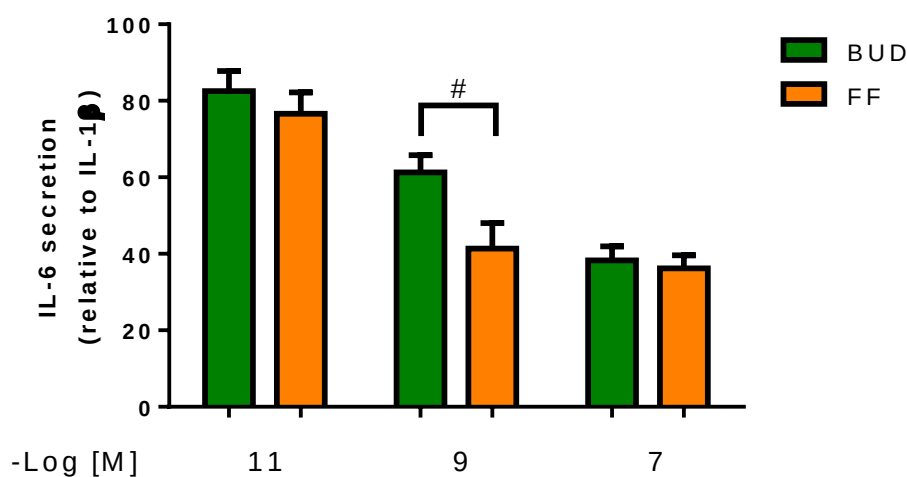


Fig. 6.3.4.1.2 Comparison of Budesonide (BUD) and fluticasone furoate (FF)-mediated suppression of IL-1 β -induced IL-6 secretion from monocytes. Monocytes were isolated from 6 healthy volunteers and stimulated with IL-1 β for 16h and with varying concentrations of BUD and FF. IL-6 levels were determined and results shown as means $\pm$ SEM of percent suppression of IL-1 β -stimulated levels. #p<0.05 vs. BUD (10⁻⁹M).

BUD and FF also suppressed IL-1 β -induced IL-6 in a concentration dependent manner. The highest concentration (10^{-7} M) of BUD and FF achieved similar levels of IL-6 suppression (62 ± 3.7 vs. $64\pm3.4\%$, $p=NS$) with IC_{50} values of $1.0\pm1.6\times10^{-8}$ and $1.4\pm2.0\times10^{-9}$ M respectively. In contrast, FF (10^{-9} M) was more effective in suppressing IL-1 β -induced IL-6 than the corresponding concentration of BUD (59 ± 6.7 vs. $39\pm3.7\%$ suppression, $^{#}p<0.05$).

6.3.4.2 Corticosteroid suppression of CXCL8 in severe asthma and COPD

So far, we have shown that BUD and FF suppress IL-1 β -induced CXCL8 to a similar extent in blood monocytes from healthy volunteers. We next explored the anti-inflammatory effects of BUD and FF in severe asthmatics and COPD patients. In blood monocytes from severe asthmatics 10^{-11} M BUD was as effective in suppressing CXCL8 as observed in healthy volunteers (19.8 ± 11.8 vs. $23.1\pm5.4\%$ suppression). In contrast, BUD-mediated suppression of IL-1 β -induced CXCL8 in blood monocytes from COPD patients was completely abolished at the lowest concentration of BUD (10^{-11} M) compared to that seen in healthy volunteers (-5.9 ± 9.2 vs. $23.1\pm5.4\%$, $p<0.05$). Additionally, BUD-mediated suppression of IL-1 β -induced CXCL8, at the highest concentration of 10^{-7} M, was reduced in COPD patients by 11 % compared to healthy volunteers (48.1 ± 6.6 vs. $59.1\pm5.1\%$) although this did not reach statistical significance ($p=0.23$). We observed an almost 15-fold increase in the concentration of BUD required to obtain a 50% inhibition of CXCL8 in blood monocytes from severe asthmatics compared to healthy volunteers. Again, this did not reach statistical significance ($1.2\pm7.6 \times 10^{-7}$ vs $7.7\pm2.6 \times 10^{-9}$ M, $p=ns$) (Fig.

6.3.4.2.3). There was no significant difference in IC_{50} values between COPD patients and healthy volunteers ($5.0 \pm 2.7 \times 10^{-8}$ vs. $7.7 \pm 2.6 \times 10^{-9}$ M, $p=0.23$).

Next, we examined FF-mediated suppression of CXCL8 in severe asthmatics and COPD patients compared to healthy volunteers. We found FF (10^{-11} - 10^{-7} M) was as effective in reducing IL-1 β -induced CXCL8 in severe asthmatics as observed in healthy volunteers for each corresponding concentration measured (Fig. 6.3.4.2.2a). The IC_{50} values were $4.5 \pm 2.2 \times 10^{-9}$ and $2.4 \pm 2.2 \times 10^{-9}$ M in severe asthmatics and healthy volunteers, respectively (Fig. 6.3.4.2.2a). In COPD patients, FF-mediated suppression of IL-1 β -induced CXCL8 was similar to that in healthy volunteers, with IC_{50} values of $9.8 \pm 2.6 \times 10^{-9}$ and $2.4 \pm 2.2 \times 10^{-9}$ M respectively (Fig. 6.3.4.2.2a). There were no significant differences at each corresponding concentration of FF between healthy volunteers, and severe asthmatics and COPD patients.

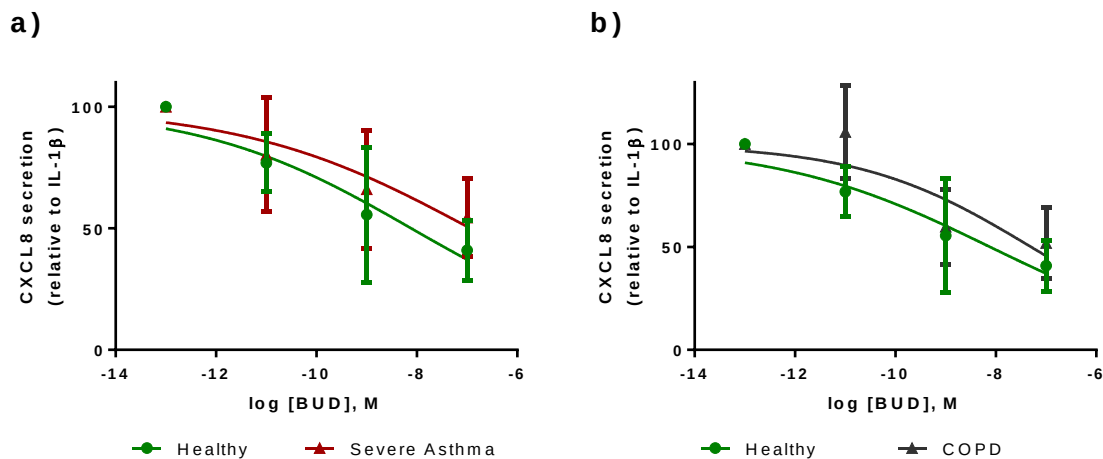


Fig. 6.3.4.2.1 Budesonide (BUD)-mediated suppression of IL-1 β -induced CXCL8 in blood monocytes from healthy volunteers, severe asthmatics and COPD patients. Monocytes were isolated from peripheral blood mononuclear cells from 6 healthy, 4 severe asthmatics and 8 COPD patients. Cells were stimulated with IL-1 β (1ng/ml) for 16h with varying concentrations of BUD. a) compares BUD-mediated CXCL8 suppression between

healthy volunteers and severe asthmatics; b) compares BUD-mediated suppression of CXCL8 between healthy volunteers and COPD patients. CXCL8 levels were determined by ELISA. Results expressed as means $\pm$ SEM of percent inhibition of the IL-1 β response.

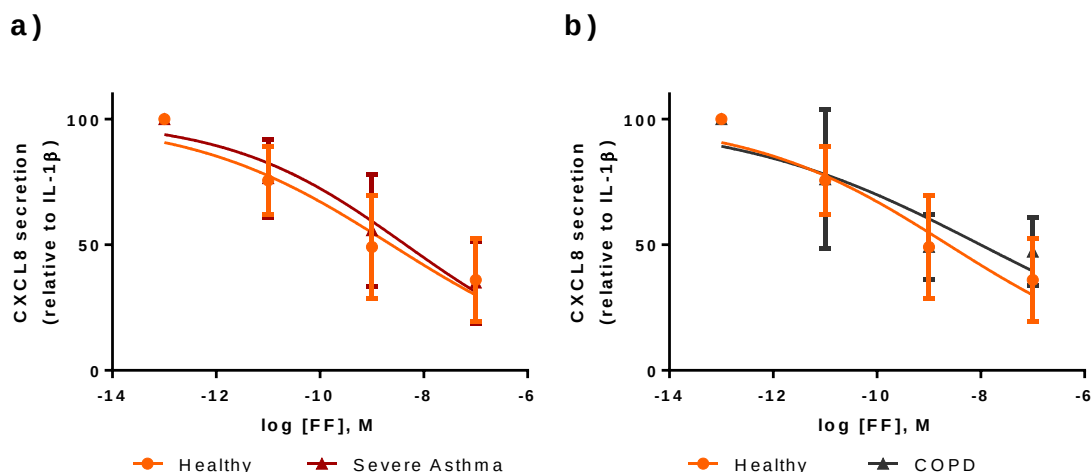


Fig. 6.3.4.2.2 Fluticasone furoate (FF)-mediated suppression of IL-1 β -induced CXCL8 in blood monocytes from 6 healthy volunteers, 4 severe asthmatics and 8 COPD patients. Monocytes were isolated from peripheral blood mononuclear cells and stimulated with IL-1 β (1ng/ml) for 16h and varying concentrations of FF. a) compares FF-mediated CXCL8 suppression between healthy volunteers and severe asthmatics and b) compares FF-mediated suppression of CXCL8 between healthy volunteers and COPD patients. CXCL8 levels were determined by ELISA. Results expressed as means $\pm$ SEM of percent inhibition of the IL-1 β response.

Corticosteroid-mediated suppression of IL-1 β -induced CXCL8		
		IC ₅₀
FF	Healthy	2.4 $\pm$ 2.2 x10 ⁻⁹
	Severe Asthma	4.5 $\pm$ 2.2 x10 ⁻⁹
	COPD	9.8 $\pm$ 2.6 x10 ⁻⁹
BUD	Healthy	7.7 $\pm$ 2.6 x10 ⁻⁹
	Severe Asthma	1.2 $\pm$ 7.6 x10 ⁻⁷
	COPD	5.0 $\pm$ 2.7 x10 ⁻⁸

Fig. 6.3.4.2.3 Comparison of Budesonide (BUD)- and fluticasone furoate (FF)-mediated suppression of IL-1 β -induced CXCL8 from peripheral blood monocytes isolated from 6 healthy volunteers, 4 severe asthmatics and 8 COPD patients.

6.3.5 Corticosteroid-mediated suppression of IL-6 secretion

We next compared BUD- and FF-induced suppression of IL-1 β -induced IL-6 in blood monocytes from the three patient groups. BUD (10⁻¹¹M) suppressed IL-1 β -induced IL-6 to a similar extent in cells from severe asthmatics as compared to those from healthy volunteers (30 $\pm$ 9.7 vs. 17 $\pm$ 5.2%, p=ns) with IC₅₀ values of 1.2 $\pm$ 5.3x10⁻⁸ and 1.0 $\pm$ 1.6 x10⁻⁸M respectively (Fig. 6.3.5.1a). Furthermore, although BUD (10⁻⁷M)-mediated suppression of IL-1 β -induced IL-6 secretion was 13% less in monocytes from severe asthmatics compared to healthy volunteers, this did not reach statistical significance (p=0.29). BUD was equally effective at suppressing IL-6 release from cells isolated from severe asthmatics at 10⁻⁹M and 10⁻⁷M (45 $\pm$ 6.0 vs 48 $\pm$ 12%, p=ns). This is in contrast to the situation in cells from healthy volunteers where there was a significant additional reduction in IL-6 secretion between the two BUD concentrations (38.7 $\pm$ 4.5 vs. 61.7 $\pm$ 3.7% repression, p<0.05) (Fig. 6.3.5.1a). In cells from COPD patients, all BUD concentrations (10⁻¹¹ to 10⁻⁷M) were all equally as effective in

suppressing IL-6 secretion as observed in healthy volunteers with IC_{50} values of $1.0 \pm 2.1 \times 10^{-8}$ and $1.0 \pm 1.6 \times 10^{-8}$ M respectively (Fig. 6.3.5.1b).

Finally, each corresponding concentration of FF suppressed IL-1 β -induced IL-6 to a similar extent in all three patient groups, with IC_{50} values of $1.4 \pm 2.0 \times 10^{-9}$ (Healthy volunteers), $5.3 \pm 2.5 \times 10^{-9}$ (severe asthmatics) and $1.7 \pm 1.8 \times 10^{-9}$ M (COPD) respectively. The effects of BUD and FF reached a maximum at 10^{-9} M with respect to suppression of IL-1 β -induced IL-6 secretion in severe asthmatics. In COPD patients, FF also suppressed IL-1 β -induced IL-6 in a concentration-dependent manner (Fig. 6.3.5.2).

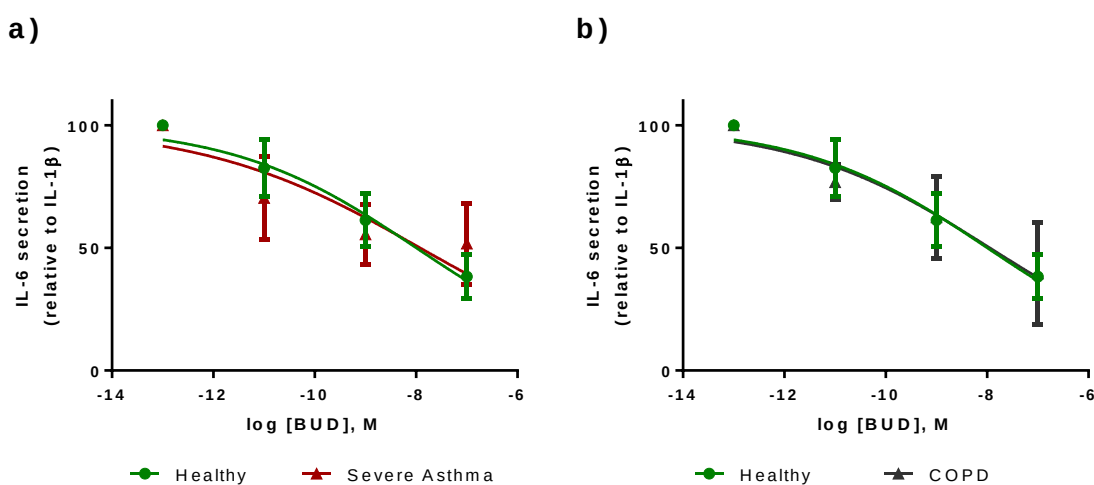


Fig. 6.3.5.1 Budesonide (BUD)-mediated suppression of IL-1 β -induced IL-6 in blood monocytes from 6 healthy volunteers, 4 severe asthmatics and 8 COPD patients. Monocytes were isolated from peripheral blood mononuclear cells and stimulated with IL-1 β (1ng/ml) for 16h and varying BUD concentrations. Graphs show a) BUD-mediated IL-6 suppression between healthy volunteers and severe asthmatics and b) BUD-mediated IL-6 suppression between healthy volunteers and COPD patients. IL-6 levels were determined and results expressed as means $\pm$ SEM of percent inhibition of the IL-1 β response.

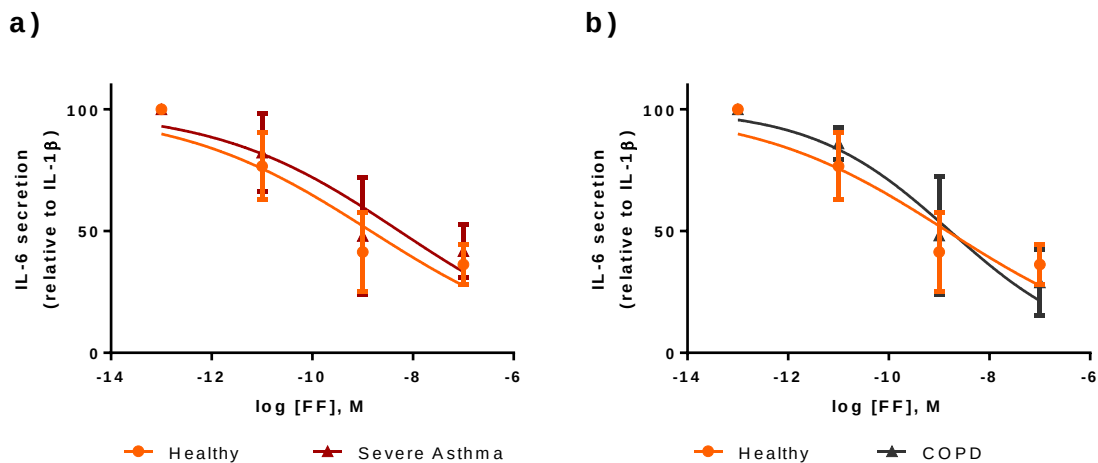


Fig. 6.3.5.2 Fluticasone furoate (FF)-mediated suppression of IL-1 β -induced IL-6 in blood monocytes from 6 healthy volunteers, 4 severe asthmatics and 8 COPD patients. Monocytes were isolated and stimulated with IL-1 β for 16h and varying concentrations of FF. Graphs show a) FF-mediated IL-6 suppression between healthy subjects and severe asthmatics and b) FF-mediated suppression of IL-6 between healthy subjects and COPD patients. IL-6 levels were determined and results expressed as means $\pm$ SEM of percent inhibition of the IL-1 β response.

Corticosteroid-mediated suppression of IL-1 β -induced IL-6		
		IC ₅₀
FF	Healthy	1.4 $\pm$ 2.0 $\times 10^{-9}$
	Severe Asthma	5.3 $\pm$ 2.5 $\times 10^{-9}$
	COPD	1.7 $\pm$ 1.8 $\times 10^{-9}$
BUD	Healthy	1.0 $\pm$ 1.6 $\times 10^{-8}$
	Severe Asthma	1.2 $\pm$ 3.3 $\times 10^{-8}$
	COPD	1.1 $\pm$ 2.1 $\times 10^{-8}$

Fig. 6.3.5.3 Comparison of Budesonide (BUD)- and fluticasone furoate (FF)-mediated suppression of IL-1 β -induced IL-6 from peripheral blood monocytes isolated from 6 healthy volunteers, 4 severe asthmatics and 8 COPD patients.

6.3.6 LABA effects on corticosteroid-mediated suppression of CXCL8

The combination of LABA with low dose corticosteroids is more effective in dampening down inflammation than monotherapy with high dose corticosteroids in mild-to-moderate asthmatics (Usmani et al., 2005b, Papakonstantinou et al., 2012, Woolcock et al., 1996). In Chapter 3, we showed low concentrations of the LABA FORM and VI significantly enhanced corticosteroid-mediated suppression of IL-1 β -induced CXCL8 in a monocyte cell line. As observed in our *ex vivo* study (section 6.2) and consistent with other investigations, CXCL8 is highly expressed in COPD patients (Keatings et al., 1996). Several studies in cells extracted from COPD patients have shown high dose corticosteroids fail to reduce pro-inflammatory markers (Culpitt et al., 1999, Keatings et al., 1997, Pauwels et al., 1997). In sputum macrophages of COPD patients, high doses of budesonide (800 mg twice daily) failed to suppress inflammatory markers (Keatings et al., 1997). We therefore explored whether FORM and VI in combination with their respective corticosteroids used in clinical practice, BUD and FF, could enhance corticosteroid-mediated suppression of CXCL8. Secondly, in COPD patients, where there is an element of corticosteroid insensitivity, whether LABA (10^{-9} M) could restore corticosteroid sensitivity.

We observed FORM and VI both at 10^{-9} M, had no significant effect on IL-1 β -induced CXCL8 (Fig. 6.3.6.1 & Fig. 6.3.6.2). In healthy volunteers, BUD at 10^{-11} M in combination with FORM (10^{-9} M) caused a greater suppression of IL-1 β -induced CXCL8 than BUD (10^{-11} M) alone, but this did not reach statistical significance (40.0 ± 7.0 vs. $23.0 \pm 5.4\%$, $p=0.09$) (Fig. 6.3.6.1a). Importantly, BUD at 10^{-11} M in

combination with FORM (10^{-9} M) was as effective as the higher concentration of BUD at 10^{-9} M alone (40.0 ± 7.0 vs. $44.4 \pm 11.4\%$, $p = \text{ns}$). Whereas in COPD patients, a lower concentration of BUD (10^{-11} M) in combination with FORM (10^{-9} M) was not as effective in reducing CXCL8 levels as the higher concentration of BUD (10^{-9} M) alone (7.4 ± 11.0 vs. $40.2 \pm 6.4\%$, $p < 0.05$) (Fig. 6.3.6.1b). Importantly in COPD patients, FORM at 10^{-9} M was unable to restore BUD (10^{-11} M) responsiveness (7.4 ± 11.0 vs. $5.9 \pm 9.2\%$, $p = \text{ns}$).

In cells from healthy volunteers, VI (10^{-9} M) did not enhance FF (10^{-11} M)-mediated suppression of IL-1 β -induced CXCL8 (40.0 ± 7.0 vs. $24.4 \pm 6.1\%$, $p = 0.19$). However, a lower concentration of FF (10^{-11} M) in combination with VI (10^{-9} M) was as effective as a higher concentration of FF (10^{-9} M) (40.0 ± 7.0 vs. $51.0 \pm 8.3\%$, $p = \text{ns}$) (Fig. 6.3.6.2a). Whereas in COPD patients, FF (10^{-11} M) in combination with VI (10^{-9} M) was not as effective as the higher concentration of FF (10^{-9} M) in suppressing CXCL8 (22.7 ± 7.7 vs. $50.8 \pm 4.5\%$, $*p < 0.05$) (Fig. 6.3.6.2b).

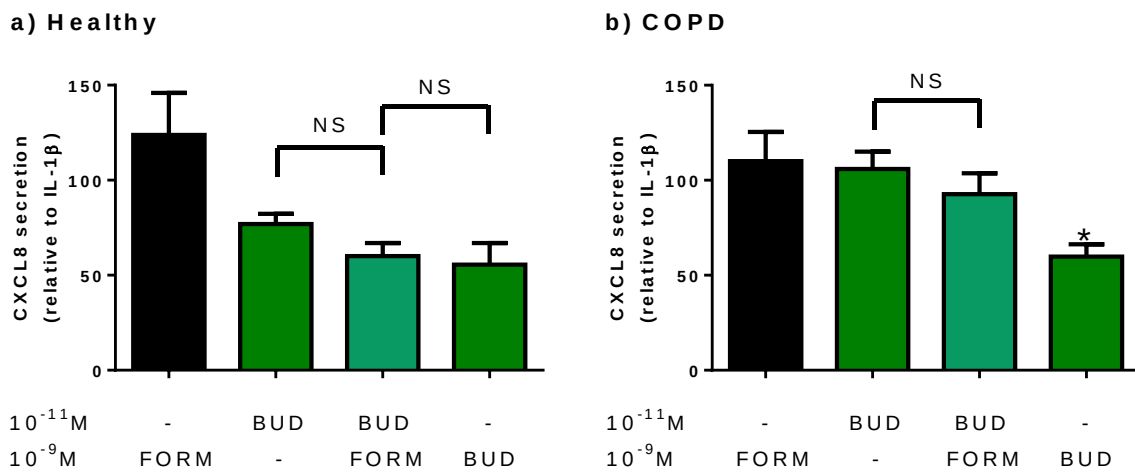


Fig. 6.3.6.1 Comparison of Budesonide (BUD) alone and in combination with formoterol (FORM). Blood monocytes from healthy volunteers and COPD patients were stimulated with IL-1 β (1ng/ml) followed by BUD alone and in combination with FORM. 16h following IL-1 β stimulation the supernatant was collected and CXCL8 levels were determined. Results are expressed as means $\pm$ SEM of percent inhibition of the IL-1 β response. *p<0.05 vs. BUD (10⁻¹¹M) and FORM (10⁻⁹M).

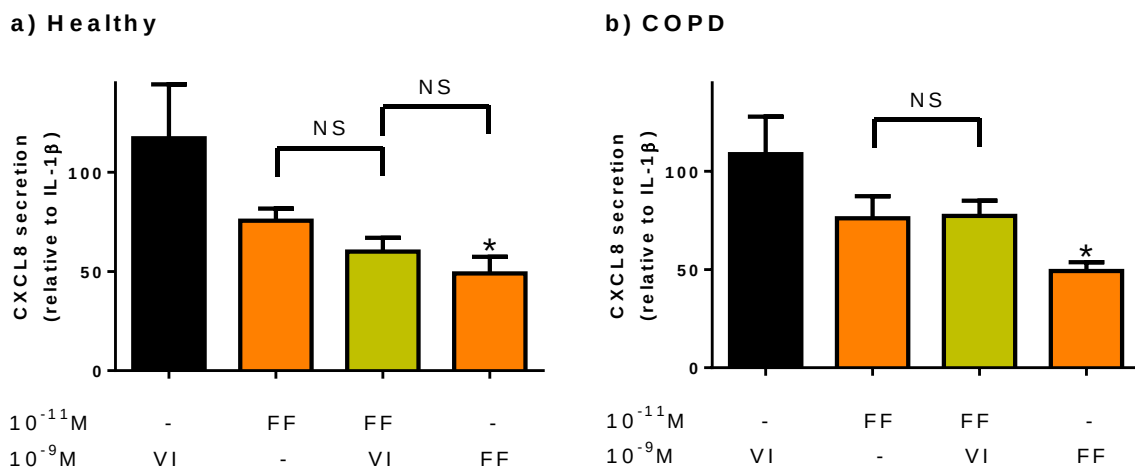


Fig. 6.3.6.2 Comparison of Fluticasone furoate (FF) alone and in combination with vilanterol trifenate (VI). Blood monocytes from healthy volunteers and COPD patients were stimulated with IL-1 β (1ng/ml) followed by FF alone and in combination with VI. 16h following IL-1 β stimulation the supernatant was collected and CXCL8 levels were determined. Results are expressed as means $\pm$ SEM of percent inhibition of the IL-1 β response. *p<0.05 vs. FF (10⁻¹¹M).

6.4 Discussion

In Chapter 4, we showed that in an *in vitro* model of oxidative stress associated with heightened inflammatory response that replicated the key pathological changes in severe asthma and COPD, BUD-induced GR nuclear localisation and suppression of CXCL8 were reduced. Based upon these results, we undertook an *ex vivo* study involving patients with severe asthma and COPD, both corticosteroid insensitive diseases, and used healthy volunteers as a control group to further investigate our findings. As expected, COPD patients had significant airway obstruction, deemed by a reduced FEV₁ percentage of predicted values, compared to healthy volunteers (Table 6.1). The pro-inflammatory profile in blood monocytes from severe asthmatics and COPD patients was consistent with previous studies (Daldegan et al., 2005, Morjaria et al., 2011).

Corticosteroids dampen down inflammation via activation of the GR, which then binds to GRE on corticosteroid-inducible genes (Barnes, 2006b). We observed no significant difference in BUD- and FF-induced GRE binding between healthy volunteers and COPD patients. Whereas in severe asthmatics, both BUD and FF were unable to induce GR-GRE binding, consistent with data reported in PBMCs of corticosteroid-insensitive severe asthmatics, where GR ligand-binding affinity in the nucleus was significantly reduced (Irusen et al., 2002).

We next compared BUD and FF suppression of IL-1 β -induced CXCL8 and IL-6 between all three patient groups. In COPD patients, BUD-mediated suppression of IL-1 β -induced CXCL8 was significantly reduced at 10⁻¹¹M, whereas FF retained its

sensitivity. This experimental finding was consistent with our previous experiments in our *in vitro* model (Chapter 4). However, there were no significant differences in IC₅₀ values for BUD in severe asthmatics and in COPD patients compared to healthy volunteers. This is in contrast to recent findings, where corticosteroid mediated suppression of pro-inflammatory mediators was severely reduced in PBMCs extracted from severe asthmatics and COPD patients (Mercado et al., 2012, To et al., 2010). This difference in corticosteroid sensitivity observed between our results and the aforementioned studies in severe asthmatics and COPD patients may be due to several reasons. Firstly, evidence suggests that the extent of corticosteroid insensitivity may correlate to the severity of disease (To et al., 2010). Patients with COPD enrolled in our study had milder airway obstruction compared to those enrolled by Ito and colleagues, where moderate-to-severe COPD patients were recruited. Secondly, Ito and colleagues demonstrated corticosteroid insensitivity in PBMCs, whereas we explored corticosteroid sensitivity in monocytes. Thirdly, a multitude of pro-inflammatory mediators are involved in driving the pathology in COPD, besides IL-1 β , CXCL8 and IL-6 (Barnes, 2008). It may be that corticosteroid suppression of other pro-inflammatory mediators may be reduced in our patient groups. This can be assessed by the use of gene expression arrays or deep sequencing of RNA samples obtained from our patients. In addition, the use of different inflammatory stimuli may result in differences in corticosteroid sensitivity. For example, Mercado and colleagues showed that TNF- α -induced CXCL8 was significantly less sensitive to dexamethasone in PBMCs of patients with severe asthma than healthy controls (Mercado et al., 2012).

In mild-to-moderate asthmatics, low doses of inhaled corticosteroids in combination with LABA have been shown to be more effective in suppressing pro-inflammatory mediators than high dose corticosteroids alone (Woolcock et al., 1996, Usmani et al., 2005b, Eickelberg et al., 1999). In U937 monocytes, Rossios and colleagues showed that the LABA FORM, but not SLM, was able to reverse corticosteroid insensitivity, via inhibition of the PI3K pathway, suggesting FORM may be able to partially reverse corticosteroid insensitivity in COPD (Rossios et al., 2012). We observed that BUD (10^{-11} M) in combination with FORM (10^{-9} M) suppressed CXCL8 to a similar extent as a higher concentration of BUD (10^{-9} M) alone in healthy volunteers (Fig. 6.3.6.1a). Similar results were observed for the combination of FF with VI (Fig. 6.3.6.2a). However, in COPD patients, both FORM and VI were unable to enhance BUD- and FF-mediated suppression of CXCL8 (Fig. 6.3.6.1b & Fig. 6.3.6.2b). This is inconsistent with the data reported by Rossios and colleagues, where FORM was able to restore dexamethasone sensitivity in PBMCs from COPD patients (Rossios et al., 2012). In this study, Rossios and colleagues used TNF- α to stimulate CXCL8 release, whereas we used IL-1 β . At the molecular level, differences in the signalling pathways activated by these two inflammatory mediators may explain our results (Nee et al., 2004). Therefore, examination of the gene profiles and signalling pathways between these two stimuli may indicate key relative corticosteroid resistant targets. In subsequent studies using an *in vitro* model of H₂O₂-induced corticosteroid insensitivity, Rossios and colleagues noted that FORM was able to partially inhibit PI3K- δ -dependent Akt phosphorylation, whereas SLM had no effect. Pre-treatment with IC87114, a PI3K δ inhibitor, restored the complementary effect of SLM on the corticosteroid (Rossios et al., 2012).

Finally, the inability of BUD and FF to induce GR-GRE binding in patients with severe asthma and the failure of BUD at a low concentration to suppress CXCL8 in COPD patients support our findings in our *in vitro* model of oxidative stress-induced corticosteroid insensitivity (Chapter 4). Some aspects of corticosteroid insensitivity observed in our *in vitro* model, not seen in blood monocytes, better reflect patterns of corticosteroid insensitivity observed in monocytes/macrophages obtained from the airways of patients with severe asthma and COPD (Culpitt et al., 2003). In Chapter 5, we were able to demonstrate that reduced GR nuclear import, under conditions of oxidative stress and heightened inflammatory response, was associated with degradation of nuclear RanGTP, an essential co-factor for importin-7 mediated GR nuclear import. This does suggest that the inability of BUD and FF to induce GR-GRE binding in patients with severe asthma may be due to impairment of pathways that regulate GR nuclear import. It would be essential to characterise the role of the importin-7 pathway in these patient samples, as a potential novel mechanism of corticosteroid insensitivity.

CHAPTER 7

GENERAL DISCUSSION & FUTURE DIRECTIONS

General Discussion

Corticosteroids are widely used in the treatment of chronic inflammatory diseases. However in COPD, cystic fibrosis and severe asthma, corticosteroids fail to improve the underlying inflammation (Suissa and Barnes, 2009, Vestbo et al., 1999, Balfour-Lynn and Welch, 2009, Davies and Richeldi, 2002). Recent advances in the cellular and molecular basis of pathology in asthma and COPD have revealed several biological targets that may contribute to the relative corticosteroid insensitivity observed in the disorders severe asthma and COPD (Barnes, 2008). These targets include impaired GR nuclear translocation, reduced HDAC-2 activity and heightened pro-inflammatory transcription factor activity.

Although *in vitro* and *in vivo* data support a role for impaired GR nuclear translocation as a potential mechanism of corticosteroid insensitivity in asthma (Irusen et al., 2002, Mercado et al., 2012, Okamoto et al., 1999), as of yet, no published data has confirmed this in COPD patients. Furthermore, the mechanisms that govern GR nuclear translocation are mostly unknown. Subsequently, not much is known about the mechanisms under conditions of corticosteroid insensitivity. There is growing evidence that excess reactive oxygen species (ROS) generated by inflammatory and immune cells of the lung leads to oxidative stress (Kirkham and Rahman, 2006), which plays a crucial role in the amplification of redox-sensitive pro-

inflammatory transcription factors that leads to corticosteroid insensitivity (Lee and Yang, 2012, Barnes, 2008). In this Discussion Chapter, I summarise my novel findings concerning the role of GR nuclear translocation under conditions of oxidative stress associated with a heightened inflammatory response.

In Chapter 3, we demonstrated that fluticasone furoate (FF) suppressed IL-1 β -induced CXCL8 to a greater extent than budesonide (BUD) in U937 monocytes (Fig. 3.3.2.1). This was associated with a greater induction of GR nuclear translocation by FF compared to BUD (section 3.3.1). There were no observed differences between FF and fluticasone propionate (FP). The combination of BUD and formoterol (FORM) and, FF and vilanterol trifenate (VI) caused a greater reduction in CXCL8 release than each corticosteroid alone (Fig. 3.3.2.2). There was no difference in CXCL8 suppression between the combination of FP and salmeterol (SLM) and FP alone. In the 20h corticosteroid washout experiments, FF maintained its ability to induce GR nuclear localisation and to suppress IL-1 β -induced CXCL8, whereas BUD and FP were unable to maintain these outputs (section 3.3.4). These differences between the corticosteroids may be partly explained by FF's greater affinity for the GR (Valotis and Hogger, 2007, Valotis et al., 2004).

In Chapter 4, we demonstrated that hydrogen peroxide (H₂O₂) caused a significant increase in intracellular and mitochondrial oxidative stress (section 4.3.1). Specifically, the significant increase in mitochondrial superoxide anion levels in our U937 model of oxidative stress mirrored data that has been shown by other investigators *ex vivo* in diaphragmatic muscle biopsies from COPD patients, where

superoxide anion levels in the mitochondria and membrane compartments was significantly greater than in healthy control subjects (Marin-Corral et al., 2009). We also observed, H₂O₂ significantly enhanced IL-1 β -induced NF- κ B activity (Fig. 4.3.4.2) and CXCL8 secretion (Fig. 4.3.4.1), key inflammatory markers that have been observed to be elevated in COPD (Barnes, 2008). This provides further experimental evidence of the relevance of our oxidative stress-driven inflammatory model as an *in vitro* model of COPD.

Subsequently, we showed that H₂O₂ significantly reduced BUD- and FP-mediated suppression of IL-1 β -induced CXCL8, consistent with data reported in peripheral blood mononuclear cells (PBMCs) of COPD patients and severe asthmatics, and in sputum macrophages of COPD patients (Mercado et al., 2012, To et al., 2010, Keatings et al., 1997). In Chapter 4, we were also able to demonstrate that oxidative stress-induced corticosteroid insensitivity was associated with impaired GR nuclear import (Fig. 4.3.6). This was consistent with experimental data reported in PBMCs of corticosteroid-insensitive asthmatics, where ligand-binding to GR was severely reduced (Irusen et al., 2002). In addition, we found FF-mediated suppression of CXCL8 and FF-induced GR nuclear localisation was resistant to the effects of H₂O₂. This highlights the relative nature of this corticosteroid insensitivity and raises the potential that novel corticosteroids may be able to reverse this effect (Crim et al., 2001, Valotis and Hogger, 2007, Rossios et al., 2012). Greater occupation of the lipophilic 17- α alpha pocket of the GR by FF over FP may contribute to FF's resistance to oxidative stress (Biggadike et al., 2008).

In Chapter 5, we demonstrated that members of the importin (section 5.3.2) and exportin (section 5.3.6) family of nuclear transport receptors are essential in the regulation of GR nucleocytoplasmic shuttling which is necessary for corticosteroid function. Specifically, for the first time in the literature we demonstrated that importin-7 knockdown significantly reduced FP-mediated suppression of IL-1 β -induced CXCL8 and impaired FP-induced GR nuclear localisation. In parallel, we were also able to see that leptomycin B (LMB), a potent inhibitor of exportin-1, enhanced BUD- and FF-induced GR nuclear localisation at 2h, although, it was unable to reverse the loss of BUD-induced GR nuclear localisation following a 20h washout. It is likely that different mechanisms regulate GR nucleocytoplasmic shuttling in the presence and absence of the ligand, and that they are time-dependent. Savory and colleagues showed that rapid nuclear import of GR (4 to 6 minutes) was mediated through the NL1 fragment of GR, whereas slow GR nuclear import (45 to 60 min) was NL2-mediated (Savory et al., 1999).

We also showed in subsequent experiments in U937 monocytes that nuclear RanGTP, an essential co-factor for importin-7 mediated nuclear import of cargo proteins, was significantly degraded in our *in vitro* model of oxidative stress-induced corticosteroid insensitivity (Fig. 5.3.5). These findings support the notion that impaired corticosteroid-induced GR nuclear import leads to corticosteroid insensitivity, which may be partly due to degradation of the importin-7 pathway. Furthermore, these findings also suggest that the importin-7 pathway, in particular nuclear RanGTP, is potentially a novel therapeutic target for restoring corticosteroid insensitivity.

In Chapter 6, we assessed corticosteroid insensitivity in blood monocytes from severe asthmatics and mild-to-moderate COPD patients, compared to healthy volunteers. In both asthmatics and COPD patients, we were unable to detect any significant difference in IC_{50} values for BUD- and FF-mediated suppression of CXCL8 (Fig. 6.3.4.2.3) and IL-6 (Fig. 3.5.3), compared to their healthy counterparts. In addition, we observed no difference in BUD or FF-induced GR-GRE binding between all three groups. However, BUD was unable to suppress CXCL8 at a low concentration in COPD patients, compared to healthy volunteers, suggesting relative corticosteroid insensitivity. Previously, it has been reported that at the cellular level, macrophages, T-cells, epithelial cells and PBMCs obtained from severe asthmatics and COPD patients are corticosteroid insensitive (Keatings et al., 1997, Rossios et al., 2012, To et al., 2010, Mercado et al., 2012). In the process of extraction of monocytes from PBMCs, we may have lost vital cellular components in PBMCs that give rise to corticosteroid insensitivity, which may explain why we did not observe a significant loss in anti-inflammatory effects of corticosteroids in our patients although there were some changes in the magnitude of the effects seen.

Secondly, the patients enrolled in our study had milder airway obstruction compared to those enrolled by Keatings and colleagues (67 vs. 35 mean FEV_1 (% pred.)) (Keatings et al., 1997). Data reported from Ito and colleagues suggests that severity of disease correlates to the extent of corticosteroid insensitivity (To et al., 2010). Corticosteroids downregulate a multitude of pro-inflammatory mediators and therefore it would be important to assess corticosteroid suppression of other pro-inflammatory mediators, in addition to CXCL8 and IL-6. Furthermore, Mercado and

colleagues used TNF- α as the inflammatory stimuli, whereas in our study we used IL-1 β (Mercado et al., 2012). Variations in the signaling pathways activated by TNF- α and IL-1 β may also contribute to differences in corticosteroid sensitivity (Nee et al., 2004). Nee and colleagues demonstrated that the differential effects of TNF- α and IL-1 β on matrix metalloproteinase-9 (MMP-9) are mediated through the TNF-receptor I (TNF-RI) and IL-1-receptor (IL-1-R) and the various signaling pathways PKC, extracellular signal-regulated protein kinase (ERK1/2) and p38MAPK.

Finally, the number of severe asthmatic patients we recruited was fewer than the number of subjects recruited by Mercado and colleagues. This may also have contributed to our inability to detect statistical significance in corticosteroid-mediated suppression of pro-inflammatory mediators (Mercado et al., 2012). Surprisingly, although we observed no significant reduction in BUD- or FF-mediated suppression of CXCL8 or IL-6 in blood monocytes from severe asthmatics, compared to healthy volunteers, both corticosteroids were unable to induce GR-GRE binding. This loss in GR-GRE binding is consistent with findings in PBMCs of severe asthmatics, where dexamethasone-induced GR nuclear translocation, was severely reduced (Irusen et al., 2002).

In summary, I have shown that importin-7 is important in ligand-mediated GR nuclear import and that this process is affected by oxidative stress in a cell line. Modulation of this mechanism may affect GR function in cells from patients with severe asthma and more severe COPD (Fig 7.1). Confirmation of this effect in patient samples from the site of disease is essential.

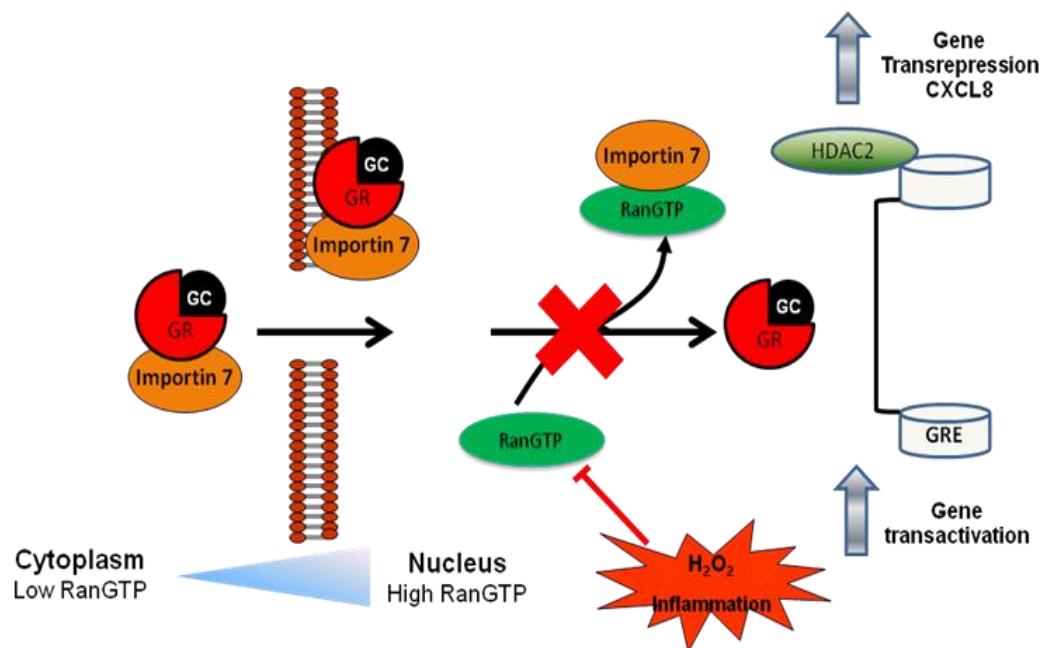


Fig. 7.1 Potential role of importin-7-RanGTP complex in oxidative stress-induced corticosteroid insensitivity. Corticosteroid-induced GR nuclear translocation is essential for corticosteroid-mediated of pro-inflammatory mediators. Importin-7 binds to and mediates nuclear translocation of the glucocorticoid receptor (GR). Once in the nucleus, nuclear RanGTP binds to importin-7 resulting in the dissociation of the importin-7 and GR complex. Consequently, the GR is able to move through the nucleus and either binds to glucocorticoid response elements on corticosteroid-inducible genes or recruits histone deacetylase (HDAC)-2 to prevent the gene transcription of pro-inflammatory mediators. Under conditions of oxidative stress, nuclear RanGTP is reduced, which consequently prevents the dissociation of importin-7 and GR leading to corticosteroid insensitivity.

Future directions

We need to show our novel findings concerning the role of importin-7 in our *in vitro* model can also be observed in primary cells and *in vivo* in airway or blood cells from patients with severe asthma and COPD. Several members of the nuclear importin family, besides importin-7, have been shown to interact and mediate GR nuclear translocation, using GR purified from rat liver and S-methionine-labelled fragments of GR in reticulocyte lysate (Freedman and Yamamoto, 2004, Vandevyver et al., 2012). It will be important to identify the role of other nuclear importin receptors in GR nucleocytoplasmic shuttling. More importantly, nuclear RanGTP levels need to be quantified in corticosteroid insensitive and sensitive individuals to determine if there is a difference in these states so as to further validate this biological entity as a potential novel therapeutic target to reverse corticosteroid insensitivity.

Indeed, nuclear RanGTP is maintained by 2 enzymes, cytoplasmic RanGTPases-activating protein (RanGAP), which hydrolyses RanGTP to RanGDP, and the nuclear RanGDP/GTP-activating protein (RANGEF), which hydrolyses RanGDP to RanGTP (Poon and Jans, 2005, Wagstaff and Jans, 2009). It will be important to determine whether reduced nuclear RanGTP levels seen under conditions of oxidative stress is due to an imbalance of these enzymes. A combination of *in vitro* enzymatic assays along with siRNA-mediated knockdown or overexpression of each enzyme may more closely define a role for these enzymes in GR nuclear import. In addition, immunoprecipitation assays and site-directed mutagenesis of GR and importin-7 may identify key interaction sites between these two proteins to allow better understanding of the mechanisms of GR nuclear import. The use of SILAC

analysis may also determine whether other proteins are involved in GR nuclear import. Genome-wide analysis using gene arrays or deep sequencing of the effect of ROS-stress on GR function would also be of interest and could be linked to alterations in GR function in primary monocytes from patients with COPD or severe asthma.

As we were unable to show corticosteroid insensitivity in blood monocytes from severe asthmatics and COPD patients, it will be necessary to extract PBMCs and alveolar macrophages which have been reported to be corticosteroid insensitive, and determine whether this loss in corticosteroid insensitivity is associated with impaired GR nuclear translocation. Indeed, recruiting further numbers of severe asthmatic patients may validate our initial results in blood monocytes and enable us to apply statistical analyses. We measured GR-GRE binding in this study as fewer cells are required for quantification. However measurement of nuclear GR levels, which directly impact on downregulation of pro-inflammatory genes by corticosteroids, will be useful as well as a readout of GR-transactivation such as *DUSP1* or *GILZ* expression.

Use of gene expression arrays or deep sequencing may be applied to RNA samples obtained from monocytes isolated in this study under the various conditions examined. Statements regarding corticosteroid function on the expression of 1 gene may not hold true for a host of other genes. It will be important to establish a genome-wide measure of Bud and FF function in these cells regarding both transactivation and transrepression functions.

There is much interest in the role of epigenetic control of GR function and in particular GR interaction with the DNA-chromatin backbone. Genome-wide analysis of GR-nucleosome interaction using ChIP-seq analysis in ROS-treated cells or primary cells from patients with disease may further elucidate mechanisms important for ROS-mediated changes in GR function.

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Abstracts

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