The role of GSK3β in regulating corticosteroid function under conditions of oxidative stress in chronic obstructive pulmonary disease
Declaration

This thesis is the result of my own work. DNA plasmids prepared by others and the microarray experiments have been appropriately acknowledged in the text. The work was carried out at the National Heart and Lung Institute, Airway Disease Section, Imperial College, Faculty of Medicine, London.
Abstract

Chronic obstructive pulmonary disease (COPD) is a progressive chronic inflammatory lung disease characterised by insensitivity to the anti-inflammatory actions of corticosteroids. Reactive oxygen species deriving from cigarette smoke or other environmental noxious particles or gases are a critical etiologic factor in the pathogenesis of COPD and contribute to the reduced corticosteroid action. In the lungs of COPD patients the increased levels of reactive oxygen species induce activation of the Phosphoinositide 3-kinase (PI3K)/Akt and mitogen activated protein kinase (MAPK) pathways which are known to be involved in regulating inflammatory responses as well as corticosteroid insensitivity. Members of these pathways have been implicated in phosphorylating glycogen synthase kinase (GSK3β) on serine 9 leading to inhibition of its activity. GSK3β is a constitutively active serine/threonine kinase that regulates a number of cellular processes, including inflammatory responses, cell cycle and metabolism. In this thesis I investigated whether GSK3β is modulated in primary peripheral macrophages and peripheral blood monocytes from COPD patients and/or under exposure to oxidative stress and how this modulation affects corticosteroid function.

The results obtained showed that the levels of p-GSK3β-Ser9 are increased in peripheral lung macrophages and peripheral blood monocytes from COPD patients compared to smokers with normal lung function and non-smokers. This is mediated by increased levels of reactive oxygen species as in response to H₂O₂-derived oxidative stress, Akt, p38 MAPK and extracellular signal regulated kinase (ERK) 1/2 MAPK phosphorylate and inactivate GSK3β in a time- and concentration-dependent manner. Inhibition of GSK3β by treatment with the specific inhibitor CT99021, gene silencing using siRNA or overexpression of a kinase dead mutant reduced the anti-inflammatory effects of corticosteroids in monocytes and led to
increased pro-inflammatory cytokine release upon LPS (Lipopolysaccharide) stimulation. In addition, overexpression of a constitutively active mutant GSK3β with a serine 9 to alanine mutation reversed H$_2$O$_2$-induced reduction of corticosteroid function.

GSK3β-regulated corticosteroid function under exposure to oxidative stress does not involve impaired glucocorticoid receptor (GR) nuclear translocation, GR binding to (nuclear factor kappa B) NF-κB/p65 or p65 DNA binding capacity. By contrast, my data showed that GSK3β regulates the key co-repressor recruited by GR, histone deacetylase 2 (HDAC2). Inhibition of GSK3β led to reduction of HDAC2 activity which correlated with an increased phosphorylation on its serine 394 residue. This finding suggests that GSK3β regulates corticosteroid function by inhibiting histone deacetylation leading to chromatin condensation at the site of NF-κB-mediated transactivation of inflammatory gene expression.

Analysis of the effects of CT99021 on global gene expression using microarrays confirmed that GSK3β is a key regulator of corticosteroid-sensitive inflammatory gene expression. Among the genes that were differentially expressed in response to CT99021, protein kinase A (PKA) was upregulated suggesting that it may play a significant role in mediating GSK3β-dependent corticosteroid function in monocytes.

My findings suggest that there are multiple parallel mechanisms that mediate the oxidative stress-induced corticosteroid unresponsiveness and GSK3β is a key member of the redox-sensitive signalling pathways in COPD. Identification of the downstream targets of GSK3β that mediate phosphorylation and inhibition of HDAC2 activity will assist identification of novel key mediators with potential for therapeutic targeting.
Acknowledgments

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I dedicate this thesis to my mum, my dad and my brother Dimitri, for the love and understanding….for always being there for me and supporting all my steps and decisions…
Publications


# Table of Contents

*Abstract* ................................................................................................................................. 3

*Publications* ............................................................................................................................ 6

*Chapter I* .................................................................................................................................. 18

*Introduction* ............................................................................................................................. 18

1.1. Chronic obstructive pulmonary disease (COPD) ................................................................. 19

1.1.1. Definition ......................................................................................................................... 19

1.1.2. Prevalence ....................................................................................................................... 20

1.1.3. Pathophysiology ............................................................................................................ 20

1.2. Inflammation and immunity ............................................................................................... 21

1.2.1. The normal inflammatory response ................................................................................. 21

1.2.2. Inflammation in COPD .................................................................................................. 22

1.2.3. The role of macrophages in driving inflammation in COPD .......................................... 22

1.3. Reactive oxygen species .................................................................................................... 26

1.3.1. Reactive oxygen species and their role in COPD pathogenesis .................................... 27

1.4.1.1. The MAPK family: members and function ................................................................. 29

1.4.1.2. Redox regulation of the MAPKs' activity ..................................................................... 30

1.4.2. The phosphatidylinositol 3-kinase (PI3K) signalling pathway ................................... 33

1.4.2.1. Activation of the PI3K signalling pathway ................................................................. 33

1.4.2.2. Classes and structure of the PI3Ks ............................................................................. 33

1.4.2.3. Akt: structure and function ....................................................................................... 35

1.4.2.4. Regulation of Akt activation ....................................................................................... 37

1.4.2.5. Redox regulation of the PI3K/Akt signalling pathway ............................................. 37

1.4.3. The Glycogen Synthase Kinase 3 (GSK3) ...................................................................... 39

1.4.3.1. Structure of GSK3 ....................................................................................................... 40

1.4.3.2. Substrate recognition by GSK3 ................................................................................ 41

1.4.3.3. Regulation of GSK3 activity ....................................................................................... 42

1.4.3.4. The role of GSK38 in regulating the Wnt/β-catenin signalling pathway ................... 46

1.4.3.5. GSK3β and its role in regulating inflammation ......................................................... 49

1.5. Signalling to the nuclear factor kappa B (NF-κB) ............................................................... 52

1.5.1. NF-κB: structure and function ....................................................................................... 52

1.6. The role of histone acetylation on driving gene transcription ........................................... 54

1.6.1. Histone deacetylases (HDACs): role and classes ............................................................ 56

1.6.2. HDACs regulate NF-κB activity in COPD .................................................................... 57
1.7. The anti-inflammatory role of corticosteroids ................................................................. 59
  1.7.1. The glucocorticoid receptor (GR): structure and isoforms ........................................ 59
  1.7.2. Ligand binding–dependent GR activation ..................................................................... 60
  1.7.3. Corticosteroids: mechanism of action .......................................................................... 61
1.8. Corticosteroid insensitivity in COPD .................................................................................. 62
  1.8.1. Molecular mechanisms of corticosteroid insensitivity in COPD ..................................... 63
    1.8.1.1. Regulation of GRβ activity ....................................................................................... 63
    1.8.1.2. Genetic variation ...................................................................................................... 64
    1.8.1.3. Oxidative stress and corticosteroid responsiveness ................................................... 64
      1.8.1.3.1. The role of HDAC2 in oxidant-induced corticosteroid insensitivity in COPD .......... 65
      1.8.1.3.2. The role of kinase signalling pathways in regulating corticosteroid insensitivity in COPD ..................................................................................................................... 67
1.9. Rationale for the study ......................................................................................................... 70
  1.9.1. Hypothesis ..................................................................................................................... 71
  1.9.2. Aims ................................................................................................................................ 71

Chapter II ................................................................................................................................... 72

2.1. Materials ................................................................................................................................ 73
  2.1.6. Transient transfections of MonoMac 6 cells ................................................................. 76
2.2. Methods ................................................................................................................................ 79
  2.2.1. Human studies ................................................................................................................. 79
    2.2.1.1. Peripheral lung sections ............................................................................................. 79
    2.2.2. Peripheral blood monocytes .......................................................................................... 79
    2.2.1.2. Lung tissue processing and immunohistochemistry ..................................................... 80
    2.2.1.3. Isolation of peripheral blood mononuclear cells (PBMCs) .......................................... 80
    2.2.1.4. Monocyte isolation from PBMC population using MACS® Cell Separation ............... 80
    2.2.1.5. Cell culture and treatment of monocytes ...................................................................... 81
    2.2.2. Culture and treatments of the human monocytic cell line MonoMac6 ....................... 82
    2.2.2.1. Cell culture and treatment of MonoMac6 ................................................................. 82
    2.2.2.2. Transient transfections of MonoMac6 cells ............................................................... 82
    2.2.2.2.1. Transfection with GSK3β siRNA .............................................................................. 82
    2.2.2.2.2. Transfection with plasmid DNA ............................................................................. 83
    2.2.3. Cell viability assay ......................................................................................................... 83
    2.2.4. Measurement of endotoxin levels in plasmid DNA constructs ..................................... 84
    2.2.5. Purification of DNA plasmid constructs using ethanol precipitation ......................... 85
2.2.7. Determination of protein expression by Western blotting ........................................... 88

Chapter III ................................................................................................................................ 100

3.1. Introduction .............................................................................................................................. 101

3.2. Results ..................................................................................................................................... 104

3.2.1. Phosphorylation of GSK3β on Ser9 is increased in peripheral lung macrophages and monocytes in patients with COPD compared to healthy subjects ............................................ 104

3.2.2. Inactivation of GSK3β suppresses dexamethasone anti-inflammatory function in human primary monocytes .................................................................................................................. 108

3.2.2.1. Inhibition of GSK3β activity by treatment with CT99021 had no effect on cell viability and basal inflammatory cytokine release .................................................................................. 108

3.2.2.2. Treatment of monocytes with CT99021 had no effect on LPS-induced cytokine release .......................................................................................................................... 109

3.2.2.3. Treatment of monocytes with CT99021 reduces dexamethasone function in a concentration-dependent manner ........................................................................................................... 112

3.2.3. Inactivation of GSK3β suppresses dexamethasone function in the human monocytic cell line MonoMac6 ......................................................................................................................... 115

3.2.3.1. The anti-inflammatory function of dexamethasone in MonoMac6 cells ....................... 115

3.2.3.2. Treatment of MonoMac6 with the GSK3β inhibitor CT99021 had no effect on cell viability or LPS-induced pro-inflammatory cytokine release .......................................................... 116

3.2.3.3. Treatment of MonoMac6 with the GSK3β inhibitor CT99021 decreased dexamethasone function in a concentration-dependent manner ............................................................... 116

3.2.3.4. Treatment of MonoMac6 with on-target GSK3β siRNA inhibited total levels of GSK3β in a concentration- and time-dependent manner ........................................................................ 119

3.2.3.5. Effect of transfection with GSK3β siRNA on MonoMac6 cell viability over time....... 120

3.2.3.6. GSK3β protein knock down had no effect on LPS-induced pro-inflammatory cytokine release in MonoMac6 cells ............................................................................................ 123

3.2.3.7. GSK3β protein knock down suppressed dexamethasone anti-inflammatory function in MonoMac6 cells .................................................................................................................. 126

3.2.4. Plasmid overexpression in MonoMac6 cells .............................................................................. 129

3.2.4.1. Transient transfection efficiency and effect on cell viability ...................................... 129

3.2.4.2. Efficiency of the overexpression of the mutant GSK3β mRNA in MonoMac6 cells following plasmid transfection ......................................................................................................... 129

3.2.4.2. Effect of transient plasmid overexpression on basal and LPS-induced pro-inflammatory cytokine release .................................................................................................................. 133

3.2.4.3. Measurement of endotoxin levels of the plasmid constructs ........................................ 135

3.2.4.4. Inhibition of LPS did not reverse plasmid DNA induced pro-inflammatory response in MonoMac6 cells ................................................................................................................. 136
3.2.4.5. Transfection of MonoMac6 cells with other plasmid DNA construct induces a pro-inflammatory response ................................................................. 138

3.2.4.5. Effect of mutant GSK3β overexpression on dexamethasone function in MonoMac6 cells ........................................................................................................ 139

3.2.4.5.1. Inactive K85R mutant GSK3β overexpression suppresses the anti-inflammatory effect of dexamethasone ......................................................... 140

3.2.4.5.2. Exposure of MonoMac6 cells to H₂O₂ inhibits dexamethasone-induced suppression of LPS-stimulated GM-CSF release ................................................................. 141

3.2.4.5.3. H₂O₂-induced inhibition of dexamethasone function is regulated by Ser9 on GSK3β: overexpression of the S9A mutant kinase restored dexamethasone function ............... 143

3.3.1. The inhibitory Ser9 phosphorylation levels of GSK3β are increased in lung macrophages and blood monocytes from patients with COPD and smokers compared to non-smokers ... 145

3.3.2. Role of GSK3β inactivation on inflammation .......................................................... 146

3.3.3. Role of GSK3β inactivation on corticosteroid anti-inflammatory function .............. 148

Chapter IV .......................................................................................................................... 154

The mechanism of GSK3β-regulated corticosteroid function in response to oxidative stress ...... 154

4.1. Introduction .................................................................................................................. 155

4.2. Results .......................................................................................................................... 158

4.2.1. Mechanism of oxidative stress induced GSK3β inactivation .................................................. 158

4.2.1.1. Effect of H₂O₂ exposure on monocyte viability ............................................................ 158

4.2.1.2. Exposure to H₂O₂-induced Ser9 phosphorylation on GSK3β in a time-and concentration-dependent manner ................................................................. 158

4.2.1.3. Effect of H₂O₂ treatment on kinase phosphorylation over time ...................................... 160

4.2.1.4. Exposure to H₂O₂-induced Ser9 phosphorylation on GSK3β in an ERK1/2-, Akt-, and p38 MAPK-dependent pathway in monocytes derived from PBMCs .................................................. 164

4.1.5. Exposure to H₂O₂ induced activation of ERK1/2 is not mediated by Akt or PI3Kδ in human monocytes .......................................................................................... 166

4.2.2. Investigation of the effects of the CT99021 compound and H₂O₂ on kinase activity .... 168

4.2.3. Mechanism of GSK3β induced impairment of glucocorticoid function ...................... 170

4.2.3.1. Effect of GSK3β knock down on GR expression ........................................................ 170

4.2.3.2. Effect of GSK3β inactivation on GR nuclear translocation ........................................ 170

4.2.3.3. Effect of GSK3β inactivation on association of GR with NF-κB/p65 ............................ 173

4.2.3.4. NF-κB p65 DNA binding capacity is not regulated by GSK3β kinase activity ............ 175

4.2.3.5. Effect of GSK3β inhibition on HDAC2 mRNA and protein expression ...................... 176

4.2.3.6. Effect of GSK3β inhibition on HDAC2 activity ........................................................ 177

4.2.3.7. Inhibition of GSK3β led to post-translational modifications on HDAC2 .................... 177
4.3. Discussion ............................................................................................................................................. 181

4.3.1. Mechanism of H$_2$O$_2$ induced GSK3β inactivation .............................................................................. 181

4.3.1.1. Exposure of monocytes to H$_2$O$_2$ leads to activation of PI3K and ERK1/2 MAPK and inactivation of GSK3β in a time-dependent manner ......................................................................................... 181

4.3.1.2. H$_2$O$_2$ induced inactivation of GSK3β is mediated by p38 MAPK, ERK1/2 MAPK and Akt in primary monocytes .............................................................................................................................. 182

4.3.2. Evaluation of the CT99021 specificity against a range of kinases ......................................................... 184

4.3.3. Mechanism of GSK3β regulated corticosteroid insensitivity in monocytes .......................................... 185

Chapter V ..................................................................................................................................................... 190

5.1. Introduction .............................................................................................................................................. 191

5.2. Methods .................................................................................................................................................. 192

5.2.1. Culture treatment of MonoMac6 cells and sample preparation ............................................................. 192

5.2.2. Agilent microarrays ............................................................................................................................ 192

5.2.3. Data analysis ....................................................................................................................................... 192

5.3. Results ................................................................................................................................................... 194

5.3.1. CT99021 regulates the Wnt signalling pathway via up-regulation of FRAT1 and FRAT2 gene expression .................................................................................................................................................. 194

5.3.2. Effect of CT99021 on regulation of inflammatory gene expression and the pathways regulated ................................................................................................................................................... 194

5.3.3. Confirmation of the effect of CT99021 on dexamethasone-induced inhibition of IL-8 and CSF2 gene expression in LPS-stimulated MonoMac6 cells ............................................................................. 200

5.4. Discussion .............................................................................................................................................. 203

5.4.1. Inhibition of GSK3β activity affects the activation of the Wnt signalling pathway via up-regulation of FRAT expression .......................................................................................................................... 203

5.4.2. Effect of CT99021 on regulation of inflammatory pathways ..................................................................... 204

Chapter VI ....................................................................................................................................................... 208

6.1. Rationale of the study .............................................................................................................................. 209

6.2. Summary of the study findings .............................................................................................................. 210

6.3. Discussion ............................................................................................................................................ 212

6.4. Future directions .................................................................................................................................... 217

7. References .................................................................................................................................................. 217
List of Figures

1.1 Macrophages orchestrate the chronic inflammation in COPD 25
1.2 The mechanisms of reactive oxygen species-induced activation of the MAPK signalling pathway 32
1.3 Activation of the PI3K/Akt signalling pathway 38
1.4 Schematic representation of the mammalian GSK3α and GSK3β isoforms 41
1.5 Regulation of GSK3β activity by serine phosphorylation 44
1.6 Regulation of GSK3β activity by phosphorylation 45
1.7 The role of GSK3β in the Wnt/β-catenin signalling pathway 48
1.8 Chromatin remodelling-controlled gene expression 57
1.9 Mechanism of corticosteroid insensitivity in COPD 69
2.1 Diagram of a basic ImmunoPrecipitation procedure 97
3.1 p-GSK3β-s9/total GSK3β is increased in COPD peripheral lung macrophages compared to smokers with normal lung function and non-smokers 106
3.2 p-GSK3β-s9/total GSK3β is increased in COPD peripheral blood monocytes compared to smokers with normal lung function and non-smokers 107
3.3 The effects of inhibition of GSK3β by treatment with CT99021 on cell viability and basal GM-CSF levels in primary human monocytes 110
3.4 The effect of inhibition of GSK3β by CT99021 on LPS-induced GM-CSF levels released in primary human monocytes 111
3.5 CT99021 reduces dexamethasone (10^-9M) anti-inflammatory function in a concentration-dependent manner 113
3.6 CT99021 inhibits dexamethasone (10^-6M) anti-inflammatory function in human peripheral blood monocytes 114
3.7 Dexamethasone induced a concentration-dependent inhibition of pro-inflammatory cytokine release 115
3.8 Effect of inhibition of GSK3β by CT99021 on cell viability and on LPS-induced CXCL8 levels released in the monocytic cell line MonoMac6 117
3.9 Inhibition of GSK3β by CT99021 suppresses dexamethasone function in human MonoMac6 cells 118
3.10 On-target GSK3β siRNA reduced total GSK3β protein levels in a time- and concentration-dependent manner 121
3.11 Electroporation affected MonoMac6 cell viability but addition of siRNAs had no impact on cell death 122
3.12 Reduction of GSK3β protein levels following 24 hours of siRNA transfection had no effect on LPS-induced pro-inflammatory cytokine release 124
3.13 Reduction of GSK3β protein levels following 48 hours of siRNA transfection had no effect on LPS-induced pro-inflammatory cytokine release 125
3.14 Inactivation of GSK3β by siRNA transfection for 24 hours reduced dexamethasone anti-inflammatory function in LPS-stimulated MonoMac6 cells 127
3.15 Inactivation of GSK3β by siRNA transfection for 48 hours had no overall significant effect on dexamethasone anti-inflammatory function in LPS-stimulated MonoMAC6 cells

3.16 Transfection efficiency was measured by levels of GFP fluorescence emitted under UV light

3.17 Effect of transient transfection with pcDNA3.1 on cell viability

3.18 Overexpression of the mutant GSK3β constructs measured by RT-PCR

3.19 Transfection of MonoMAC6 cells with plasmid DNA increases both basal and LPS-induced GM-CSF levels of release

3.20 Endotoxin levels of the plasmid constructs

3.21 Effect of plasmid transfection on basal and LPS-induced GM-CSF release following plasmid endotoxin removal in MonoMAC6 cells

3.22 Inhibition of LPS by Polymyxin B did not reverse the pro-inflammatory effect of plasmid transfection in MonoMAC6 cells

3.23 Transfection of MonoMAC6 cells with a different plasmid DNA construct induced Polymyxin B-independent release of GM-CSF release

3.24 Overexpression of the kinase dead K85R GSK3β mutant suppresses dexamethasone function in LPS-stimulated MonoMAC6 cells

3.25 H₂O₂-induced suppression of dexamethasone anti-inflammatory function in MonoMAC6 cells

3.26 H₂O₂-induced suppression of dexamethasone function is reversed in cells expressing the S9A mutant GSK3β kinase

4.1 High concentrations of H₂O₂ induced reduction of cells viability in primary monocytes

4.2 H₂O₂ induced phosphorylation of GSK3β (Ser9) in a concentration-dependent manner

4.3 H₂O₂ exposure leads to Akt phosphorylation in primary monocytes

4.4 H₂O₂ exposure leads to ERK1/2 activation in primary monocytes

4.5 H₂O₂ exposure leads to GSK3β inactivation in primary monocytes in a time-dependent manner

4.6 H₂O₂ exposure inhibits GSK3β in peripheral blood monocytes via activation of the Akt, ERK1/2 and p38 MAPK kinases

4.7 H₂O₂-induced activation of ERK1/2 is not mediated by PI3K

4.8 Effect of H₂O₂ exposure and treatment with the CT99021 compound on kinase activation

4.9 GSK3β knock down results in reduction of total GRα protein levels in MonoMAC6 cells

4.10 GRα nuclear translocation is not affected by GSK3β inhibition

4.11 Levels of GRα bound to NF-κB/p65 are not affected by GSK3β inhibition

4.12 NF-κB/p65 DNA binding capacity is not affected by CT99021 in monocytes from PBMCs
4.13 Inactivation of GSK3β does not affect HDAC2 protein or mRNA expression in peripheral blood monocytes

4.14 Inactivation of GSK3β leads to reduction of HDAC2 enzymatic activity in primary monocytes

4.15 Inactivation of GSK3β leads to increase p-Ser394-HDAC2 levels in primary monocytes

5.1 Principal Component Analysis of microarray data

5.2 Diagrammatic illustration of the effect of CT99021 on regulating expression of the genes involved in regulating the Wnt/β-catenin pathway

5.3 Effect of CT99021 on dexamethasone-dependent suppression of LPS-induced inflammatory gene expression

5.4 Effect of CT99021 on expression of genes involved in regulating the chemokine signalling pathway

5.5 Effect of CT99021 on expression of genes that regulate the JAK/STAT signalling pathway

5.6 Effect of CT99021 on dexamethasone function on LPS-induced IL-8 mRNA expression

5.7 Effect of CT99021 on dexamethasone function on LPS-induced CSF2 mRNA expression

6.1 Mechanism of GSK3β-regulated corticosteroid function in the presence of reactive oxygen species in monocytes

List of Tables

2.1.1 Cell culture materials 73
2.1.2 Molecular biology reagents 73
2.1.3 Buffers and solutions 75
2.1.4 Kits and assays 76
2.1.5 Primers’ sequences used for Real-Time qPCR 76
2.1.6 Antibodies used for Western blotting 77
2.1.7 Compounds and stimuli 78
2.1.8 Equipment 78
2.2.1 Antibodies and conditions used for ELISA 94
3.1 Characteristics of subjects for peripheral lung sections 105
3.2 Characteristics of subjects for isolation of peripheral blood monocytes 105

Abbreviations

A Alanine
AC Adenylyl cyclase
ACD Acid citrate dextrose
AF-1 Activation function1
AKAP220  A kinase anchoring protein 220
AP-1  Activator protein 1
APC  Antigen presenting cell
APC  Adenomatous polyposis coli
AR  Androgen
Arg  Arginine
ASK  Apoptosis signal-regulating kinase
ATF-2  Activating transcription factor 2
BAD  Bcl-2 associated death promoter protein
BAL  Bronchoalveolar lavage
BAX  Bcl-2 associated X protein
Bcl  B cell lymphoma
cAMP  Cyclic adenosine monophosphate
CBP  CREB-binding protein
CCL  CC chemokine ligands
CDP  Cysteine-dependent phosphates
CK  Casein kinase
c-Myc  Avian myelocytomatosis virus oncogene cellular homolog
COPD  Chronic obstructive pulmonary disease
CREB  cAMP response element-binding protein
CRM1  Chromosome region maintenance
CXCR  C-X-C chemokine receptor
DBD  DNA binding domain
DLK  Dual-leucine-zipper-bearing kinase
DNA  Deoxyribonucleic acid
Dvl  Dishevelled
EGF  Epidermal growth factor
EGFR  Epidermal growth factor receptor
ER  Oestrogen
ERK  Extracellular signal-regulated kinase
FEV₁  Forced expiratory volume in one second of expiration
FRAT  Frequently rearranged in advanced T-cell lymphomas
FVC  Forced vital capacity
Fz1  Frizzled
GBP  GSK3β binding protein
GCPR  G coupled protein receptor
GILZ  Glucocorticoid-inducible leucine zipper
GLUT  Glucose transports
GM-CSF  Granulocyte-macrophage colony stimulating factor
GR  Glucocorticoid receptor
GSH  Glutathione
GSK3  Glycogen synthase kinase 3
GTP  Guanosine triphosphate
HAT  Histone acetyltransferase
HDAC  Histone deacetylase
HM  Hydrophobic motif
Hop  Hsp-organising protein
<table>
<thead>
<tr>
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<th>Full Name</th>
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<tbody>
<tr>
<td>Hsp</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IKK</td>
<td>Inhibitor of kappa B kinase</td>
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<tr>
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<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<tr>
<td>K</td>
<td>Lysine</td>
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<tr>
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<td>Lymphoid enhancer factor</td>
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<td>Monocyte chemotactic protein</td>
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<td>Murine double minute</td>
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<td>Mixed-lineage kinase</td>
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<td>Matrix metalloproteinase</td>
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<td>Manganese superoxide dismutase</td>
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<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
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<tr>
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<td>Myeloid differentiation protein 88</td>
</tr>
<tr>
<td>NADPH</td>
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<tr>
<td>NEMO</td>
<td>NF-κB essential modifier</td>
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<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
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<td>Nuclear localisation site</td>
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<td>Nuclear retention signal</td>
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<td>Polymyxin B</td>
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<td>PDK</td>
<td>Phospho-inosite-dependent kinase</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal Component Analysis</td>
</tr>
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<td>Pleckstrin homology</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
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<td>PKC</td>
<td>Protein kinase C</td>
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<tr>
<td>PP2A</td>
<td>Phosphatase 2A</td>
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<td>PR</td>
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<td>PRR</td>
<td>Pattern recognition receptors</td>
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<tr>
<td>Ptdln</td>
<td>Phosphatidylinositol</td>
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Chapter I

Introduction
**1.1. Chronic obstructive pulmonary disease (COPD)**

**1.1.1. Definition**

COPD is a lung disease characterised by abnormal chronic inflammatory response of the respiratory tract involving the peripheral airways and the lung parenchyma (Barnes 2000; Chung and Adcock 2008). The chronic inflammation in COPD is associated with remodelling and narrowing of the small airways, which results in airflow limitation and a gradual decline of the lung function which is progressive and not fully reversible (Hogg, Chu et al. 2004; Rabe, Hurd et al. 2007). COPD is clinically diagnosed by a post-bronchodilator forced expiratory volume in 1 second (FEV$_1$)/forced vital capacity (FVC) ratio of <70% and a FEV$_1$ of ≥80% (Mannino and Buist 2007; Rabe, Hurd et al. 2007). COPD is considered a preventable and treatable disease with some extrapulmonary effects that may contribute to its severity in individual patients (Rabe, Hurd et al. 2007; GOLD 2011). The inflammatory response in the lungs of COPD patients is a result of chronic exposure to noxious particles or gases. The main etiologic factor associated with COPD development is cigarette smoking (Mannino and Buist 2007; Chung and Adcock 2008) but other risk factors are also involved such as exposure to fossil fuels and environmental traffic pollution (Mannino and Buist 2007). The chronic inflammatory response in COPD persists even when the main exogenous driving factors are removed, contributing to the progression of the disease and lung function decline (Barnes 2000; Chung and Adcock 2008).
1.1.2. Prevalence

COPD is a leading cause of morbidity and mortality worldwide. The World Health Organisation (WHO) has estimated that 3 million people died from COPD in 2005 and 210 million people had diagnosed disease in 2007. COPD is the fifth most common cause of death and its mortality rates are expected to rise by 30% in the next decade. It has been now predicted that it will become the third leading cause of death worldwide by 2030 (World Health Statistics, W.H.O. 2008).

1.1.3. Pathophysiology

COPD is a complex disease characterised by three distinct disease processes that contribute to the airflow obstruction; chronic bronchitis, emphysema and small airways disease (Barnes 2000; Chung and Adcock 2008). The increased mucus production in chronic bronchitis and the reduction of mucociliary clearance documented in COPD patients contributes to bacterial infections and colonisation during the disease exacerbations (Sethi 2000; Patel, Seemungal et al. 2002). Emphysema is characterised by destruction of the lung parenchyma and enlargement of the alveoli and alveolar ducts distal to the terminal bronchioles resulting in loss of the lung elastic recoil thus contributing to airflow limitation (Kim, Eidelman et al. 1991). In addition, the degree of small airway narrowing correlates with the decline in lung function suggesting that it may be a predominant factor in the development of the airflow obstruction documented in COPD (Hogg, Chu et al. 2004).
1.2. Inflammation and immunity

1.2.1. The normal inflammatory response

Inflammation is the body’s response to infection or injury and plays a key role in both innate and adaptive immunity. In response to infection, the resident tissue cells, primarily epithelial cells, macrophages, dendritic cells, natural killer cells and mast cells, secrete cytokines, chemokines, lipid mediators and bioactive amines. These stimulate capillary permeability, local increase of blood flow and recruitment of neutrophils, blood circulating monocytes that differentiate into macrophages at the site of inflammation and dendritic cells. This acute inflammatory process involves the stimulation of several signalling pathways and pro-inflammatory mediators (Steinke and Borish 2006; Commins, Borish et al. 2010). The most well studied pro-inflammatory responses are initiated by the activation of the tumor necrosis factor receptor (TNFR), the interleukin 1 receptor (IL1R), and Toll-like receptors (TLRs) (Lawrence and Fong 2010). TLRs are activated by pathogen associated molecular patterns (PAMPs) or virulence factors and in response they induce the expression of inflammatory mediators that drive pathogen-specific adaptive immune responses (Kumar, Kawai et al. 2009). Mononuclear phagocytic cells and other antigen-presenting cells (APCs) release cytokines that induce cellular infiltrate and tissue damage.

Monocytes are activated via pattern recognition receptors (PRRs) and they release pro-inflammatory cytokines such as tumor necrosis factor (TNF), Interleukins, IL1, IL-6, CXCL8 and chemokines including IL-12, IL-15, IL-18, IL-23 and IL-27 (Steinke and Borish 2006). In addition to the cytokines that stimulate cytotoxic, cellular, humoral and allergic inflammation, others such as IL-1Ra (Interleukin 1 receptor a), TGF-β (transformation growth factor-β), IL-10 and IL-35 have anti-inflammatory properties (Commins, Borish et al.
Introduction

2010). Under normal circumstances, the immune system employs several mechanisms to resolve inflammation and clear the tissues from inflammatory cells leading to normal tissue function. Failure of reversing the inflammatory responses leads to the pathogenesis of inflammatory diseases (Lawrence and Gilroy 2007).

1.2.2. Inflammation in COPD

Inflammation in COPD is characterised by an accumulation of neutrophils, macrophages, B cells, lymphoid aggregates and CD8+ T cells (Barnes 2008; Cosio, Saetta et al. 2009). The degree of inflammation correlates with disease severity and represents both innate and adaptive immune responses to toxic gases and particles (Hogg, Chu et al. 2004). The chronic inflammation in COPD is orchestrated by pro-inflammatory chemokines (CC-chemokine ligands such as CCL2 and C-X-C motif ligands such as CXCL1 and CXCL8); cytokines (TNF-α, IL-1β, IL-6 and Interferon γ (INFγ) and proteases such as neutrophil elastase and metalloproteinases-9 (MMP9) (Barnes 2008). COPD severity correlates with an increase in B cell numbers indicating the role of adaptive immunity in the later stages of disease progression (Hogg, Chu et al. 2004; Gosman, Willemse et al. 2006). In addition, autoimmunity has been also documented in COPD, where autoantibodies, such as anti-elastin, anti-epithelial, tobacco anti-idiotypic and anti-carbonyl antibodies are present in smoking patients with COPD (Koethe, Kuhnmuench et al. 2000; Lee, Goswami et al. 2007).

1.2.3. The role of macrophages in driving inflammation in COPD

Alveolar macrophages are activated by a number of stimuli, including cigarette smoke, pro-inflammatory cytokines (such as TNF-α and IL-1β), bacterial endotoxin (such as LPS) and
other immune stimuli (Barnes 2004). Once activated, they secrete inflammatory mediators such as chemokines, cytokines, growth factors, lipid mediators and reactive oxygen species. Chemokine release leads to recruitment of monocytes from the blood circulation, neutrophils and T-lymphocytes. CXCL1, CXCL8 chemokines act via CXCR2 to attract neutrophils and monocytes; and CCL2 (monocyte chemotactic protein1 or MCP-1) acts via CCR2 to attract monocytes. CXCL9, CXCL10, and CXCL11 act via CXCR3 to attract Th1 cells and Tc1 cells, both of which release IFN-γ, which induce the release of more CXCR3-binding chemokines (Rot and von Andrian 2004; Lazzeri and Romagnani 2005).

Proteases such as neutrophil elastase and metalloproteinases-9 are also secreted by macrophages leading to destruction of the alveolar wall (emphysema) and mucus hypersecretion ( Hubbard, Fells et al. 1991; Nagase 1997). Mucus hypersecretion is also stimulated by EGF (epidermal growth factor) and TGF-α, whereas TGF-β and the connective tissue growth factor (CTGF) regulate small airway fibrosis (Figure 1.1) (Fairlie, Moore et al. 1999; Takizawa, Tanaka et al. 2001). TGF-β is overexpressed in alveolar macrophages from COPD patients compared to normal subjects contributing to the fibrosis documented in the small airways of COPD patients (de Boer, van Schadewijk et al. 1998).

Inflammatory cytokines, such as TNF-α and the granulocyte-macrophage colony stimulating factor (GM-CSF), IL-1β and IL-6 are also released in response to inflammatory stimuli and cigarette smoking and correlate with disease severity (Keatings, Collins et al. 1996; Culpitt, Rogers et al. 2003; Churg, Wang et al. 2004; Marwick, Kirkham et al. 2004; Lambert, Li et al. 2007; Mortaz, Adcock et al. 2010; Mortaz, Henricks et al. 2011; Winkler, Nocka et al. 2012). Alveolar macrophages also release the anti-inflammatory cytokine IL-10 upon inflammatory stimuli, the secretion of which is markedly decreased in the sputum of patients with COPD (Takanashi, Hasegawa et al. 1999).
Due to their key modulator role in inflammation and the diversity of the responses they drive, macrophages may account for most of the known features of COPD pathophysiology (Shapiro 1999; Tetley 2002). There is a 5 to 10-fold increase in macrophage numbers in the airways, lung parenchyma, BAL (bronchoalveolar lavage) fluid and sputum of smokers and patients with COPD correlating with disease severity (Di Stefano, Capelli et al. 1998; Retamales, Elliott et al. 2001). One reason for this is the increased recruitment of monocytes from the circulation in response to monocyte-attracting chemokines that are released in the lungs (Barnes 2004). MCP-1 (CCL2) is found elevated in sputum and PAL of patients with COPD (Capelli, Di Stefano et al. 1999; de Boer, Sont et al. 2000; Traves, Culpitt et al. 2002) and is found to be overexpressed in alveolar and small airway macrophages (de Boer, Sont et al. 2000). CXC chemokines are also elevated in sputum and BAL of patients with COPD (Traves, Culpitt et al. 2002).

The increased number of macrophages in COPD is also a result of their increased proliferation and survival in the lungs. Macrophages in the lungs of smokers have been shown to have increased proliferation rate compared to normal macrophages (Tomita, Caramori et al. 2002). Oxidative stress leads to increased expression of anti-apoptotic proteins (B cell lymphoma (Bcl)-X<sub>L</sub> and p21) leading to prolonged survival of alveolar macrophages in the lungs of both smokers and COPD patients (Tomita, Caramori et al. 2002; Marwick, Kirkham et al. 2004). Macrophages in the airways are cleared by the mucociliary clearance which is also shown to be impaired in COPD (Lourenco, Klimek et al. 1971; Foster, Langenback et al. 1980). The lymphatic drainage which also clears macrophages from the airways might be impaired in COPD patients due to emphysema and small airway fibrosis leading to increased number of macrophages in the lungs of COPD patients lungs (Barnes 2004).
Macrophages orchestrate the chronic inflammation in COPD. Macrophages are activated by cigarette smoke extract, bacterial pathogens and inflammatory stimuli and in response they secrete inflammatory mediators that orchestrate the chronic inflammation in COPD. They secrete pro-inflammatory mediators (such as TNF-α, IL-6 and IL-1β) and reactive oxygen species. They also release chemoattractants, such as CXCL1, CXCL8, CCL2 which attract neutrophils and monocytes to the site of inflammation respectively. The release of elastolytic enzymes such as neutrophil elastase and matrix metalloproteinase 9 (MMP9) drives the destruction of the alveolar wall and mucus hypersecretion. In addition, they release CXCL9, CXCL10 and CXCL11 that activate Th1 and Tc1 cells to release IFN-γ. Release of TGF-α activates epidermal growth factor receptors (EGFR) which stimulate mucus hypersecretion and release of TGF-β and CTGF induces small airway fibrosis. Adapted from (Barnes 2004).
1.3. Reactive oxygen species

Reactive oxygen species are small, highly reactive molecules including the superoxide anion radical (‘O₂⁻), hydroxyl radicals (‘OH) and hydrogen peroxide (H₂O₂). The high reactivity of ROS, results from the presence of one or more unpaired electrons in their atomic or molecular orbitals. Although H₂O₂ is not a free radical, it can be oxidised to the highly reactive ‘OH via the Fenton reaction in the presence of transition metals including iron or copper (Valko, Leibfritz et al. 2007). ROS are generated as by-products of cellular metabolic processes in mitochondria and peroxisomes, but also through the activity of cytosolic enzyme systems. The normal release of ROS in the cell is augmented by the activation of oxidative enzymes such as the nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (Finkel and Holbrook 2000).

Reactive oxygen species that are generated normally by cellular events, such as aerobic respiration, are counteracted in the cells under normal conditions by the ubiquitously expressed antioxidant proteins. These include superoxide dismutase (SOD), catalase, glutathione (GSH), glutathione peroxidase, glutaredoxin and thioredoxin (Valko, Leibfritz et al. 2007). An imbalance between oxidant production and antioxidant capacity of the cell to prevent oxidative injury is called ‘oxidative stress’ and can be induced by pro-inflammatory processes, ionising radiation and exposure to chemotherapeutic drugs (Thannickal and Fanburg 2000; Valko, Leibfritz et al. 2007). Low levels of reactive oxygen species cause changes in the intracellular redox state and can alter the enzymatic function of redox-sensitive proteins (Reth 2002; Forman 2007). High levels of reactive oxygen species can lead to impaired physiological functions via cellular damage of DNA and proteins’ lipid peroxidation, all implicated to be involved in human pathologies.
Oxidative stress has been implicated in many human diseases such as atherosclerosis, cancer, neurodegenerative diseases, ageing and COPD (Valko, Leibfritz et al. 2007). Due to the lack of our understanding of the exact mechanisms by which reactive oxygen species function in both normal and disease states, it remains uncertain whether oxidative stress is a cause or a result of these human pathologies. For that reason, there has been extensive investigation focused on addressing this question. Most studies use H$_2$O$_2$ as an agent to mimic the actions of reactive oxygen species due to its relatively long half-life and good membrane permeability (Reth 2002; Forman 2007).

1.3.1. Reactive oxygen species and their role in COPD pathogenesis

Reactive oxygen species are released from macrophages in response to inflammatory stimuli or cigarette smoke contributing to the increased oxidative stress documented in the airways of COPD patients (Montuschi, Collins et al. 2000; Rahman, van Schadewijk et al. 2002; Stoner, Wang et al. 2008). This is mediated by the activation of the NADPH oxidase in the cell membrane which produces superoxide anions (O$_2^-$) which are then converted to H$_2$O$_2$ by the enzyme superoxide dismutase (SOD) (Iles and Forman 2002). This elevated release of reactive oxygen species regulates the macrophage microbial killing function and is also critical for activation of signal transduction pathways and transcription factors which orchestrate the inflammatory gene expression in COPD (Barnes 2004).

Reduced gene expression of the antioxidant manganese superoxide dismutase (MnSOD) in the epithelium of smokers and COPD patients suggest amplification of the injurious effects of cigarette smoke derived reactive oxygen species (Pierrou, Broberg et al. 2007). The direct oxidative effect of cigarette smoke ($10^{15}$ radicals per puff) on the epithelium is shown by the increased nitrotyrosine levels detected in the lung epithelial lining fluid from chronic
smokers (Ichinose, Sugiura et al. 2000). Apart from its direct effects, cigarette smoke also induces the release of endogenous oxidants as suggested by increased NADPH oxidase (Nox) activity in macrophages and neutrophils from smokers (Noguera, Batle et al. 2001; Rahman, Biswas et al. 2006). Therefore, migration of neutrophils and macrophages into the lungs of chronic smokers is accompanied by elevated levels of ROS and subsequently cellular damage, which is reflected by increased H$_2$O$_2$ and lipid peroxidation products in exhaled breath condensate (Montuschi, Collins et al. 2000).

Oxidative stress in COPD could also affect smaller airways since catalase gene expression is reduced in peripheral lung tissue from COPD subjects compared to smokers and non-smoking healthy controls (Tomaki, Sugiura et al. 2007). Increased release of ROS by peripheral blood neutrophils in COPD possibly contributes to systemic oxidative stress, evident from the increased lipid and protein peroxidation products in plasma and urine of COPD patients (Noguera, Batle et al. 2001; Santus, Sola et al. 2005; Hanta, Kocabas et al. 2006). Systemic oxidative stress is thought to be involved in the development of some of the systemic effects of COPD (Langen, Korn et al. 2003).
1.4. Oxidative stress induced regulation of kinase signalling pathways

1.4.1. The mitogen activated protein kinases (MAPKs)

1.4.1.1. The MAPK family: members and function

The MAPKs comprise a family of redox-sensitive protein-serine/threonine kinases that play a key role in mediating the transduction of biological signals from the cell membrane to the nucleus (Boutros, Chevet et al. 2008; Brown and Sacks 2009). They regulate cell growth and fate, therefore their sustained activation leads to uncontrolled proliferation and cell death (Winter-Vann and Johnson 2007). There are three well studied subgroups of MAPKs: the extracellular signal regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs) and the p38 MAPKs (including p38α, p38β, p38γ and p38δ isoforms). Activation of MAPKs is mediated by activation of the upstream MAPK kinase kinases (MAP3Ks). The latter phosphorylate and activate their downstream targets, the dual specificity MAPK kinases (MAP2Ks) which subsequently phosphorylate the threonine and tyrosine residues of the MAPKs leading to their activation (Boutros, Chevet et al. 2008; Brown and Sacks 2009). Upon ligand binding to growth factor receptors, ligand induced dimerisation and oligomerisation leads to phosphorylation and activation of receptor tyrosine kinases (RTKs). These activate the Ras-GTPase (Guanosine triphosphatase) which leads to activation of Raf and the MAP/ERK Kinase (MEK). MEK is responsible for the activation of the ERK pathway via phosphorylation (Aslan and Ozben 2003; Winter-Vann and Johnson 2007).

The p38 MAPK pathway is activated in response to inflammatory cytokines, ligands for G protein-coupled receptors (GPCRs), heat shock, oxidative and osmotic stress (Cuadrado and Nebreda 2010). The p38 MAPK family members are activated by the two MEK family members MEK3 and MEK6. MEK6 can phosphorylate all four p38 isoforms whereas MEK3
activates p38α, p38γ and p38 δ. The members of the MAP3K family shown to stimulate p38 MAPK activation include the apoptosis signal-regulating kinase-1 (AKS1 or MAP3K5), the dual-leucine-zipper-bearing kinase 1 (DLK1), the transforming growth factor β-activated kinase 1 (TAK1 or MAP3K7), the thousand and one amino acid (TAO), the mixed-lineage kinase 3 (MLK3 or MAP3K11), the tumor progression loci 2 (TPL2 or MAP3K8), the MEKK3, MEKK4 and the leucine zipper and sterile-α motif kinase 1 (ZAK1) (Son, Cheong et al. 2011). Some of the MAP3Ks that stimulate p38 MAPK activation can also activate the JNK pathway. MEK4 and MEK7 have been implicated in phosphorylation of JNKs in response to cytokines, agents that interfere with DNA and protein synthesis, growth factors and transforming agents (Bogoyevitch, Ngoei et al. 2010).

1.4.1.2. Redox regulation of the MAPKs’ activity

Reactive oxygen species have been shown to activate RTKs (Nakashima, Takeda et al. 2005) but studies have also shown that H₂O₂ is a critical mediator for ligand-independent phosphorylation of growth factor receptors in response to oxidative stress (Meves, Stock et al. 2001). Although direct exposure to exogenous H₂O₂ has been shown to lead to activation of MAPK pathways, the exact mechanism is still not known. Reactive oxygen species can alter protein structure and function by modifying critical amino acid residues of proteins (Meves, Stock et al. 2001). Oxidation of the cysteine-rich motifs present in several growth factors and cytokine receptors have been implicated in mediating oxidative stress induced activation of the MAPKs. In addition, reactive oxygen species can activate MAPKs via oxidative modification of intracellular kinases such as the MAP3Ks (Liu, Nishitoh et al. 2000). Studies focused on elucidating the mechanisms by which oxidative stress activates p38 and JNK MAPKs have revealed the critical role of the redox-sensitive proteins, thioredoxin and
glutaredoxin (Ichijo 1999). Reactive oxygen species can oxidise thioredoxin leading to dissociation of the latter from ASK-1 leading to the subsequent activation of the JNK and p38 pathways (Matsuzawa and Ichijo 2008). Besides ASK-1, there are other MAP3Ks or MAP2Ks that are redox sensitive and regulate oxidant stress-induced activation of MAPKs (Son, Cheong et al. 2011). In addition to the above mechanisms, H₂O₂ induced inactivation of the MKPs (MAPK phosphatases) by oxidation of their catalytic cysteine can also lead to MAPK activation (Kamata, Honda et al. 2005; Hou, Torii et al. 2008). Reactive oxygen species can lead to up-regulation of MKP-1 expression which correlates with inactivation of JNK and p38 activity and reduction of MKP-1 expression has been shown to lead to sustained activation of the JNK pathway (Kuwano and Gorospe 2008) (Figure 1.2).
Figure 1.2. The mechanisms of reactive oxygen species-induced activation of the MAPK signalling pathway. Production of reactive oxygen species is stimulated by growth factors, cytokines, and stresses. Under normal conditions these are counteracted by the ubiquitously expressed endogenous antioxidant proteins. When the antioxidant capacity is exceeded by the levels of reactive oxygen species, then oxidative stress leads to activation of the MAPKs via oxidative modifications of the MAPK signalling proteins (RTKs and MAP3Ks). Oxidative stress induced inhibition of the MKPs is also a mechanism involved in oxidant stress induced MAPKs activation. Continuous arrows represent activating/increasing effects and dashed arrows represent decreasing/inhibiting actions. Adapted from (Son, Cheong et al. 2011).
1.4.2. The phosphatidylinositol 3-kinase (PI3K) signalling pathway

1.4.2.1. Activation of the PI3K signalling pathway

The PI3K signal transduction pathway plays an important role in regulating cell growth, proliferation, survival and motility (Leslie 2006). The cascade is activated by phosphorylation of RTKs leading to its direct binding to PI3K or its indirect binding to adaptor proteins such as insulin receptor substrate (IRS) in the case of insulin-like growth factor (IGF-1) signalling. Upon activation PI3K converts the membrane phosphatidylinositol 4,5-biphosphate [PI(4,5)P₂] to the lipid phosphatidylinositol 3,4,5-triphosphate [PI(3,4,5)P₃]. PIP3 in turn recruits Akt and 3'-phospho-inosite-dependent kinase-1 (PDK1) to the membrane via binding to its pleckstrin homology (PH) domains (Vanhaesebroeck and Alessi 2000) and PDK1 phosphorylates Akt on Ser473 leading to its activation (Bhaskar and Hay 2007). Activation of Akt can also be mediated by the mTOR (mammalian target of rapamycin) complex 2 (TROC2) and it is a Ser/Thr kinase that regulates the function of downstream kinases (Sarbassov, Guertin et al. 2005). The PI3K/Akt signalling pathways are tightly regulated by phosphatases. The phosphatase and tensin homologue (PTEN) is responsible for the enzymatic conversion of PIP3 back to PI(4,5)P2. In addition, protein tyrosine phosphatases (PTPases) regulate PI3K activation via inactivation of receptor tyrosine kinases (Dillon, White et al. 2007) (Figure 1.3).

1.4.2.2. Classes and structure of the PI3Ks

There are three different classes of phosphatidylinositol 3-kinases based on their substrate preference and sequence homology (Engelman, Luo et al. 2006). Class I PI3Ks primarily generate (phosphatidylinositol) PtdIns(3,4,5)P3 from PtdIns(4,5)P2. Class IA and IB PI3Ks
differ primarily on their regulatory subunits. Class IA PI3Ks are heterodimers consisting of a regulatory subunit (p85α, p85β or p55γ) and a catalytic subunit (p110) (Fruman, Meyers et al. 1998). Each of the regulatory subunits of the Class I of PI3Ks is encoded by a distinct gene (PIK3R1, PIK3R2 and PIK3R3, respectively). The basic structure of the p85 regulatory units contains 2 Src (Rous carcinoma associated cellular homologue)-homology 2 (SH2) domains that flank one p110-binding domain (Songyang, Shoelson et al. 1993). Through their SH2 domains, the p85 regulatory subunits bind to phosphorylated tyrosine residues on activated RTKs or adaptor molecules such as IRS1. This binding is fundamental for the obstruction of the inhibitory action that p85 exerts on the p110 catalytic subunit of the PI3K molecule (Yu, Wjasow et al. 1998). Three different genes encode the p110 catalytic subunit isoforms p110α, p110β and p110δ (PIK3CA, PIK3CB and PIK3CD, respectively) (Fruman, Meyers et al. 1998).

While p110α and p110β are ubiquitously expressed, the p110δ subunit is predominantly expressed in leukocytes (Engelman, Luo et al. 2006). The p110 catalytic subunit contains 5 distinct domains: an N-terminal p85-binding domain, a GTPase Ras-binding domain (RBD), a C2 domain, a PIK homology domain and the catalytic domain located in the C-terminal end of the subunit (Fruman, Meyers et al. 1998). Class IA PI3Ks are activated by the growth factor RTKs. While the insulin and IGF-1 receptors utilise the IRS family of adaptor molecules to recruit Class IA PI3Ks to the plasma membrane and trigger their activity (Myers, Backer et al. 1992; Yamamoto, Lapetina et al. 1992; Giorgetti, Ballotti et al. 1993), other RTKs such as the EGFR recruit Class IA PI3K directly (Moscatello, Holgado-Madruga et al. 1998).

The small GTPase Ras can also directly activate Class IA PI3K by interaction with the RBD domain of the p110 catalytic subunit. Class IB PI3Ks have a p101 regulatory subunit and a p110γ catalytic subunit. Since class IB PI3Ks do not possess a p85 regulatory subunit, they
are typically not regulated by RTKs, and rather activated by the G-protein-coupled receptors (Stephens, Smrcka et al. 1994; Stoyanov, Volinia et al. 1995).

Class II PI3Ks consist of a single p110-like catalytic subunit, in three different isoforms (PI3K C2α, β and γ). Although it is generally assumed that class II PI3Ks can phosphorylate both PtdIns and PtdIns(4)P \textit{in vitro}, they seem to have a preferential activity for PtdIns, transforming it into PtdIns(3)P. \textit{In vivo} studies also suggest that PtdIns(3)P might be the most abundant product generated by this class of PI3K (Falasca and Maffucci 2007). There is still a considerable lack of knowledge regarding the function and regulation of this class of PI3Ks.

Class III is represented solely by the mammalian homolog of the vacuolar protein-sorting defective 34 (Vps34) molecule, first identified in \textit{Saccharomyces cerevisiae}. PtdIns is the only substrate of this class of PI3K identified and the only product it generates is PtdIns(3)P. In both yeast and mammalian cells, Vps34 is required for the induction of autophagy during nutrient deprivation (Backer 2008). However, as Vps34 has also been implicated in the positive regulation of mTOR/raptor signaling through nutrient sensing (Byfield, Murray et al. 2005; Nobukuni, Joaquin et al. 2005), its role in autophagy might be context-dependent. More recently, Src has been demonstrated to phosphorylate and activate Vps34, in mammalian cells, leading to cellular transformation (Hirsch, Shen et al. 2010).

\textbf{1.4.2.3. Akt: structure and function}

Akt consists of a central kinase domain flanked by an N-terminal pleckstrin homology (PH) domain and a carboxyl-terminal regulatory domain which contains the hydrophobic motif (HM) which is characteristic of the AGC (protein kinases A, G and C) kinases including also the p70 ribosomal S6 kinase and the serum-glucocorticoid-inducible kinase (SGIC) (Hanad et
Akt catalytic activity is regulated by phosphorylation on its Thr308 and Ser473 residues located on its catalytic and HM domains respectively (Liao and Hung 2010). Akt regulates a number signalling cascades that control apoptosis, cell cycle, angionegenesis and tissue invasion and thus plays a critical role in regulating malignant neoplastic transformation (Hanahan and Weinberg 2000). The kinase inhibits apoptosis by inactivating pro-apoptotic proteins and/or activating anti-apoptotic ones. One of its many targets is the pro-apoptotic member of the Bcl-2 family, BAD (Bcl-2 associated death promoter protein). Akt-induced phosphorylation of BAD on Ser136 disrupts the BAD-Bcl-2-mediated cell death (Datta, Dudek et al. 1997). Another member of the Bcl family targeted by Akt is BAX (Bcl-2 associated X protein), whose translocation from the cytoplasm to mitochondria is regulated by phosphorylation by the kinase (Tsuruta, Masuyama et al. 2002). Akt is also known to phosphorylate and inactivate pro-caspase 9 on Ser196 (Cardone, Roy et al. 1998) and induce p53 degradation via phosphorylation of Mdm2 (murine double minute) and subsequent increase of the latter’s ability to ubiquitinate p53 (Ogawara, Kishishita et al. 2002). The kinase has been also shown to activate IKK (Inhibitor of Kappa B kinase) leading to activation of the NF-κB (Salminen and Kaarniranta 2010).

One the most critical roles of Akt signalling is regulation of cell cycle progression. Akt phosphorylates and inactivates GSK3, thereby rescuing cyclin D1 and c-Myc (avian myelocytomatosis virus oncogene cellular homolog) from ubiquitination and degradation by the proteasome and positively regulating G1/S cell cycle progression (Liang and Slingerland 2003). Its role in metabolism derives from its regulatory function on glucose transport, protein and glycogen synthesis and gluconeogenesis suppression (Whiteman, Cho et al. 2002). Active Akt mediates the effects of insulin signaling in enhancing cell growth. Akt promotes glucose uptake into adipocytes by inducing the translocation of the glucose transporters GLUT1 and GLUT4.
transports 4 (GLUT4) to the plasma membrane (Kohn, Summers et al. 1996; Cong, Chen et al. 1997). In addition, it phosphorylates and inhibits glycogen synthase kinase 3 (GSK3) leading to promotion of glycogen synthesis. Akt also promotes protein synthesis via GSK3 mediated regulation of the eukaryotic initiation factor 2B (eIF2B) (Proud 2006).

1.4.2.4. Regulation of Akt activation

Akt activity in mammalian cells is controlled by reversible phosphorylation of serine and threonine residues in response to serum stimulation or inhibition of phosphatase 2A (PP2A) activity (Andjelkovic, Zolnierowicz et al. 1996). PDK1 is responsible for activating Akt in response to insulin and growth factors (Alessi, Deak et al. 1997) via phosphorylation on its Thr308 residue. By contrast, PDK2 or mTOR targets the Ser473 residue of Akt1 (Figure 1.3). Full activation of Akt requires Thr308 and subsequent Ser473 phosphorylation (Liao and Hung 2010).

1.4.2.5. Redox regulation of the PI3K/Akt signalling pathway

Oxidative stress regulates the activity of PI3K via oxidative modification of Cys (cysteine)-dependent phosphatases (CDPs) and protein kinases. CDPs are a large family of enzymes that share a catalytic domain with a conserved and highly reactive Cys residue, which at physiological pH exists as a thiolate anion. Oxidative modification of the Cys residue leads to inactivation of CDPs’ enzymatic function (Salmeen and Barford 2005). The CDPs that control the PI3K/Akt signalling pathway include PI3-phosphatase, PTEN and PTPases. S-nitrosylation of the active Cys of PTEN is an key mechanism controlling redox regulating PI3K activity (Yu, Li et al. 2005). Under normal condition, PTEN is sustained in a reduced state by the NADPH/Trx (thioredoxin) system which is redox sensitive. Any decrease in NADPH, as in the
case of mitochondrial dysfunction, leads to oxidative modifications of PTEN and subsequent activation of the PI3K/Akt signalling pathway (Pelicano, Xu et al. 2006).

![Diagram of the PI3K/Akt signalling pathway](image)

**Figure 1.3. Activation of the PI3K/Akt signalling pathway.** Upon activation and phosphorylation of RTKs, their tails become docking sites for the p85 PI3K subunit which dissociates the p110 subunit of PI3K. This phosphorylates PIP2 generating PIP3. PIP3 recruits Akt and PDK1 to the plasma membrane via its PH domain. PDK1 phosphorylates Akt on Thr308 priming it for phosphorylation of Ser473 via PDK2 or mTOR. PP2A and PHLPP phosphatases are responsible for dephosphorylating Akt and inactivating it. In addition, PI3K activation is counteracted by PTEN which targets PIP3 and generates PIP2. Adapted from (Liao and Hung 2010).

In addition, protein phosphatases are also known to be targets of ROS-species mediated oxidation. These include the PTP1B, SHP-2 (SH2 domain containing tyrosine phosphatase 2) and TC45 (T-cell protein tyrosine phosphatase 45), which are all negative regulators of RTKs causing activation of the receptor regulated activation of the PI3K/Akt pathway (Lee, Kwon et al. 1998; Meng, Fukada et al. 2002; Wang, Zeigler et al. 2007). In addition to phosphatases, the PI3K and Akt protein kinases can be also modified by redox modification.
leading to downregulation of the PI3K/Akt pathway. Oxidant stress stimulates the formation of a bisulphide bridge between Cys297 and Cys311 in the Akt kinase domain. This event assists the binding of Akt to PP2A leading to dephosphorylation of the former and its inactivation (Murata, Ihara et al. 2003).

The p85 subunit of PI3K is also known to be a direct target for tyrosine nitration which also leads to inactivation of the Akt pathway (el-Remessy, Bartoli et al. 2005). Receptor-induced activation of PI3K can also activate Rac-NAD(P)H oxidase (NOX) leading to intracellular generation of reactive oxygen species (Welch, Coadwell et al. 2003). It was recently demonstrated that NOX-induced production of ROS regulates monocyte/macrophage survival via activation of Akt and inhibition of the p38 MAPK pathway (Wang, Zeigler et al. 2007).

1.4.3. The Glycogen Synthase Kinase 3 (GSK3)

GSK3 was first discovered as one of the kinases responsible for the phosphorylation and inactivation of glycogen synthase (Embi, Rylatt et al. 1980). Apart from its role in glycogen metabolism, GSK3 acts as a downstream regulator that determines the output of numerous signalling pathways initiated by diverse stimuli (Frame and Cohen 2001; Grimes and Jope 2001). Dysfunction of the pathways in which GSK-3 acts as a key regulator have been implicated in the development of human diseases such as diabetes, Alzheimer's disease, bipolar disorder and cancer (Doble and Woodgett 2003).
1.4.3.1. Structure of GSK3

There are two mammalian isoforms of GSK3 (GSK3α and GSK3β) which share a 98% homology in their kinase domains but differ substantially in their N-terminal and C-terminal domains (Force and Woodgett 2009). GSK3α has a glycine rich N-terminal sequence (Frame and Cohen 2001), which accounts for its higher molecular weight compared to GSK3β (52kDa versus 47kDa) (Figure 1.4). Homologues of GSK3 exist in all eukaryotes examined to date and display a high degree of homology; isoforms from species as distant as flies and humans display >90% sequence similarity within the kinase domain (Ali, Hoeflich et al. 2001). Both isoforms are redundant in regulating Wnt (wingless/integrated)/β-catenin signalling, where GSK3α regulates glycogen storage in the liver and GSK3β has the same function but in the skeletal muscle (Force and Woodgett 2009). GSK3α knockout mice are viable, although they show increased insulin sensitivity and reduced fat mass (MacAulay, Doble et al. 2007).

Embryos carrying homozygous deletions of exon 2 of GSK3β die around embryonic day 16 due to liver degeneration caused by extensive hepatocyte apoptosis. The inability of GSK3α to rescue the GSK3β-null mice indicates that the degenerative liver phenotype is specific to the beta isoform (Hoeflich, Luo et al. 2000). The differences in the function of the two isoforms are probably due to differences in affinity that they have towards interacting molecules such as Akt isoforms (Brognard, Sierecki et al. 2007). Another possible explanation is the tissue-specific scaffolds that mediate competitive binding of one of the two isoforms to specific targets (Force and Woodgett 2009).

Another splice variant of GSK3β has been recently identified which accounts for ~15% of total GSK3. GSK3β2 contains a 13-residue insert located in the kinase domain (Mukai,
Ishiguro et al. 2002). This variant has less activity towards the microtubule-associated protein tau compared to the unspliced isoform and is localised primarily to neuronal cell bodies. Due to the location of the insert, the splice variant has probably different affinity to scaffolding proteins and hence exerts its function to different subset of target proteins (Mukai, Ishiguro et al. 2002).

**Figure 1.4. Schematic representation of the mammalian GSK3α and GSK3β isoforms.** GSK3α and GSK3β share a conserved kinase domain (in purple) where the Y279 and Y216 are respectively located. The glycine-rich N-terminal domain is unique to GSK3α. The serine residues that regulate GSK3α and GSK3β activity are shown by the blue arrowheads accordingly. Taken from Doble and Woodgett (2003).

### 1.4.3.2. Substrate recognition by GSK3

GSK3 targets primed phosphorylated molecules, where the ‘priming’ residue is located four amino acids C-terminal to the site of GSK3 phosphorylation (Fiol, Mahrenholz et al. 1987). The consensus sequence for GSK3 substrates is Ser/Thr-X-X-Ser/Thr-P, where the first Ser or Thr is their target residue, X is any amino acid (but often Pro) and the phospho-Ser/Thr is the primed site. Although priming phosphorylation increases the efficiency of the kinase activity of GSK3 (by 100-1000 fold), it is not strictly required for its function (Thomas, Frame et al. 1999; Hagen and Vidal-Puig 2002). As shown in Figure 1.5, the phosphorylated residue of the primed substrate binds to the positively charged pocket including the R (Arginine) 96,
R180 and K (Lysine) 205 residues. This binding optimises the orientation of the kinase domains and places the substrate at the correct position at the catalytic groove promoting its phosphorylation. The substrates of GSK3 that lack this priming site have negatively charged residues on or near this position which act like ‘pseudo’ phospho-residues (Doble and Woodgett 2003).

1.4.3.3. Regulation of GSK3 activity

The crystal structure of GSK3 has been recently identified providing important insights into its regulation and its preference for primed, pre-phosphorylated substrates (Bax, Carter et al. 2001; Dajani, Fraser et al. 2001; ter Haar, Coll et al. 2001). Activation of GSK3 requires phosphorylation in its activation loop (T-loop) on Y279 or Y216 for GSK3α and GSK3β respectively (Doble and Woodgett 2003). Y216/Y279 phosphorylation enhances GSK3 kinase activity by >200 fold by forcing open the substrate site (Hughes, Nikolakaki et al. 1993) but there is no disruption to the open conformation in the unphosphorylated protein thus it is not essential for its kinase activity (Dajani, Fraser et al. 2001).

Negative regulation of GSK3 is the key mechanism controlling its signal transduction since the kinase is constitutively active in most resting cells. Downregulation of GSK3 activity is regulated by inactivating its kinase domain, by disturbing its ability to bind to its substrates or by altering its ability to recognise them (Doble and Woodgett 2003). Phosphorylation of the Ser21 or Ser9 residue of GSK3α or GSK3β, respectively, transforms the molecule’s amino terminus into a ‘pseudosubstrate’. This phosphoserine occupies the same binding site that the priming phosphate of the substrate of GSK3 would occupy thereby blocking the
substrate’s access to the active site of the kinase (Cohen and Frame 2001) (Figure 1.5). There are several kinases that target these serine sites including AKT (Cross, Alessi et al. 1995), p90 ribosomal kinase (p90RSK), p70 ribosomal S6 kinase (p70S6K) (Sutherland and Cohen 1994), PKA (Liu, Zhang et al. 2004) and protein kinase C (PKC) (Fang, Yu et al. 2002).

PKA regulates GSK3β activity in response to GPCR activation. GPCRs are activated upon hormonal stimulation and activate adenylyl cyclase (AC) mainly via the Gsα subunit of G proteins (Krupinski, Coussen et al. 1989). This leads to production of cyclic adenosine monophosphate (cAMP) which activates PKA (Kuo and Greengard 1969; Walsh, Perkins et al. 1971). PKA forms a complex with A kinase anchoring protein (AKAP220) which assist PKA-induced phosphorylation of GSK3β and subsequent inhibition of its activity (Tanji, Yamamoto et al. 2002). c-AMP has multiple effects on cellular processes such as gene expression, cell cycle control and cell survival/death decision (Dumont, Jauniaux et al. 1989; Glass, Lundquist et al. 1989; Meyer-Franke, Kaplan et al. 1995; von Knethen, Lotero et al. 1998) and regulation of GSK3β may contribute to these effects establishing the role of the kinase on controlling cell proliferation and survival (Hughes, Nikolakaki et al. 1993; Cui, Meng et al. 1998). GSK3β regulates cell proliferation/survival and glycogen synthase activity in response to growth factors (i.e. insulin and IGF-1) via activation of the PI3K/Akt signalling pathway (Sutherland, Leighton et al. 1993; Cross, Alessi et al. 1994; Stambolic and Woodgett 1994; Cross, Alessi et al. 1995; Pap and Cooper 1998).

As mentioned in the previous section, the insulin receptor or IGF-1 receptor can activate PI3K via utilisation of IRS1 and IRS2 mediators. The insulin receptor has intrinsic tyrosine kinase activity and upon ligand binding it phosphorylates its own tyrosine residues. This
leads to the recruitment and subsequent phosphorylation of IRS which is recognised by the p85 subunit of PI3K leading to activation of Akt (Antonetti, Algenstaedt et al. 1996; Valverde, Lorenzo et al. 1998; Shaw 2001). Akt phosphorylates GSK3β on Ser9 and inactivates it, resulting in dephosphorylation of glycogen synthase, suppression of its activity and stimulation of glycogen synthesis (Parker, Caudwell et al. 1983; Welsh and Proud 1993; Cohen, Alessi et al. 1997) (Figure 1.6).

Figure 1.5. Regulation of GSK3β activity by serine phosphorylation. When GSK3β is constitutively active in resting cells, substrates that have been either primed by a priming kinase (PK) or unprimed substrates can be phosphorylated by the kinase. In the case of primed substrates, the phospho-residue on N+4 position binds the positively charged pocket comprising of the arginine (R) and lysine (K) residues. The serine or threonine on position N then binds to the catalytic site (C.S.). In stimulated cells that GSK3β is phosphorylated on Ser9 by an inactivating kinase (IK), the phosphorylated N-terminus becomes a pseudosubstrate, occupying the binding pocket and acting as a competitive inhibitor for the target substrates. This leads to inhibition of GSK3β kinase activity. Adapted from (Doble and Woodgett 2003).
Regulation of GSK3β activity via phosphorylation. Two major signalling pathways that regulate GSK3β activity via Ser9 phosphorylation include the insulin-regulated activation of PI3K/Akt pathway and the GCPR-mediated activation of PKA. (a) Upon insulin stimulation, the receptor phosphorylates itself on tyrosine residues (pY). These are then recognised by the PTB domains of the IRS1 and IRS2, which are recruited and phosphorylated by the insulin receptor. They interact with the SH2 domain of p85 of PI3K, recruiting the p110 subunit leading to activation of Akt. Akt phosphorylates GSK3β on Ser9 and inactivates it, resulting in dephosphorylation of glycogen synthase. (b) Ligand binding to GCPR results in AC activation and increased production of c-AMP leading to PKA activation. AKAP220 binds both PKA and GSK3β and allows PKA-induced phosphorylation of GSK3β on Ser9 blocking its activity. Adapted from (Cohen and Frame 2001; Jope and Johnson 2004; Rayasam, Tulasi et al. 2009).
1.4.3.4. The role of GSK3β in regulating the Wnt/β-catenin signalling pathway

The canonical Wnt signalling pathway regulates cell fate and axis specification. Apart from regulating the cell’s ‘decision’ to proliferate or differentiate, Wnt signalling controls several cellular processes such as apoptosis, survival and cell motility (Sokol and Melton 1992; Moon, DeMarais et al. 1993; Gieseler, Mariol et al. 1995; Huguet, Smith et al. 1995; Edwards 1999; Ellies, Church et al. 2000; Bonner, Lemon et al. 2003; Tepera, McCrea et al. 2003; Simon, Grandage et al. 2005). Recent studies have identified that Wnt5a signalling is upregulated in response to pathogens and enhances NF-κB-driven inflammatory gene expression in human monocytes and macrophages (Nau, Richmond et al. 2002; Chaussabel, Semnani et al. 2003; Blumenthal, Ehlers et al. 2006). Although, Wnt5a is known to activate the non-canonical pathway, recent studies have shown that it can also activate the canonical Wnt pathway (Mikels and Nusse 2006), which is regulated by GSK3β. However, regulation of GSK3β activity in the Wnt canonical pathway does not utilise the same phosphorylation event as in PI3K/Akt signalling (Ding, Chen et al. 2000; Wu and Pan 2010).

Under normal conditions, β-catenin is anchored to GSK3β by Axin and adenomatous polyposis coli (APC) which form the ‘destruction complex’. Casein kinase 1α (CK1α) phosphorylates β-catenin on Ser45 priming it for phosphorylation by GSK3β on Ser37 and Ser33 and Thr41 which in turn prepares the molecule for ubiquitination by the β-Trcp (beta-transducing-repeat-containing protein) E3 ubiquitin ligase subunit and degradation by the proteasome (Clevers 2006). Phosphorylation of APC is involved in regulating the release of ubiquitinated β-catenin from the Axin/APC/GSK3β complex (Ha, Tonozuka et al. 2004). Unphosphorylated β-catenin and APC bind to different sites on Axin and are both targeted for phosphorylation by CK1α and GSK3β. CK1α-induced phosphorylation of APC allows it to
bind to β-catenin with high affinity, and hence Axin is displaced and free to bind to new unphosphorylated β-catenin molecules (Xing, Clements et al. 2003; Xing, Clements et al. 2004). β-Catenin is thus maintained at a low level and the DNA-bound T cell factor/lymphoid enhancer factor (TFC/LEF) protein family suppresses the transcription of Wnt target genes (Seidensticker and Behrens 2000).

Binding of Wnt ligand to its receptor complex, composed of the seven-span transmembrane receptor Frizzled (FzI) and the single-pass transmembrane protein low-density lipoprotein (LDL)-related protein 5/6 (LRP5/6) leads to disruption of the APC/Axin/GSK3β complex as shown on Figure 1.7. Upon Wnt binding, CK1γ and GSK3β phosphorylate LPR6 which then binds with high affinity to Axin (Davidson, Wu et al. 2005; Zeng, Tamai et al. 2005). Axin is therefore re-located to the plasma membrane releasing β-catenin from the disrupted complex (Lee, Salic et al. 2003). The cytoplasmic Dishevelled (Dvl) protein is also required for Wnt signalling. Dvl is recruited to the LRP5/6 receptor and binds Axin and CK1α, thus titrating them away from the destruction complex. In addition, upon stimulation with Wnt, the Dvl-FRAT1/2 (frequently rearranged in T cells lymphomas or GSK3β binding protein-GBP) complex inhibits GSK3β induced β-catenin inhibition as FRAT1/2 competes with Axin for binding to GSK3β (Li, Yuan et al. 1999; Thomas, Frame et al. 1999) (Figure 1.7).
Figure 1.7. The role of GSK3β in the Wnt/β-catenin signalling pathway. In the absence of the Wnt ligand, GSK3β is bound to Axin forming a complex with CKIα and APC called the destruction complex which anchors β-catenin (β-cat). CKIα phosphorylates β-catenin on Ser45, priming it for phosphorylation by GSK3β on Thr41, Ser37 and Ser33 which target β-catenin for ubiquitination and proteosomal degradation. Stimulation of the Frizzled receptor and LRP5/6 receptor by Wnt results in recruitment of FRAT1/2 and Dvl in the destruction complex. GSK3β and CKIγ phosphorylate LRP6 and enhance its binding to Axin and the latter relocates to the plasma membrane. In addition, FRAT1/2 competes with Axin for binding to GSK3β leading to disruption of the destruction complex. GSK3β fails to phosphorylate β-catenin, which accumulates in the nucleus and transactivates genes regulated by LEF/TCF transcription factors. Adapted from (Doble and Woodgett 2003; Jope and Johnson 2004; Rayasam, Tulasi et al. 2009).
A number of studies have documented a role for GSK3β on regulating inflammatory responses (Cortes-Vieyra, Bravo-Patino et al. 2012). Inhibition of GSK3β enhanced LPS-induced IL-10 release from monocytes. In contrast, LPS-stimulated production of IL-1β, IL-6, TNF-α and IL-12, was attenuated by GSK3β inhibition. This effect was mediated through modulation of the interaction of p65/NF-κB with CREB (cAMP response element-binding protein) and the co-activator CREB-binding protein (CBP) (Martin, Rehani et al. 2005). The same group also demonstrated that GSK3β modulated LPS-induced production of IL-1Ra by human monocytes via affecting ERK1/2 activity downstream of targeting the inhibitory Ser71 residue on Rac (protein kinase Bα) (Rehani, Wang et al. 2009). Moreover, LPS-induced TNF-α expression in cultured neonatal mouse cardiomyocytes is associated with GSK3β activation. Inhibition of GSK3β led to increased TNF-α production, whereas its activation or overexpression attenuated LPS-induced TNF-α production (Shen, Fan et al. 2008).

Inhibition of GSK3β activity has also been shown to induce inflammatory responses in LPS stimulated macrophages. In the study conducted by Wang, Garcia et al. (2008), LPS stimulation of macrophages enhanced IFNβ production in a MyD88 (Myeloid differentiation protein 88)-dependent manner. Suppression of GSK3β activity in these cells resulted in enhanced IFNβ expression via inhibition of c-jun function. INFγ augments TNF-α production by TLR ligands in human primary monocytes and macrophages as a result of suppressing IL-10 production. Both GSK3β activation and suppression of MAPK activation were linked to INFγ-mediated suppression of IL-10. This process could be reversed by inactivation of GSK3β activity through the downstream effectors, CREB and AP-1 (activator protein 1) (Hu, Paik et
al. 2006). Hence, there is increasing evidence that GSK3β plays a key role in regulating the inflammatory responses upon pathogen stimulation.

Interestingly, there is evidence that pre-stimulation of macrophages with TNF-α induced LPS tolerance in a GSK3β-dependent pathway. According to Park, Park-Min et al. (2011), GSK3β activation upon TNF-α stimulation, suppressed chromatin accessibility and NF-κB-mediated transactivation of inflammatory genes. Additionally, GSK3β has been shown to regulate mTORC1-induced pro- or anti-inflammatory cytokine production. This was mediated by the downstream target of mTORC1, S6K1, which in response to LPS phosphorylates GSK3β leading to increased production of IL-12 and IL-10 in human monocytes (Wang, Brown et al. 2011). In addition in a recent study by Baarsma, Meurs et al. (2011), GSK3β mediated cigarette smoke- and IL-1β- induced production of CXCL-8 in airway smooth muscle cells by enhancing p65 transactivation activity. The different effects that GSK3β exerts depend on the type of cell, the nature of the pathogen and the physiological state of the cell and the means by which the kinase is inhibited. Most studies have been focused on the role of GSK3β in response to bacterial LPS, but not much work has been conducted to evaluate the role of GSK3β activity in response to exogenously derived reactive oxygen species. In addition, most of the pro- or anti-inflammatory effects of GSK3β inhibition have been tested via inhibition of the kinase with small molecule compounds that are known to have off-target effects and regulate other kinases with known inflammatory roles such as histone deacetylases, CDK2-cyclin A, ERK8 and inositol monophosphate. There is an emerging need to evaluate the role of GSK3β on regulating inflammation upon corticosteroid treatment and investigate how is that modulated by reactive oxygen species which are known to be elevated in inflammatory cells in the lungs of COPD patients. The existence of such different
published functions of GSK3β, summarised on Table 1.1, derives from the different means (different compounds known to inhibit GSK3β function) that the authors have used to regulate GSK3β function in each study. Further investigation is required to elucidate the mechanisms that GSK3β employs to control the pathogenesis of infection and the expression of pro- or anti-inflammatory genes (Cortes-Vieyra, Bravo-Patino et al. 2012).

Table 1.1. GSK3β modulation of inflammatory responses

<table>
<thead>
<tr>
<th>Cell type</th>
<th>GSK3β Inhibition/activation</th>
<th>Pro- or anti-inflammatory</th>
<th>Inflammatory mediators</th>
<th>Signalling effectors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Monocytes</td>
<td>Inhibition</td>
<td>Anti</td>
<td>LPS induced: IL-6, TNF-α, IL1β</td>
<td>CREB, CBP</td>
</tr>
<tr>
<td>Mouse cardiomyocytes</td>
<td>Inhibition</td>
<td>Pro-</td>
<td>LPS induced: TNF-α</td>
<td>Not studied</td>
</tr>
<tr>
<td>Human monocytes</td>
<td>Inhibition</td>
<td>Pro-</td>
<td>LPS induced: IL1-Ra</td>
<td>ERK1/2</td>
</tr>
<tr>
<td>Human monocytes</td>
<td>Activation</td>
<td>Pro-</td>
<td>IFNγ induced: IL-10</td>
<td>CREB, AP-1</td>
</tr>
<tr>
<td>Human monocytes</td>
<td>Inhibition</td>
<td>Anti</td>
<td>LPS induced: IL10, IL12</td>
<td>mTORC1, S6K1</td>
</tr>
<tr>
<td>Airway smooth muscle cells</td>
<td>Inhibition</td>
<td>Pro-</td>
<td>CSE- and IL-1β induced: IL8 and eotaxin</td>
<td>p65-dependent gene transactivation</td>
</tr>
<tr>
<td>Human monocytes</td>
<td>Inhibition</td>
<td>Pro-</td>
<td>Decreased GR-mediated transrepression</td>
<td>Ser404 GR phosphorylation</td>
</tr>
</tbody>
</table>
1.5. Signalling to the nuclear factor kappa B (NF-κB)

1.5.1. NF-κB: structure and function

NF-κB signalling regulates multiple aspects of innate and adaptive immunity (Silverman and Maniatis 2001; Bonizzi and Karin 2004). The mammalian NF-κB family consists of five members, p65 (RelA), RelB, c-Rel, p50/p105 (NF-κB1) and p53/p100 (NF-κB2). In unstimulated cells these exist as homo- or heterodimers bound to the inhibitor of NF-κB (IκB) which prevents their translocation to the nucleus, thereby maintaining the transcription factor inactive (Duckett, Perkins et al. 1993; Campbell and Perkins 2006) NF-κB proteins contain an N-terminal conserved 300 amino acids Rel homology domain (RHD) which is responsible for dimerisation, interactions with IκBs and DNA binding (Hayden and Ghosh 2004; Perkins 2006). NF-κB signalling occurs either through the classical or the alternative pathway (Bonizzi and Karin 2004). The alternative (non-canonical) pathway was not investigated in this thesis so only the classical pathway is discussed.

In the classical pathway, in response to cellular stimulation via regulatory inducers, such as TNF-α, IL-1β or LPS, IκB is phosphorylated, ubiquitinated and degraded by the proteasome. Degradation of IκB is initiated upon specific phosphorylation by active IKK. IKK activity can be purified as a 700-900 kDa complex consisting of two kinase subunits, IKKα (IKK1) and IKKβ (IKK2) and the regulatory subunit NF-κB essential modifier (NEMO) or IKKγ (Rothwarf and Karin 1999; Ghosh and Karin 2002). In the classical NF-κB pathway, IKKβ is necessary for IκBα phosphorylation on Ser32 and Ser34, and IκBβ on Ser19 and Ser23. Once phosphorylated, the IκB proteins are recognised by the Skp1-Culin-Roc1/Rbx1/Hrt-1-F-Box (SCRF) family of ubiquitin ligases (Ben-Neriah 2002) which bind to the phosphorylated E3
recognition sequence on IκB (Yaron, Gonen et al. 1997; Wu and Ghosh 1999). Polyubiquitination of IκBα takes place on the conserved Lys21 and Lys22 residues (Alkalay, Yaron et al. 1995; Scherer, Brockman et al. 1995; DiDonato, Mercurio et al. 1996) and targets the molecule for proteasomal degradation.

Both p65 and c-Rel have a consensus PKA site at Ser276 (Mosialos and Gilmore 1993; Neumann, Grieshammer et al. 1995). Degradation of IκB activates the catalytic subunit of p65 and phosphorylates it on Ser276 (Zhong, SuYang et al. 1997; Zhong, Voll et al. 1998). p65 phosphorylation is required for promoting its binding to DNA and it also provides a site for interaction with the co-activator (cAMP-response element binding protein/adenoviral protein E1A) CBP/p300 (Zhong, Voll et al. 1998; Zhong, May et al. 2002).

NF-κB binds to promoter and enhancer regions containing κB sites with the consensus sequence GGGRNYYCC (N=any base, R=purine and Y=pyrimidine). The N-terminal Ig-like domain of the RHD is responsible for sequence specificity, whereas the C-terminal domain forms the dimerisation interface (Chen, Huang et al. 1998; Chen, Ghosh et al. 1998; Huxford, Huang et al. 1998). RelB, c-Rel and p65 contain the transactivation domain (TAD) which is located towards the C-terminus. The TADs promote transcription via dissociation of repressor molecules recruitment of co-activators such as CBP/p300, CBP, the CBP/p300 associated factor (P/CAF) and the activating transcription factor-2 (ATF-2). These have intrinsic histone acetyltransferase (HAT) activity and lead to acetylation of core histones and also recruit other HAT enzymes to the transactivation site. These enzymes acetylate lysine (K) residues on histone tails, which facilitates access to other transcription factors and initiation of transcription (Grunstein 1997; Li, Carey et al. 2007).
1.6. The role of histone acetylation on driving gene transcription

Nucleosomal DNA is tightly packaged to varying degrees leading to chromatin compaction as small as 10nm fibre to high order structures such as the mitotic chromosomes. Condensation of DNA in chromatin leads to coiling of DNA on the surface of the nucleosome core and the folding of the nucleosome assemblies resulting in decreased accessibility to transcription factors and gene silencing (Wu 1997; Rahman, Marwick et al. 2004). The chromatin structure consists of DNA (165 base pairs) wrapped around an octamer of core histones composed of two molecules of each the histones H2A, H2B, H3 and H4. Post-translational modifications (PTMs), such phosphorylation, acetylation and methylation on histones regulate gene expression (Urnov and Wolffe 2001). The N-terminal region of the histone tail is where the highest density of PTM possibilities exists. These dynamic modifications regulate the structure and the function of chromatin through two main mechanisms (Kouzarides 2007); causing a change in chromatin dynamics (euchromatin vs heterochromatin) (Shogren-Knaak, Ishii et al. 2006) or serving as a docking site for non-histone proteins including chromatin remodelling engines leading to regulation of chromatin organisation via recruitment of transcription factors and co-regulator complexes (Ruthenburg, Allis et al. 2007; Gardner, Allis et al. 2011).

The combination of PTMs in the appropriate context gives rise to a histone code or language which is interpreted by an array of diverse proteins (Gardner, Allis et al. 2011; Oliver and Denu 2011). These can be classified as ‘writers’, ‘erasers’ and ‘readers’. Writers and erases are the enzymes such as HATs, HDACs, kinases, phosphatases, methyltransferases and demethylases. Readers are proteins that recognise modified histones, such as those belonging to the bromodomain family, which identify acetylated lysine residues in histones and non-histone proteins (Mujtaba, Zeng et al. 2007; Sanchez and Zhou 2009). Among all
different PTMs on histones that regulate gene transcription, here we focus the discussion on acetylation since it is the one examined in this thesis. Acetylation of $\varepsilon$-group on the lysine (K) residues of histones leads to neutralisation of the positive charge on the histone tails leading to weakening of the DNA backbone and facilitates access to transcription factors (Grunstein 1997) (Figure 1.8).

As described in the previous section, transcriptional coactivators, such as CBP/p300, CBP, P/CAF and ATF-2 have intrinsic HAT activity enabling initiation of DNA uncoiling and allowing binding of transcription factors (Chen, Fischle et al. 2001). There are several acetylation targets in each histone as shown on Figure 1.8. NF-κB activation results in acetylation of specific lysine residues (primarily K8 and K12, and K5 and K16 at a lower rate) on histone H4 correlating with increased expression of inflammatory genes in human airway epithelial cells (Ito, Barnes et al. 2000). In addition, histone acetylation is associated with other modifications on histones. H3 Ser10 phosphorylation inhibits H3 K9 methylation which blocks the acetylation of this residue (Richards and Elgin 2002; Hirota, Lipp et al. 2005).

Methylation of H4 Arg3 stimulated CBP/p300 induced acetylation of H4 K5, K8, K12 and K16. This event enhances H3 methylation on Arg residues (Arg2, Arg17 and Arg27) (Daujat, Bauer et al. 2002; Huang, Litt et al. 2005). It is therefore clearly evident that both positive and negative crosstalk regulates histone acetylation patterns and the associated state of the chromatin (Gardner, Allis et al. 2011).
1.6.1. Histone deacetylases (HDACs): role and classes

The transcriptional activity of inflammatory genes is regulated by reversal of histone acetylation which is controlled by the enzymes HDACs. These act by removing acetyl groups on amino terminal K residues of histones resulting in DNA condensation into chromatin and gene silencing (Henikoff, Strahl et al. 2008; Smith and Workman 2012) (Figure 1.8). HDACs can also inhibit gene transcription via acetylation of non-histone proteins, such as NF-κB by disrupting their activity (Sengupta and Seto 2004). HDACs are therefore critical for regulation of cellular processes, such as cell cycle progression and differentiation (McLaughlin, Finn et al. 2003).

There are 18 members of the HDAC family identified in humans (HDACs 1-11) and 7 members of the silent information regulator (SIRT) family (SIRTs 1-7) (de Ruijter, van Gennip et al. 2003; Michan, Li et al. 2010). HDACs are classified into four classes; Class I HDACs include HDAC1, 2, 3, and 8 and predominantly located in the nucleus. Class II HDACs include HDACs 4-7, 9 and 10 which are expressed in a cell-specific pattern. The Class II HDACs include SIRT1-7 NAD⁺ family and control cellular functions such as inflammation, cell senescence and target non-histone substrates. Finally, Class IV HDACs include HDAC11 (Chen, Mu et al. 2002; Sengupta and Seto 2004; Michan and Sinclair 2007).

HDACs are required to exist within a co-repressor complex in order to attain their full deacetylase activity. HDAC2, for example, can associate with three co-repressor complexes; Sin3, NuRD/Mi2 and CoREST. Sin3 and Mi2 contain a core of four proteins HDAC1, RbAp46 and RbAp48 (Retinoblastoma protein associated protein 46/48) and the mSin3A protein in the Sin3 complex and Mi2 in the NuRD/Mi2 complex (Sengupta and Seto 2004).
Introduction

**Figure 1.8. Chromatin remodelling-controlled gene expression.** Acetylation of the lysine residues of histone tails is mediated by histone acetyltransferases leading to the opening up of the chromatin structure allowing the binding of transcription factors and RNA polymerase II (RNA Pol II). Removal of the acetyl groups is mediated by the histone deacetylases (HDACs). Histone deacetylation leads to repacking of chromatin and lack of gene expression or gene silencing. Adapted from (Ito, Yamamura et al. 2006).

1.6.2. HDACs regulate NF-κB activity in COPD

The activity of NF-κB is also regulated by its acetylation status. CBP/p300 acetylates p65 at lysines 218, 221 and 310 (Chen, Fischle et al. 2001). Acetylation of K221 within p65 inhibits its association with IκBα and thus promotes DNA binding of the transcription factor, whereas acetylation of the K310 residue is necessary for its function (Chen, Mu et al. 2002; Furia, Deng et al. 2002). By contrast, acetylation of K122 and 123 reduces p65 DNA binding affinity (Kiernan, Bres et al. 2003). The pattern of p65 acetylation may also play a critical role in selective gene transactivation and this may be a plausible mechanism of the selective gene induction documented in COPD (Ito 2007). The p50 subunit has been also found to be
acetylated on K431, K440 and K441 in vitro by CBP/p300 (Furia, Deng et al. 2002). Although the functional role of p50 acetylation is not very clear, it has been suggested to increase DNA binding affinity and increase recruitment of p300 on the NF-κB transactivation promoter sites (Deng and Wu 2003; Deng, Zhu et al. 2004).

HDAC1, HDAC2 and HDAC3 have been shown to interact with RelA/p65, regulate its activity and control inflammatory gene transcription (Ashburner, Westerheide et al. 2001; Chen, Fischle et al. 2001; Zhong, May et al. 2002). Deacetylation of p65 on K221 (by HDAC3) and K310 (by SIRT1) has been shown to inhibit NF-κB transactivation function. Reduction of HDAC activity in alveolar macrophages of smokers compared to non-smokers correlates with increased expression of NF-κB regulated pro-inflammatory genes (Ito, Lim et al. 2001). In addition, a reduction in total HDAC activity in the peripheral lung, bronchial biopsies and alveolar macrophages from COPD patients, compared to healthy subjects, correlated with disease severity and increased pro-inflammatory gene expression of CXCL8 and increased histone acetylation at the NF-κB binding site (Ito, Ito et al. 2005; Szulakowski, Crowther et al. 2006). Cigarette smoke-induced reduction in HDAC2 activity has been linked with increased RelA/p65 activity and subsequent increase of pro-inflammatory gene transcription (Yang, Chida et al. 2006). Binding of HDAC2 to NF-κB does not involve interaction with the DNA binding domain of p65, since the HDAC inhibitor trichostatin A (TSA) does not interfere with NF-κB DNA binding capacity, hence the domains remain to be identified (Yu, Zhang et al. 2002).
1.7. The anti-inflammatory role of corticosteroids

1.7.1. The glucocorticoid receptor (GR): structure and isoforms

The glucocorticoid receptor is part of the nuclear receptor (NR or steroid hormone receptor SHR) superfamily comprising also of oestrogen (ER), androgen (AR) and progesterone receptors (PR) (Evans 1988). GR consists of an N-terminal domain comprising of a first TAD (activation function 1/AF-1) responsible for association with basal transcription factors (Hollenberg, Weinberger et al. 1985; Kumar and Thompson 1999), a DNA-binding domain (DBD) which facilitates dimerisation and DNA-binding (Hard, Kellenbach et al. 1990; Dahlman-Wright, Wright et al. 1991; Luisi, Xu et al. 1991; van Tilborg, Bonvin et al. 1995; Kumar and Thompson 1999) and a C-terminal ligand binding domain (LBD) which comprises of a second TAD (AF-2) and protein binding sites (Darimont, Wagner et al. 1998; Hong, Darimont et al. 1999; Robinson-Rechavi, Escriva Garcia et al. 2003; Ricketson, Hostick et al. 2007). Close to the DBD and at the end of the LBD, lie two nuclear localisation sites, the ligand-independent NL1 and ligand-dependent NL2 which are responsible for promoting GR nuclear translocation (Savory, Hsu et al. 1999).

GR exists in multiple isoforms as a result of alternative splicing (GRα, GRβ, GRγ, GR-A, GR-P) and different sites of initiation of translation (GRα-A, GRα-B, GRα-C1, GRα-C2, GRα-C3, GRα-D1, GRα-D2 and GRα-D3. GRα is the predominant isoform found in most human cell types and it consists of 777 amino acids, whereas the truncated form GRβ (742 amino acids) does not bind to corticosteroids and it is not ubiquitously expressed. It can however act as a negative regulator of GRα (Duma, Jewell et al. 2006; Lu and Cidlowski 2006). In addition, a number of polymorphisms have been identified in the human GR gene, which affect its functions (Charmandari, Kino et al. 2008; Gross, Lu et al. 2009).
1.7.2. Ligand binding–dependent GR activation

In resting cells, GR is located in the cytoplasm and it is associated with a molecular chaperone complex which disrupts its ligand-binding pocket and inactivates the NL site. The chaperone complex consists of heat shock proteins, Hsp90, Hsp70, the Hsp90-binding protein p23, the hsp-organizing protein (Hop), the tetratricopeptide repeat proteins that bind Hsp90 as well as the C-terminus of Hsp70-interacting protein (CHIP) (Pratt, Galigniana et al. 2004; Grad and Picard 2007).

Corticosteroids are small hydrophobic molecules that diffuse through the membrane or enter the cell via the steroid hormone recognition and effector complex (Haller, Mikics et al. 2008). Upon ligand binding, GR undergoes conformational changes (Bledsoe, Montana et al. 2002; Bledsoe, Stewart et al. 2004), which allow it to dissociate from the chaperone complex and free its NL site. Nuclear import of GR is regulated by importin-α and importin 13 via interaction with the NL1 and NL2 sites (195, 217, 218). Nuclear export of GR is regulated by the calreticulin-bases and chromosome region maintenance 1 (CRM1) mechanism, and its location is also determined by the nuclear retention signal (NRS) (Savory, Hsu et al. 1999; Holaska, Black et al. 2002; Walther, Lamprecht et al. 2003; Kumar, Chaturvedi et al. 2004; Carrigan, Walther et al. 2007).

GR translocates into the nucleus where it exerts its positive or negative transcriptional effects (Savory, Hsu et al. 1999; De Bosscher, Vanden Berghe et al. 2003; De Bosscher, Vanden Berghe et al. 2006; Beck, Vanden Berghe et al. 2009). The GR homodimers bind in the major groove of DNA via the DBD targeting the consensus GR responsive elements (GREs) (5′ GGT ACA nnn TGT TCT 3′) found on the promoter region of GR-responsive genes (Schena, Freedman et al. 1989; La Baer and Yamamoto 1994; Rhen and Cidlowski 2005).

Activation of GR-responsive genes can be also mediated via the interaction of
transcriptional coactivator molecules (including CREB) and the DNA-bound GR (Roth, Johnson et al. 2002). This is associated with local acetylation of histones induced by the intrinsic histone acetyltransferase activity that the coactivators possess. Chromatin remodelling engines (such as the SW1/SNF proteins) are then recruited at the site of transcription via bromodomains with subsequent recruitment of RNA polymerase II resulting in the initiation of transcription (Adcock and Ito 2000). GR-responsive genes include the β2-adrenergic receptor gene (Clark and Lasa 2003) and anti-inflammatory genes such as the glucocorticoid-inducible leucine zipper (GILZ) (Clark 2003; Barnes 2006; Ayroldi and Riccardi 2009).

1.7.3. Corticosteroids: mechanism of action

The major anti-inflammatory function of corticosteroids is exerted via repression of genes that encode for cytokines, chemokines, adhesion molecules, inflammatory mediators and receptors (Barnes and Adcock 2003; Beck, Vanden Berghe et al. 2009) whose transcription is regulated by NF-κB and AP-1 (Adcock, Ito et al. 2004). Upon ligand binding and nuclear translocation, GR recruits HDAC2 in the activated inflammatory gene complex leading to deacetylation of histones and suppression of inflammatory gene transcription (transrepression). Acetylation of GR (on K492-495) upon ligand binding allows it to associate with NF-κB and be targeted by HDAC2 (Ito, Yamamura et al. 2006). GR function can be also regulated by HDAC6 via acetylation of Hsp90 and regulation of GR nuclear translocation (Kovacs, Murphy et al. 2005).

In addition to the mechanisms described above, GR can exert its anti-inflammatory actions via induction of MKP1 which in turn inhibits the MAP-kinase signalling pathway and the expression of pro-inflammatory genes (Clark and Lasa 2003). Moreover, corticosteroids have
been shown to reverse the ribonuclease-induced mRNA degradation of TNF-α upon inflammatory stimulation via transactivation of proteins (such as tristetrapolin) that destabilise mRNAs of inflammatory proteins (Smoak and Cidlowski 2006). Another mechanism employed by corticosteroids and suppress inflammatory gene expression is by interfering with the phosphorylation status of the C-terminal domain of RNA polymerase II leading to interruption of initiation of transcription of pro-inflammatory genes (Nissen and Yamamoto 2000).

1.8. Corticosteroid insensitivity in COPD

Corticosteroids are the most effective anti-inflammatory treatments available. Inflammatory and immune diseases such as asthma, rheumatoid arthritis, autoimmune diseases and inflammatory bowel disease are treated by corticosteroids. By contrast, other inflammatory diseases, including, interstitial pulmonary fibrosis, acute respiratory distress syndrome and cystic fibrosis are relatively resistant to the anti-inflammatory actions of corticosteroids (Culpitt, Rogers et al. 2003).

Most COPD patients respond poorly to high doses of inhaled oral corticosteroids leading to the concept that COPD is a relatively corticosteroid insensitive disease (Barnes and Adcock 2009). A number of studies have documented that corticosteroids have no effect on inflammatory cell number or the release of pro-inflammatory mediators in sputum or airways of COPD patients (Keatings, Jatakanon et al. 1997; Culpitt, Maziak et al. 1999; Bourbeau, Christodoulopoulos et al. 2007). This observation is also reflected ex vivo, where corticosteroids fail to suppress inflammatory cytokine release in alveolar macrophages derived BAL from patients with COPD (Culpitt, Rogers et al. 2003). Interestingly, only 10% of COPD patients show clinical response to inhaled corticosteroids and their increased
numbers of airway eosinophils suggests that they possibly have concomitant asthma (Papi, Romagnoli et al. 2000; Brightling, McKenna et al. 2005).

1.8.1. Molecular mechanisms of corticosteroid insensitivity in COPD

The molecular mechanisms that regulate corticosteroid insensitivity in COPD remain unclear. There is still a variation in corticosteroids responsiveness among different cell types and tissues (Gross and Cidlowski 2008) and its unlikely that an intrinsic relative corticosteroid insensitivity of one particular cell type or of the lung accounts for the overall reduction of corticosteroid function documented in COPD (Marwick and Chung 2010).

1.8.1.1. Regulation of GRβ activity

One mechanism suggested to lead to corticosteroid unresponsiveness in COPD is increased expression of the dominant negative GRβ isoform (Strickland, Kisich et al. 2001). However, since levels of GRβ are not found to be elevated in COPD, while GRα expression is shown to be reduced, the ratio of GRβ to GRα in the lung or inflammatory cells may account for the impaired corticosteroid function. Very few studies have assessed this ratio in COPD, particularly in cells from the lung or small airways, and there is no functional effect assigned to increased GRβ ratio identified to be able to conclude that this mechanism is responsible for the developments of corticosteroid insensitivity in COPD (Marwick and Chung 2010).
1.8.1.2. Genetic variation

Genetic susceptibility is likely to play a role in the development of COPD but in contrast with corticosteroid insensitive asthma, no direct link between genetic mutation and corticosteroid unresponsiveness has been documented in COPD (Hakonarson, Bjornsdottir et al. 2005). However, reduced antioxidant capacity due to genetic variations, such as the 213Gly variant of the antioxidant SOD3 protein, may contribute to oxidant stress mediated development of relative corticosteroid insensitivity (Juul, Tybjaerg-Hansen et al. 2006; Young, Hopkins et al. 2006; Mak, Ho et al. 2007; Smolonska, Wijnega et al. 2009).

1.8.1.3. Oxidative stress and corticosteroid responsiveness

As described earlier, the elevated oxidant burden in the lungs of COPD patients derives from both exogenous (cigarette smoking, pollutants) and endogenously (respiratory burst of inflammatory cells) and plays a central role in COPD. It also plays a key role in the development of corticosteroid insensitive inflammation (Kirkham and Rahman 2006; Rahman and Adcock 2006; Cienciewicki, Trivedi et al. 2008). Cigarette smoking induces corticosteroid insensitivity in several experimental models and the clinical observation that smoking asthmatics respond poorly to corticosteroids compared to non-smoking asthmatics strongly supports the role of oxidative stress in regulating corticosteroid function (Ito, Lim et al. 2001; Tomlinson, McMahon et al. 2005; Marwick, Caramori et al. 2009). One mechanism that has been suggested to mediate this oxidant stress-mediated affect links the redox status of the cells with GR nuclear translocation (Okamoto, Tanaka et al. 1999).
Introduction

1.8.1.3.1. The role of HDAC2 in oxidant-induced corticosteroid insensitivity in COPD

The primary mechanism leading to reduced activity of HDAC2 in the alveolar macrophages and peripheral lung tissue from smokers and patients with COPD compared to healthy non-smokers is post-translational modifications, such as phosphorylation, carbonylation and nitration (Ito, Lim et al. 2001; Marwick, Kirkham et al. 2004; Yang, Chida et al. 2006). Of all HDACs, HDAC2 activity is affected the most, but reductions in HDAC3 and HDAC5 have been also documented. Furthermore, the mRNA expression of other HDACs, such as HDAC5 and HDAC8 are decreased in lung tissue from COPD patients, correlating with disease severity (Ito et al. 2005). Patients with severe COPD have less than 5% of HDAC2 in their lungs of what found in the lungs of healthy subjects. The mRNA levels of HDAC2 have been also found to be reduced in COPD but the mechanism that regulates transcriptional regulation of the HDAC2 gene remain still unclear (Barnes 2009). Decreased HDAC2 activity has been also documented in vitro, where cigarette smoke extract resulted into modifications by aldehydes and protein nitration (Yang, Chida et al. 2006). These post-translational modifications on HDAC2 impair its catalytic activity and can target the enzyme for proteasomal degradation (Galasinski, Resing et al. 2002; Ito, Hanazawa et al. 2004; Rahman, Marwick et al. 2004; Marwick, Ito et al. 2007; Meja, Rajendrasozhan et al. 2008; Osoata, Yamamura et al. 2009).

The exact mechanisms by which reactive oxygen and nitrogen species regulate post-translational modifications on HDAC2 have not yet been elucidated (Ito, Hanazawa et al. 2004). Recent studies have implicated CK2α-mediated HDAC2 phosphorylation as being important as it leads to ubiquitination and subsequent degradation of HDAC2 by the proteasome (Adenuga, Yao et al. 2009; Adenuga and Rahman 2010). Ser421 and Ser423 on
HDAC2 are critical for the interaction with the co-repressor complex Mi2/mSin3A (Sengupta and Seto 2004). Phosphorylation on Ser394, Ser422 and Ser424 also regulates HDAC2 interactions with transcription factors, co-repressor complex formation, CBP recruitment, acetylation on lysine residues and consequently increased transrepression activity (Adenuga and Rahman 2010). Thus, although basal phosphorylation of HDAC2 is critical for its deacetylase activity, hyperphosphorylation may lead to complex dissociation and inhibit deacetylase activity (Galasinski, Resing et al. 2002).

As previously discussed, the acetylation/deacetylation balance is critically important for regulation of gene transcription (Li, Carey et al. 2007). Since HDAC2 activity is fundamental for GRα transrepression of inflammatory genes, reduction of its activity can impair corticosteroid function (Figure 1.9). In agreement with this, restoration of HDAC2 activity correlates with restoration of corticosteroid function in experimental models of COPD (Cosio, Tsaprouni et al. 2004; Marwick, Caramori et al. 2009; Zijlstra, Ten Hacken et al. 2012). In addition to HDAC2, HDAC1 and HDAC3 protein levels are reduced in macrophages in response to cigarette smoking and this reduction has been associated with tyrosine nitration and α-β unsaturated aldehyde adduct formation (Yang, Chida et al. 2006).

Since HDACs are involved in repressing pro-inflammatory genes, activation of HDAC2 may have therapeutic potential, and studies using the bronchodilator theophylline documented that its anti-inflammatory properties are associated with reversal of HDAC2 activity (Ito, Lim et al. 2002). Similarly, the dietary polyphenol compound, curcumin restored corticosteroid efficacy by reversing cigarette smoke induced reduction of HDAC2 in monocytes (Meja, Rajendrasozhan et al. 2008). The exact mechanisms that theophylline or curcumin restore
HDAC2 function have not been fully elucidated yet. In contrast, there is evidence that HDAC inhibitors have anti-inflammatory functions on their own (Grabiec, Tak et al. 2011).

1.8.1.3.2. The role of kinase signalling pathways in regulating corticosteroid insensitivity in COPD

Increased activation of the p38 MAPK in peripheral lungs and alveolar macrophages correlated with reduction in FEV₁ and FEV₁/FVC in COPD patients (Renda, Baraldo et al. 2008). Increased p38 MAPK activity has been shown to induce phosphorylation of GRα resulting in disruption of its ligand binding ability (Irusen, Matthews et al. 2002) (Figure 1.9). Corticosteroids inhibit the p38 MAPK induced inhibition of GRα via induction of the p38 MAPK-negative regulator MKP-1. In support with the hypothesis that MKP-1 signalling is linked with corticosteroid insensitivity in COPD, MKP-1 levels were found reduced in response to the corticosteroid dexamethasone in patients with severe corticosteroid insensitive asthma compared to those with non-severe asthma (Bhavsar, Hew et al. 2008).

Oxidant mediated activation of the PI3K/Akt pathways has also been implicated in regulating corticosteroid function via modulation of HDAC2 activity (Figure 1.9). Inhibition of the PI3Kδ isoform restored HDAC2 activity correlating with restoration of corticosteroid function in oxidative stress-induced corticosteroid experimental murine models (Marwick, Caramori et al. 2009). However, PI3Kγ had no effect on steroid responsiveness. In agreement with this, selective inhibition of PI3Kδ restored corticosteroid regulated anti-inflammatory mediator expression in PBMCs from patients with COPD compared to smokers with normal lung function (Marwick, Caramori et al. 2010). HDAC2 activity has been shown to be involved in mediating PI3K induced regulation of corticosteroid function in these
studies, however the exact mechanism involved is not known yet. Modulation of post-translational modifications on HDAC2 and other co-repressors such as Mi2 and mSin3a in response to activated PI3K have been suggested to play a role in steroid insensitivity (Marwick, Caramori et al. 2009; Marwick, Stevenson et al. 2010). Tyrosine nitration and hyperphosphorylation on the enzyme in response to oxidant stress is a plausible explanation but the exact signalling mediators downstream the PI3K/Akt pathway that control this event have not been identified yet (Marwick, Caramori et al. 2010). Interestingly, recent studies have proposed that the downstream effector of the PI3K/Akt pathway, GSK3β mediates hormone-dependent phosphorylation of GRα on Ser404. GSK3β regulated phosphorylation of GRα disrupts its interaction with its transcriptional co-regulators (such as CBP/p300) and p65 and impairs its function on inhibiting the NF-κB dependent inflammatory gene transactivation (Blind and Garabedian, 2008; Galliher-Beckley et al., 2008; Ismaili and Garabedian, 2004; Wang et al., 2002). Several studies have also suggested the involvement of ERK1/2 and JNK in regulating GRα phosphorylation and subsequently its transrepression properties (Galliher-Beckley and Cidlowski 2009) (summarised in Figure 1.9).
**Figure 1.9. Mechanism of corticosteroid insensitivity in COPD.** Reactive oxygen species derived from cigarette smoking, pollution or released from inflammatory cells, lead to post-translational modifications on HDAC2 that interrupt the enzyme’s ability to bind to co-repressor and exert its deacetylase function on the NF-κB site of pro-inflammatory gene transcription. Oxidant stress induced activation of MAPKs has been associated with increased phosphorylation on GRα disrupting its ligand ability. Activation of the PI3K/Akt pathway has been associated with reduction on HDAC2 activity leading to increased histone acetylation of pro-inflammatory genes; and inhibition of the PI3Kδ isoform restores corticosteroid insensitivity in cigarette smoking experimental models. The bronchodilator, theophylline has also been shown to restore corticosteroid function by protecting HDAC2 from oxidant stress induced reduction on its activity.
**1.9. Rationale for the study**

The relatively corticosteroid unresponsive inflammation in the airways of COPD patients is a central factor in the development and progression of the disease (Barnes 2008; Barnes and Adcock 2009). The increased oxidant burden in the lungs of COPD patients has a profound impact on inflammation and has been implicated to drive the corticosteroid unresponsiveness. Oxidant stress leads to activation of several kinase pathways including p38 MAPK, ERK1/2 and Akt and pro-inflammatory transcription factors such as NF-κB leading to an enhanced inflammatory response in human macrophages (Irusen, Matthews et al. 2002; Marwick, Ito et al. 2007). The constitutively active serine/threonine kinase GSK3β is also modulated by oxidative stress and has been linked to several inflammatory diseases (Henriksen and Dokken 2006; Billadeau 2007; Jope, Yuskaitis et al. 2007). GSK3β activity is negatively regulated by phosphorylation of Ser9 which can be mediated by ERK1/2, Akt and p38 MAPK (Grimes and Jope 2001). Since these kinase pathways have been linked to oxidant-mediated induction of corticosteroid insensitivity, GSK3β may represent an important downstream effector molecule of oxidant-mediated signalling.
1.9.1. Hypothesis

Oxidant stress-induced regulation of GSK3β activity plays a role in corticosteroid responsiveness during inflammatory responses of immune cells.

1.9.2. Aims

In order to determine the above hypothesis, the following aims were set:

I. Measure levels of p-Ser9-GSK3β in primary macrophages and peripheral blood monocytes from COPD patients, smokers with normal lung function and non-smokers as a marker of GSK3β activity. Determine whether exposure of primary healthy peripheral blood monocytes to H$_2$O$_2$-derived oxidant stress affects GSK3β activity and identify the kinases involved in mediating this effect.

II. Investigate how modulation of GSK3β activity regulates corticosteroid-mediated suppression of inflammatory cytokine release in human peripheral monocytes.

III. Identify the mechanism that mediates GSK3β-regulated modulation of corticosteroid function, with respect to GR nuclear translocation, GR binding to p65, p65 transactivation and HDAC2 activity.

IV. Investigate whether GSK3β inhibition regulates global mRNA expression of corticosteroid responsive pro-inflammatory genes.
Chapter II

Materials and Methods
### 2.1. Materials

#### Table 2.1.1. Cell culture materials

<table>
<thead>
<tr>
<th>MATERIAL</th>
<th>SUPPLIER</th>
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<tbody>
<tr>
<td>Ficoll Paque™ Plus</td>
<td>GE Healthcare, Buckinghamshire, UK</td>
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<tr>
<td>Hank’s Buffered Saline Solution (HBSS)</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Heat-inactivated, fetal bovine serum (FBS)</td>
<td>PAA Laboratories Ltd, UK</td>
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<td>L-Glutamine</td>
<td>Sigma-Aldrich, Dorset, UK</td>
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<tr>
<td>OPI Media Supplement</td>
<td>Sigma-Aldrich, Dorset, UK</td>
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<tr>
<td>Phosphate-buffered saline (PBS)</td>
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<tr>
<td>Roswell Park Memorial Institute (RPMI) 1640 medium</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>MEM Non-essential Amino Acid Solution (100×)</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Sterile tissue culture grade water</td>
<td>Sigma-Aldrich, Dorset, UK</td>
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#### Table 2.1.2. Molecular biology reagents

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<th>REAGENT</th>
<th>SUPPLIER</th>
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<tr>
<td>5x siRNA buffer</td>
<td>Dharmacon, Colorado, USA</td>
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<td>AMV reverse transcriptase enzyme</td>
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<tr>
<td>AMV reverse transcriptase reaction buffer</td>
<td>Promega, Southampton, UK</td>
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<tr>
<td>Bovine Serum Albumin (BSA)</td>
<td>Sigma-Aldrich, Dorset, UK</td>
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<tr>
<td>Complete Protease Inhibitor Cocktail Tablets</td>
<td>Roche Diagnostics Ltd, Burgess Hill, UK</td>
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<td>Deoxy nucleotide triphosphates (dNTPs)</td>
<td>Bioline, London, UK</td>
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<tr>
<td>Dimethylsulphoxide (DMSO)</td>
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<tr>
<td>Dithiothreitol (DTT)</td>
<td>Active Motif, Rixensart, Belgium</td>
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<tr>
<td>Sodium acetate</td>
<td>Sigma-Aldrich, Dorset, UK</td>
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<tr>
<td>Full-Range Rainbow Molecular Weight Marker</td>
<td>GE Healthcare, Buckinghamshire, UK</td>
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<tr>
<td>Hank’s Buffered Saline Solution (HBSS)</td>
<td>Sigma-Aldrich, Dorset, UK</td>
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**Materials and methods**

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
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</thead>
<tbody>
<tr>
<td>Luminata Forte Western HRP substrate</td>
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<tr>
<td>Marvel Milk Powder</td>
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<tr>
<td>Methylthiazolydiphenyl-tetrazolium bromide (MTT)</td>
<td>Sigma-Aldrich, Dorset, UK</td>
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<td>NP-40</td>
<td>Sigma-Aldrich, Dorset, UK</td>
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<tr>
<td>NuPAGE® LDS Sample Buffer (4x)</td>
<td>Invitrogen, Paisley, UK</td>
</tr>
<tr>
<td>NuPAGE® MOPS SDS Running Buffer (20x)</td>
<td>Invitrogen, Paisley, UK</td>
</tr>
<tr>
<td>NuPAGE® Novex® Bis-Tris 4-12% mini gels</td>
<td>Invitrogen, Paisley, UK</td>
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<td>Phenylmethylsulfonyl fluoride (PMSF)</td>
<td>Sigma-Aldrich, Dorset, UK</td>
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<td>Phosphatase inhibitor cocktail</td>
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</tr>
<tr>
<td>Phosphate-buffered saline (PBS) solution</td>
<td>Sigma-Aldrich, Dorset, UK</td>
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<tr>
<td>Phosphate-buffered saline (PBS) tablets</td>
<td>Sigma-Aldrich, Dorset, UK</td>
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<tr>
<td>Radioimmunoprecipitation assay (RIPA) buffer</td>
<td>Millipore, MA, USA</td>
</tr>
<tr>
<td>Random primers</td>
<td>Promega, Southampton, UK</td>
</tr>
<tr>
<td>ReBlot™ Plus antibody stripping solution</td>
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</tr>
<tr>
<td>Recombinant RNasin ribonuclease inhibitor</td>
<td>Promega, Southampton, UK</td>
</tr>
<tr>
<td>RNase-free water</td>
<td>Bioline, London, UK</td>
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<tr>
<td>Sodium Chloride (NaCl)</td>
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<td>Tween® 20</td>
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<td>β-mercaptoethanol</td>
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<td>PureProteome Protein A agarose beads</td>
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<tr>
<td>FUJIFILM medical X-ray film</td>
<td>Genetic Research Instrumentation Ltd, Rayne, UK</td>
</tr>
<tr>
<td>HDAC activity substrate (Bis-(BOC-(Ac)-Lys)-Rhodamine110)</td>
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<tr>
<td>Trypsin</td>
<td>Sigma-Aldrich, Dorset, UK</td>
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### Materials and methods

#### Table 2.1.3. Buffers and solutions

<table>
<thead>
<tr>
<th>BUFFER/SOLUTION/STAIN</th>
<th>Description</th>
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<tbody>
<tr>
<td>5M NaCl</td>
<td>292g NaCl; up to 1L with deionised H₂O.</td>
</tr>
<tr>
<td>Acid citrate dextrose solution (ACD-anti-coagulant)</td>
<td>4.2g di-sodium hydrogen citrate; 5g glucose; up to 100ml with deionised H₂O; filter-sterilise before use.</td>
</tr>
<tr>
<td>Kimura Stain</td>
<td>11ml Toluidine Blue; 0.8ml Light Green; 0.5ml Saponin saturated in 50% (v/v) ethanol; 5ml phosphate-buffered saline solution; filter-sterilise before use.</td>
</tr>
<tr>
<td>Stop Solution (for ELISA)</td>
<td>11ml Sulphuric Acid; up to 100ml with deionised H₂O.</td>
</tr>
<tr>
<td>TBS-Tween (for Western blotting)</td>
<td>10ml Tris-HCl; 30ml 5M NaCl; up to 1L with deionised H₂O; 500µl Tween® 20.</td>
</tr>
<tr>
<td>Tris-HCl (pH 7.4)</td>
<td>121g Tris base; adjust to pH 7.4 with HCl; up to 1L with deionised H₂O.</td>
</tr>
<tr>
<td>Wash Buffer (for ELISA)</td>
<td>5 phosphate buffer tablets diluted in 1L deionised H₂O; 500µl Tween® 20.</td>
</tr>
<tr>
<td>TE buffer</td>
<td>10 mM Tris-Cl, pH 7.6. 1 mM EDTA pH 8.</td>
</tr>
<tr>
<td>IP Elution buffer</td>
<td>0.2 M Glycine HCL pH 3</td>
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<tr>
<td>IP Neutralization buffer</td>
<td>1M Tris pH 9</td>
</tr>
<tr>
<td>IP Wash buffer</td>
<td>50 mM Tris HCL pH7.4, 150 mM NaCl, 1% NP-40, 1% CHAPS, 1mM EDTA</td>
</tr>
<tr>
<td>Lysis Buffer</td>
<td>As indicated in the corresponding sections</td>
</tr>
<tr>
<td>HDAC activity buffer</td>
<td>25mM TRIS-HCl pH 8, 137mM NaCl, 2.7mM KCl, 1mM MgCl₂, 0.1% CHAPS</td>
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</tbody>
</table>
Materials and methods

Table 2.1.4. Kits and assays

<table>
<thead>
<tr>
<th>KIT/ASSAY</th>
<th>SUPPLIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bicinchoninic acid (BCA) protein assay</td>
<td>Pierce Biotechnology, Rockford, IL, USA</td>
</tr>
<tr>
<td>Duoset ELISA Kits</td>
<td>R&amp;D Systems, Abingdon, UK</td>
</tr>
<tr>
<td>Human Monocyte Nucleofector® Kit</td>
<td>Lonza, Cologne, Germany</td>
</tr>
<tr>
<td>MACS Monocyte Isolation Kit II</td>
<td>Miltenyi Biotec Ltd., Surrey, UK</td>
</tr>
<tr>
<td>Nuclear Extraction Kit</td>
<td>Active Motif, Rixensart, Belgium</td>
</tr>
<tr>
<td>QiShredder Spin Columns</td>
<td>Qiagen, Crawley, UK</td>
</tr>
<tr>
<td>QuantiTect SYBR Green PCR Kits</td>
<td>Qiagen, Crawley, UK</td>
</tr>
<tr>
<td>RNase-Free DNase Set</td>
<td>Qiagen, Crawley, UK</td>
</tr>
<tr>
<td>RNeasy Mini Kit</td>
<td>Qiagen, Crawley, UK</td>
</tr>
<tr>
<td>TransAM NF-κB/p65 DNA Binding Assay</td>
<td>Active Motif, Rixensart, Belgium</td>
</tr>
<tr>
<td>Human Phospho-Kinase Array Kit</td>
<td>R&amp;D Systems, Abingdon, UK</td>
</tr>
<tr>
<td>ToxinSensorTM Chromogenic LAL</td>
<td>Genscript, Piscataway, NJ, USA</td>
</tr>
</tbody>
</table>

Table 2.1.5. Primers’ sequences used for Real-Time qPCR

<table>
<thead>
<tr>
<th>GENE</th>
<th>Primer sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18s forward</td>
<td>CTAGAGGGACAAGTGCCG</td>
</tr>
<tr>
<td>18s reverse</td>
<td>AGCTGAGGCTAGTGTA</td>
</tr>
<tr>
<td>HDAC2 forward</td>
<td>TCATTGGAAAAATTGACAGCATAGT</td>
</tr>
<tr>
<td>HDAC2 reverse</td>
<td>CATGGTGATGGTGGTAGGAAG</td>
</tr>
<tr>
<td>GSK3β forward</td>
<td>CAGGCCGCCAGACCCACCT</td>
</tr>
<tr>
<td>GSK3β S9A mutant</td>
<td>CAGGCCGCCAGACCACCG</td>
</tr>
<tr>
<td>GSK3β reverse</td>
<td>TACAGATCCACAAAGGTCTCG</td>
</tr>
<tr>
<td>IL-8 forward</td>
<td>AACTTCTCCACAACCTCTG</td>
</tr>
<tr>
<td>IL-8 reverse</td>
<td>TTGGCATCCCTCGATTTC</td>
</tr>
<tr>
<td>GM-CSF forward</td>
<td>CATGCTGCTGAGATGAATGAAA</td>
</tr>
<tr>
<td>GM-CSF reverse</td>
<td>GTCTGAGCGAGTCGGCTC</td>
</tr>
</tbody>
</table>

2.1.6. Transient transfections of MonoMac 6 cells

ON-TARGETplus SMARTpool GSK3β was from Dharmacon. The GSK3 beta S9A pcDNA3.1 plasmid (Addgene plasmid 14754), the GSK3 beta K85A pcDNA3.1 plasmid (Addgene plasmid 14755) and the negative control pcDNA3.1 plasmid lacking the insert were from Addgene (Cambridge MA, USA)(Stambolic and Woodgett 1994). The eGFPpcDNA3.1 plasmid DNA was a kind gift of Prof. A. Oosterhout from University of Groningen, Netherlands.
### Table 2.1.6. Antibodies used for Western blotting

<table>
<thead>
<tr>
<th>1° Antibody</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospho- Akt (Ser473) (#4060)</td>
<td>1/500</td>
<td>Cell Signaling, Danvers, MA, USA</td>
</tr>
<tr>
<td>Total Akt (#9272)</td>
<td>1/500</td>
<td>Cell Signaling, Danvers, MA, USA</td>
</tr>
<tr>
<td>Phospho- GSK3β (Ser9) (#9336)</td>
<td>1/1000</td>
<td>Cell Signaling, Danvers, MA, USA</td>
</tr>
<tr>
<td>Phospho-GSK3β (Tyr216) (sc-135653)</td>
<td>1/400</td>
<td>Santa Cruz, Heidelberg, Germany</td>
</tr>
<tr>
<td>P- GSK3β (Ser9) and total GSK3β for IHC (sc-11757, sc-9166)</td>
<td>1/100</td>
<td>Santa Cruz, Heidelberg, Germany</td>
</tr>
<tr>
<td>Total GSK3β (#9832)</td>
<td>1/1000</td>
<td>Cell Signaling, Danvers, MA, USA</td>
</tr>
<tr>
<td>Phospho ERK1/2 (Thr202/Tyr204) (#9101)</td>
<td>1/2000</td>
<td>Cell Signaling, Danvers, MA, USA</td>
</tr>
<tr>
<td>Total ERK1/2 (#9102)</td>
<td>1/1000</td>
<td>Cell Signaling, Danvers, MA, USA</td>
</tr>
<tr>
<td>Total NF-κB/p65 (C-20) (IP) (sc-372)</td>
<td>2µg</td>
<td>Santa Cruz, Heidelberg, Germany</td>
</tr>
<tr>
<td>Total NF-κB/p65 (WB) (#6956)</td>
<td>1/100</td>
<td>Cell Signaling, Danvers, MA, USA</td>
</tr>
<tr>
<td>Total GR (E-20) (sc-1003)</td>
<td>1/1000</td>
<td>Santa Cruz, Heidelberg, Germany</td>
</tr>
<tr>
<td>Phospho-HDAC2 (Ser394) (ab75602)</td>
<td>1/1000</td>
<td>AbCam, Cambridge, UK</td>
</tr>
<tr>
<td>Total HDAC2 (H2663)</td>
<td>1/10000</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Lamin A/C (ab49721)</td>
<td>1/1000</td>
<td>AbCam, Cambridge, UK</td>
</tr>
<tr>
<td>β-actin (ab8227)</td>
<td>1/10000</td>
<td>AbCam, Cambridge, UK</td>
</tr>
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<table>
<thead>
<tr>
<th>2° Antibody</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyclonal Goat Anti-Rabbit (P0448)</td>
<td>1/4000</td>
<td>DAKO, Cambridgeshire, UK</td>
</tr>
<tr>
<td>Polyclonal Goat Anti-Mouse (P0447)</td>
<td>1/2000</td>
<td>DAKO, Cambridgeshire, UK</td>
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Materials and methods

Table 2.1.7. Compounds and stimuli

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Stock Solution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC87114/ PI3Kδ inhibitor</td>
<td>10^{-2}M in DMSO</td>
<td>GSK, Stevenage, UK</td>
</tr>
<tr>
<td>CT99021/ GSK3β inhibitor</td>
<td>10^{-2}M in DMSO</td>
<td>Pfizer, Cambridge, MA, USA</td>
</tr>
<tr>
<td>SB239063/ p38 MAPK inhibitor</td>
<td>10^{-3}M in DMSO</td>
<td>GSK, Stevenage, UK</td>
</tr>
<tr>
<td>U0126/ MEK inhibitor</td>
<td>10^{-3}M in sterile H₂O</td>
<td>Cell Signaling, Danvers, MA, USA</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>1M in DMSO</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>LPS from Escherichia coli</td>
<td>1mg/mL in PBS</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>3% wt in H₂O</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Polymyxin B</td>
<td>50 mg/mL in H₂O</td>
<td>Sigma-Aldrich, Dorset, UK</td>
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Table 2.1.8. Equipment

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>96-well MicroWell™ NUNC plates</td>
<td>Fisher Scientific UK Ltd, Loughborough, UK</td>
</tr>
<tr>
<td>Gel Doc-It™ imaging system and LabWorks software</td>
<td>Ultra-Violet Products Ltd., Cambridge, UK</td>
</tr>
<tr>
<td>Inverted light microscope</td>
<td>Leica Microsystems, Buckinghamshire, UK</td>
</tr>
<tr>
<td>NanoDrop™ 8000</td>
<td>Labtech International, East Sussex, UK</td>
</tr>
<tr>
<td>Nucleofector® II Device</td>
<td>Lonza, Cologne, Germany</td>
</tr>
<tr>
<td>RotorGene RG3000 (for Realtime PCR)</td>
<td>Qiagen, (Corbett Research), Crawley, UK</td>
</tr>
<tr>
<td>BioTek Instruments microplate reader</td>
<td>Winooski, VT, USA</td>
</tr>
<tr>
<td>Storm 840 phosphoimager</td>
<td>Molecular Dynamics Div, Amersham Pharmacia Bioteck Inc, New Jersey, USA</td>
</tr>
<tr>
<td>Corning® Clear Bottom 96 well plate</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
</tbody>
</table>
2.2. Methods

2.2.1. Human studies

2.2.1.1. Peripheral lung sections

Subjects for the peripheral lung sections were recruited from the Section of Respiratory Medicine of the University Hospital of Ferrara, Italy. Peripheral lung tissue was collected from 21 patients with COPD, 19 smokers, and 14 non-smokers. Subjects’ characteristics are shown on table 3.1 (chapter III). All subjects were undergoing elective surgery for lung cancer, and COPD was diagnosed retrospectively; these subjects were not taking bronchodilator, theophylline, antibiotic, antioxidant, and/or glucocorticoid therapy in the last month before surgery. The study was approved by the local ethics committee of the University Hospital of Ferrara, and all patients were provided written informed consent.

2.2.2. Peripheral blood monocytes

Peripheral venous blood was collected from 4 patients with COPD, 3 smokers with normal lung function and 4 non-smokers for measurement of p-Ser9-GSK3β/total GSK3β ratio. COPD was defined according to the international guidelines as the presence of a post bronchodilator FEV₁/FVC ratio of less than 70%. Subjects’ characteristics are shown on Table 3.2 (chapter III). For the rest of the experiments, healthy subjects volunteered for peripheral venous blood donation (60ml). Pulmonary function tests were performed as previously described (Varani, Caramori et al. 2006). All participants gave informed consent to a protocol approved by the ethics committee of the Royal Brompton and Harefield NHS Trust/National Heart and Lung Institute.
2.2.1.2. Lung tissue processing and immunohistochemistry

Lung tissue processing and immunohistochemistry were performed as previously described (Varani, Caramori et al. 2006). The total numbers of macrophages were counted in 20 non-consecutive fields (magnification×40). Macrophages were identified by means of morphologic staining. Normal nonspecific IgG from the animals in which the primary antibodies were raised was used for negative controls (Santa Cruz Biotechnology, Santa Cruz, CA). All staining and cell counting was performed in a blind manner. The number of positively stained cells was expressed as a percentage of the total cells counted.

2.2.1.3. Isolation of peripheral blood mononuclear cells (PBMCs)

80ml of venous blood was collected into syringes containing acid citrate dextrose (ACD; an anti-coagulant) to give a final concentration of 1mM. The blood was divided into 4x20ml aliquots each of which were then diluted 1:1 with Hanks’ Buffered Saline Solution, HBSS. 20ml of this mixture was then layered onto 15ml of Ficoll-Hypaque® in a 50ml Falcon tube. After centrifugation, 400 x g for 30 minutes at 22°C, PBMCs were collected from the interface between the Ficoll® and plasma, washed with HBSS and centrifuged again at 400 x g for 10 minutes at 22°C. After this spin, the PBMCs were resuspended in RPMI 1640 GlutaMAX media with 10% FBS and 2mM L-Glutamine at 37°C with 5% CO₂ and 90% humidity. Cell count was conducted using Kimura staining and a haemocytometer.

2.2.1.4. Monocyte isolation from PBMC population using MACS® Cell Separation

Monocytes were isolated from PBMCs using the MACS® Cell Separation Technology by depletion of non-monocytes by a process referred to as ‘negative selection’. The principle works on the basis of indirect magnetic labelling of non-monocytes to a cocktail of biotin-conjugated monoclonal antibodies, as a primary labelling reagent and anti-biotin...
Materials and methods

Monoclonal antibodies conjugated to microbeads, as secondary labelling reagent. The magnetically labelled non-monocytes are depleted by retaining them on a MACS® Column in the magnetic field of a MACS Separator, while the unlabelled monocytes pass through the column. Cells were re-suspended in 30µl of separation buffer per 1x10⁷ cells in total. 10µl of FCR blocking reagent and biotin–antibody cocktail were also added per 1x10⁷ cells and the mixture was incubated on ice for 10 minutes. After this time, 30µl of separation buffer and 20µl of anti-biotin microbeads were added per 10⁷ cells in total. Cells were then incubated for 15 minutes on ice followed by washing with the separation buffer and then re-suspended in 500µl of buffer. An LS-MACS column was attached to a magnet and rinsed with 3ml of buffer. The cell suspension was then added to the column and the run-through from the column (the negative fraction, representing the enriched monocytes) was collected. A further 9ml of buffer was run through the column and collected. The resulting effluent was centrifuged and re-suspended in complete medium. Macrophage purity (>95%) was determined using FACS by staining the cells with anti-CD14 antibody.

2.2.1.5. Cell culture and treatment of monocytes

Isolated monocytes were cultured in RPMI 1640 GlutaMAX phenol red free media with 1% FBS and 2 mM L-glutamine at 37°C (5% CO₂ and 90% humidity) and plated (0.5x10⁶ cells per ml) into 96-well plates for supernatant collection and 6-well plates for protein extraction. Cells were pre-treated with the small molecule inhibitors/compounds for 30 minutes and/or with dexamethasone for 30 minutes and treated with LPS for 16 hours prior to collection of cell supernatants for ELISA or treated with H₂O₂ prior to protein extraction for Western blotting, ImmunoPrecipitation or kinase array assay.
2.2.2. Culture and treatments of the human monocytic cell line MonoMac6

2.2.2.1. Cell culture and treatment of MonoMac6

Human monocyte-macrophage cells (MonoMac6) were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 1% nonessential amino acids and 1% sodium pyruvate (5% CO₂ and 90% humidity) at a density of $0.2 \times 10^6$ cells/ml. Cells were cultured in starvation media containing only 1% FBS for 24 hours prior to being subjected to treatments.

2.2.2.2. Transient transfections of MonoMac6 cells

MonoMac6 cells were transiently transfected with siRNA and plasmids using Amaxa nucleofection. This process, developed by Amaxa AG biosystems, is a combination of electroporation and cell specific solutions which allows the introduction of RNA or DNA directly into the nucleus of cells (Hamm et al., 2002).

2.2.2.2.1. Transfection with GSK3β siRNA

For siRNA transfections, the experimental design included three controls: untransfected cells, cells subjected to nucleofection in the absence of siRNA (mock transfection control) and cells transfected with functional non-targeting siRNA (scramble control). Briefly, MonoMac6 cells were centrifuged at 1200 rpm for 3 minutes and resuspended in HBSS to remove any serum resides from the cells. $2 \times 10^6$ cells were aliquotted into sterile tubes and were then centrifuged again at 1200 rpm for 3 minutes. The cells were then resuspended in RPMI with 10% FBS (untransfected control), in nucleofector solution alone (mock transfection control), nucleofector solution containing 200nM or 400nM non-targeting siRNA (scramble control) and 200nM or 400nM on target GSK3β siRNA at a total
Materials and methods

A concentration of 100µl. All samples, apart from the untransfected control, were transferred into electroporation cuvettes and nucleofected with program W-01 on the nucleofector device. The transfected cells were transferred into 1ml of pre-warmed RPMI with 10% FBS and re-seeded in both 6-well plates and 96 well plates for 24, 48 or 72 hours. The seeding density was empirically determined as 500µl of cell suspension in 6-well plates and 50µl in 96-well plates. The cells from 6-well plates were used for protein extraction and determination of GSK3β protein knock down whereas the cells from 96 well plates were used for treatment with dexamethasone +/- LPS for the times and concentrations indicated in the specific experiments.

2.2.2.2. Transfection with plasmid DNA

For plasmid transfections, the same protocol was used but included the following controls: 1) untransfected cells, 2) mock transfected cells and 3) cells transfected with the empty vector (pcDNA3.1) 4) cells transfected with the eGFPpcDNA3.1 vector and 5) cell transfected with either the S9ApDNA3.1 or the K85RcDNA3.1 vectors. MonoMac6 cells were cultured as described and transfected for 18 or 24 hours with plasmid DNA at concentrations varying from 1-3µg. Plasmid expressions were verified using wild type primers and S9A mutant primers that recognise both endogenous wild type and mutant GSK3β. Following optimisation, the cells were transfected with 1µg of eGFPpcDNA3.1 and 2µg of the S9ApDNA3.1 or the K85RcDNA3.1 for 18 hours before subjected to treatments as described in the specific experiments.

2.2.3. Cell viability assay

Cell viability was determined by MTT assay. The principle of the assay is based on the ability of the yellow MTT dye to become reduced in the mitochondria of living cells, producing a
purple formazan product. This product is dissolved in DMSO and the absorbance of the resulting solution can give an indication of the number of viable cells present.

For determining cell viability, cells were seeded in 96-well tissue culture plates and stimulated according to the specific experimental procedure. At the end of the stimulation period, the supernatants were removed and MonoMac 6 cells or primary monocytes were incubated with 150 μl of 1 mg/ml MTT solution in serum-free RPMI at 37°C for 30 minutes. Staining of the cells was confirmed by observation under a light microscope and 150 μl DMSO were then added to each well and mixed thoroughly to ensure the product is dissolved. The resulting solution was then transferred in 96-well microtitre plates and the absorbance at 550nm was measured using a microplate reader. The effect of a treatment on cell viability was determined as fold change in absorbance compared to the unstimulated control.

2.2.4. Measurement of endotoxin levels in plasmid DNA constructs

The endotoxin levels in the plasmid constructs were measured using the Toxin Sensor™ Chromogenic LAL Endotoxin Assay kit (Genscript, Piscataway, NJ, USA), a quantitative endpoint test which utilises a modified Limulus Amebocyte Lysate (LAL) and a synthetic colour producing substrate to detect endotoxin chromogenically. Serial endotoxin standard solutions were prepared of 1, 0.5, 0.25, 0.1, 0.05, 0.025 and 0.01 EU/ml by diluting the 1EU/ml provided endotoxin stock solution with LAL Reagent Water. 100μl of each standard sample (in duplicate) and the test samples (diluted 1 in 10 in LAL Reagent Water) were dispensed into the endotoxin-free vials provided with the kit. The samples were mixed thoroughly for 30 seconds with a vortexer. A blank sample vial containing only 100μl of LAL Reagent Water was also used as a negative control. LAL stock solution was reconstituted
with 1.7ml of LAL Reagent Water and 100µl of the working solution was added into the vials. The vials were mixed well by swirling gently and incubated for 45 minutes at 37°C. The chromogenic substrate stock solution was reconstituted by addition of 1.7ml of LAL Reagent Water to give a final concentration of 2mM. 100µl of the reconstituted chromogenic solution was added to each vial and the samples were incubated for 5 minutes at 37°C. The Color-stabilizer #1 (Stop Solution) was added (500µl) to each vial and the samples were swirled gently to mix well. The Color-Stabilizer #2 was then added to the samples (500µl) and the samples were mixed well before addition of the Color-Stabilizer #3 (500µl). The samples were swirled gently avoiding bubble formation. The absorbance of each reaction vial was measured at 545nm using the microplate reader from BioTek Instruments, Inc (Winooski, VT, USA). The endotoxin levels in each vial were calculated graphically according to the linear relationship of the standard curve.

2.2.5. Purification of DNA plasmid constructs using ethanol precipitation

Ethanol precipitation accomplished with sodium acetate (CH₃COONa) was used to purify the DNA plasmid constructs and remove endotoxin and impurities (Maniatis 1982). 100% ice cold ethanol (1ml) and Sodium acetate (40µl) were added to 400µl TE buffer containing the plasmid constructs. The solution was left on dry ice for 15 minutes before centrifugation at 12,000 rpm for 2 minutes. The supernatant was discarded and the tubes containing the precipitated DNA were left open on the bench for 10 minutes for any remaining traces of ethanol to evaporate. The constructs were re-suspended in 500µl TE buffer and DNA purity was analysed using the NanoDrop™ 8000 spectrophotometer by the ratio of absorbance at 260nm and 280nm.
2.2.6. Determination of mRNA expression by real-time PCR

For the determination of mRNA expression, total RNA was extracted from the cells and complimentary DNA (cDNA) was prepared by reverse transcription polymerase chain reaction (RT-PCR). mRNA levels were quantified by real-time quantitative PCR amplification of the cDNA using SYBR Green dye.

2.2.6.1. RNA extraction

Cells (primary monocytes or MonoMac6 cells) were washed with 1 ml HBSS solution and then incubated with 350 μl/well of RLT buffer containing 1% β-mercaptoethanol for 5 minutes at room temperature, for cell lysis to take place. RNA extraction was immediately performed or cell lysates were stored at -80°C. Total RNA was isolated using the RNeasy Mini Kit according to the manufacturer’s instructions. Briefly, cell lysates were homogenised by centrifugation through QIAshredder spin columns at 12,000 rpm for 2 minutes. An equal amount (350μl) of 70% ethanol was then mixed with the lysate and the resulting solution was applied to the RNeasy Mini spin column by centrifugation at 10,000 rpm for 15 seconds, in order for total RNA to be retained on the silica-based membrane. The membrane bound RNA was washed to remove contaminants and incubated with a DNAse enzyme for 15 minutes at room temperature to remove any DNA contamination. RNA was eluted from the column by applying 30μl H₂O and centrifuging at 10,000 rpm for 1 minute. RNA purity was determined by determining the ratio of absorbance at 260nm over the absorbance at 280nm (A260/A280) using the NanoDrop™ 8000 spectrophotometer. Furthermore, RNA concentration in μg/μl was calculated using the relationship: RNA concentration (μg/μl) = (A260 x 40 μg/ml x dilution factor) / 1000.
2.2.6.2. Reverse-transcriptase PCR

Total RNA (0.5μg) in a final volume of 10μl was incubated for 5 minutes at 70°C on a thermal cycler in order to denature the RNA strands. At the end of the denaturation step the reverse-transcription reaction was set-up in a total volume of 20μl, which contained 1mM dNTPs, 1X AMV reverse transcriptase reaction buffer, 1 μg random primers, 40U recombinant RNasin ribonuclease inhibitor, 10U AMV reverse transcriptase enzyme and denatured RNA. The reaction mixture was incubated at 42°C for 60 minutes followed by incubation at 90°C for 4 minutes to inactivate the reverse transcriptase enzyme using the Rotor-Gene 3000 apparatus (Corbett Research). The final cDNA product was then diluted 3-fold in nuclease-free water.

2.2.6.3. Real-time quantitative PCR

mRNA expression was quantified by means of a two-step quantitative real-time PCR, using the fluorescent DNA binding dye SYBR Green. Briefly, 2.5 μl of cDNA were added to capillary tubes containing 7.5 μl of PCR reaction mixture, which consisted of SYBR Green PCR Master Mix Reagent (containing HotStarTaq DNA polymerase, dNTPs and SYBR Green I dye) and gene specific primers, and the PCR reaction was carried out on the Rotor-Gene 3000 PCR apparatus (Corbett Research). The reaction involved incubation at 95°C for 15 minutes to activate the heat-activated DNA polymerase followed by 30-40 cycles consisting of a denaturation step (94°C for 20 sec), an annealing step (55-60°C for 20 sec) and an extension step (72°C for 20 sec). Fluorescence emitted from DNA-bound SYBR Green was detected at 510nm after excitation at 470nm during the extension step. Data from the reaction were analysed using the computer software Rotor-Gene 6 v.6. CT values were converted into copies of the gene per reaction, by using a standard curve created by serial dilution of a
suitable sample. Gene expression was normalised to 18S rRNA expression. Specific primers for GSK3β and mutant sequences were designed according to their published sequences using the GenScript online primer design software and synthesised by Sigma-Genosys (Haverhill, Suffolk, UK). Primer specificity was assessed by using the online sequence analysis software BLAST (www.ncbi.nlm.nih.gov/BLAST/). Melting curve analysis and agarose gel electrophoresis were carried out in order to ensure the presence of one specific PCR product. For CXCL-8 and HDAC2 pre-designed and optimised primers (QuantiTect primer assays) from Qiagen were used.

2.2.7. Determination of protein expression by Western blotting

Human primary monocytes, PBMCs or MonoMac6 cells were plated at a density of 0.5×10^6/ml on a 6-well plate, were kept on ice, scraped in media and transferred into pre-chilled 2ml eppendorfs. The cells were pelleted by centrifugation at 5000 rpm for 5 minutes at 4°C and the supernatant was removed. After washing the cells once with ice cold PBS, containing 1X phosphatase inhibitor cocktail (to retain the phosphorylation status of the proteins of interest), the supernatant was removed and the cell pellet was resuspended in 100µl of RIPA buffer containing 1mM PMSF and 1X Complete protease inhibitor cocktail. After leaving on ice for 30 minutes, the lysates were centrifuged at 13,000 rpm for 15 minutes at 4°C and the resulting supernatant (the whole cell extract) was transferred in new pre-chilled tubes and stored at -80°C.

2.2.7.1. Cytoplasmic and nuclear protein extraction

Primary monocytes or MonoMac6 cells were plated at 2 x 10^6 /well before being subjected to treatments and were then lysed with 100µl of ice-cold lysis buffer (10 mM TrisHCl pH 7.4, 150 mM NaCl, 0.75% (v/v) NP40, 1mM PMSF, 1mM DTT and 1X phosphatase inhibitor
cocktail) for 10 minutes on ice and centrifuged at 13,000 rpm for 5 minutes at 4°C. The supernatant (the cytoplasmic fraction) was collected into a fresh eppendorf. The pellet was re-suspended in 20µl of nuclei extraction buffer (20 mM HEPES, 1.5 mM MgCl$_2$, 0.42 M NaCl, 0.2 mM EDTA, 25% Glycerol, 1mM PMSF, 1mM DTT and 1X phosphatase inhibitor cocktail) and the tube was scraped against a tube rack to help break the nuclear membrane. The lysate was left on ice for 30 minutes then centrifuged at 13,000 rpm for 5 minutes at 4°C. The supernatant (the nuclear fraction) was collected into a fresh eppendorf and mixed with 40µl of neutralising buffer (20 mM HEPES, 50 mM KCl, 0.2 mM EDTA, 1mM PMSF, 1mM DTT and 1X phosphatase inhibitor cocktail). The cytoplasmic and nuclear fractions were stored at -80°C until used for Western blotting.

2.2.7.2. Bicinchoninic acid (BCA) protein assay

Protein concentration of whole cell extracts, cytoplasmic extracts or nuclear extracts was determined by using a BCA protein assay kit, in a 96-well plate according to the manufacturer’s instructions. Briefly, protein extracts were diluted 2-fold in a total volume of 10µl cell-culture grade water in the plate. BSA standards (0.2, 0.4, 0.8, 1.2, 1.6 and 2 mg/ml) were also prepared in cell culture grade water to a total volume of 10µl in each well. The BCA protein assay reaction mixture was then prepared by mixing reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide) and B (4% cupric sulphate). 200µl of the mixture was added to the protein extracts and standards and the reaction was incubated for 30 minutes at 37°C. The absorbance of the product was measured at 562nm using a microplate reader and the protein concentrations were determined by comparison to the standard curve.
2.2.7.3. SDS PAGE and Western blotting

10-50 μg of protein extracts were transferred into tubes before NuPAGE® LDS Sample Buffer (4X) containing 3.8% β-mercaptoethanol was added. The samples were boiled for 5 minutes at 95°C to denature the proteins. The proteins were then loaded onto a 4-12% Bis-Tris Novex® pre-cast mini-gel. The gels were placed in an electrophoresis cell connected to a power supply and were electrophoresed at 200V for 50 minutes in 1X NuPAGE® MOPS SDS running buffer. The gel cassette was opened using a gel knife and the gel was placed into the iBlot™ device. The iBlot™ Dry Blotting system uses a semi-dry method to blot proteins from polyacrylamide gels onto a nitrocellulose membrane in 7 minutes. The gel was placed on top of a ‘Bottom’ stack with a copper anode (negative) layer and a nitrocellulose membrane. A mini-roller was used to remove any bubbles between the gel and the membrane. A piece of filter paper soaked in deionised H₂O was placed onto the gel and again, bubbles rolled out. A ‘Top’ stack consisting of a copper cathode (positive) layer was placed on top of the sandwich in addition to a layer of sponge and the iBlot™ device was tightly closed and the transfer commenced. After the transfer had completed, the sandwich was disassembled and the nitrocellulose membrane, onto which the proteins had now transferred, was cut to size using a scalpel. To check that the transfer of protein was successful, the membrane was incubated in Ponceau-S stain for 5 min before rinsing with deionised H₂O.

To prevent any non-specific binding the membrane was rinsed in TBS-Tween (0.1% Tween) and blocked by incubating in 5% milk for 1 hour at 4°C on a shaker. The membrane was then rinsed in TBS-Tween and incubated with a suitable dilution of the primary antibody for 16 hours at 4°C on a shaker. After the primary antibody incubation, the membrane was washed
three times in TBS-Tween for 5 minutes each time then incubated with the suitable secondary antibody for 45 minutes at room temperature. At the end of the incubation, the membrane was washed 3 times for 10 minutes each time and then covered with 2 ml of Luminata™ Western HRP solution for 5 minutes at room temperature. Excess Luminata™ solution was then removed and the membrane was wrapped in cling-film and exposed to a medical X-ray film in a dark room. Exposed films were then developed and the relevant band intensities were quantified using the Gel Doc-It™ imaging system by scanning densitometric analysis using LabWorks™ software. For re-probing, the membranes were stripped by incubating in mild antibody stripping solution for 15-20 minutes then blocked in 5% milk and TBS-tween solution. Densitometric data of phosphorylated proteins were normalised to levels of total protein and the levels of the endogenous control, β-actin.

2.2.8. Determination of cytokine release by ELISA

Measurement of cytokine (CXCL8, TNF-α and GM-CSF) concentration in cell supernatants was performed by means of a sandwich ELISA. Primary monocytes or MonoMac6 cells were plated in 96-well plates at a density of 0.5×10^6 cells/ml and subjected to treatments in a final volume of 200µl. At the end of the stimulation period supernatants were removed and stored at -20°C or assayed directly. The concentration of CXCL8, TNF-α and GM-CSF in the supernatants was determined using specific DuoSet ELISA kits according to the manufacturer’s instructions. The antibody working concentrations, standard concentrations and supernatant dilutions for each assay are summarised in Table 2.2.3. Briefly, 96-well microtitre plates were coated by dispensing 100µl of capture antibody solution at the working concentration in PBS and incubating for 16 hours at room temperature. The capture
antibody solution was aspirated and then the plate washed three times with 300μl wash buffer using an ELISA plate washer (Skatron, Norway) and dried by blotting against paper towels. The plate was then blocked by dispensing 300μl of Blocking Buffer (1% BSA and 0.05% NaN3 in PBS) in each well and incubating for 45 minutes at room temperature followed by another wash step as described above. Seven recombinant protein standards were then prepared by carrying out 2-fold serial dilutions of the stock protein standard in Reagent Diluent buffer (0.1% BSA and 0.05% Tween 20 in PBS). 100μl of the standards and diluted supernatants were then dispensed in the plate and incubated for 2 hours at room temperature. The plate was then washed and 100μl of detection antibody diluted to the working concentration in Reagent Diluent buffer were dispensed in each well and incubated for 2 hours at room temperature. The plate was then washed and 100μl of streptavidin-Horseradish Peroxidase (HRP) solution, diluted 1/200 in Reagent Diluent buffer, were dispensed in each well and incubated for 20 minutes at room temperature protected from light. After the final wash step, 50μl of the substrate solution, prepared by mixing equal volumes of Colour Reagents A (H2O2) and B (Tetramethylbenzidine), were dispensed to each well and incubated until the product developed. The substrate reaction was then stopped by adding 50μl of stop solution (2N H2SO4). The absorbance of each well was then measured at 450nm with a 540nm correction using the microplate reader from BioTek Instruments, Inc (Winooski, VT, USA) and a four-parameter logistic standard curve was automatically generated using the microplate reader software. The chemokine concentration of each sample was determined from the standard curve and corrected for the dilution by multiplying by the dilution factor.
Table 2.2.3. Antibodies and conditions used for ELISA

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Capture Ab (Working conc.)</th>
<th>Detection Ab (Working conc.)</th>
<th>Standard conc. range</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL8</td>
<td>Mouse anti-human (4 µg/ml)</td>
<td>Biotinylated goat anti-human (40 ng/ml)</td>
<td>0 – 1600 pg/ml</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Mouse anti-human (2 µg/ml)</td>
<td>Biotinylated mouse anti-human (500 ng/ml)</td>
<td>0 – 1000 pg/ml</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Mouse anti-human (4 µg/ml)</td>
<td>Biotinylated goat anti-human (500 ng/ml)</td>
<td>0 – 1000 pg/ml</td>
</tr>
</tbody>
</table>

Ab: Antibody; conc: concentration

2.2.9. NF-κB p65 DNA binding assay

Human primary monocytes were seeded into 6-well plates at 2x10⁶/ml in RPMI-1540 supplemented and 1% FBS and were subjected to treatments as indicated on the specific experiment. The cells were harvested and the nuclear proteins were extracted as discussed previously. The activity of p65 in the nuclear extracts was determined using the TransAM™ NF-κB p65 Transcription Factor Assay Kit (Rixensart, Belgium). The kit uses an ELISA-base method to detect and quantify transcription factor activation and includes a 96-well plate that is coated with immobilised oligonucleotide containing the NF-κB/p65 consensus binding site (Complete Binding Buffer). The activated p65 subunit contained in the nuclear extracts binds to the oligonucleotides and is then detected by addition of an antibody that recognises the p65 subunit. The secondary antibody conjugated with HRP is the added to the wells and provides the colometric readout that is quantified by spectophotometry. The reagents used included Complete Lysis Buffer, Complete Binding Buffer, 1X Wash Buffer and 1X Antibody Binding Buffer and were prepared according to the manufacturer’s recommendations. Following the addition of Complete Binding Buffer (30µl) to each well to
be used, 10μg of the nuclear protein samples (20μl) appropriately diluted in Complete Lysis Buffer were added to the wells and were incubated for 1hour at room temperature with mild agitation (100rpm on a rocking platform). Each well was then washed 3 times with 200μl 1X Wash Buffer and 100μl of NF-κB/p65 antibody was added (1:1,000 dilution in 1X Antibody Binding Buffer). The plate was incubated for 1hour at room temperature and the wells were washed again 3 times with 200μl 1X Wash Buffer. The HRP-conjugated antibody (100μl of 1:1,000 dilution in 1X Antibody Binding Buffer) was added to the wells and incubated for 1hour at RT. The wells were washed 4 times with 200μl 1X Wash Buffer and the Developing Solution was added (100μl/ well) for 2 minutes at room temperature protected from direct light. The reaction was stopped by addition of the acidic Stop Solution (100μl) and within 5 minutes the absorbance was read at 450nm with a reference wavelength of 655nm on a Synergy™ HT multi-detection microplate reader from BioTek Instruments, Inc (Winooski, VT, USA). Nuclear extracts from Jurkat cells stimulated with TPA and calcium ionophore were used in the assay as a positive control for p65 activation. The corresponding absorbances for the different treatments were plotted as a percentage of the nuclear extracts corresponding to untreated cells.
2.2.10. The Proteome Profiler™ Array using the Human Phospho-Kinase Array kit

For parallel determination of the relative levels of protein phosphorylation, we used the human phospho kinase array. This assay uses a nitrocellulose membrane where capture and control antibodies of 46 kinases have been spotted in duplicate. The cell lysates are incubated with the array and following streptavidin-HRP and chemoluminescence detection a signal is produced at each capture spot corresponding to the amount of phosphorylated protein bound.

Monocytes were seeded at a density of $2 \times 10^6$ cells/ml in a 6-well plate and were subjected to the indicated treatments. Following stimulation, the cells were rinsed with PBS and solubilised with the lysis buffer provided by the manufacturer at $1 \times 10^7$ cells/ml. The lysates were resuspended and incubated with agitation on a rocking platform for 30 minutes at 4°C. Cell lysates were then centrifuged at 14,000 rpm for 5 minutes and the supernatants (protein extracts) were transferred into a new pre-chilled eppendorf. The protein concentration of the lysates was determined using the BCA assay and 500µg of total protein was resuspended in lysis buffer at a final volume of 334µl.

The nitrocellulose membranes were blocked with provided Array buffer (1ml) for 1 hour on a rocking platform at room temperature. The protein lysates (334µl) were diluted in block buffer (Array buffer 1) to give a final volume of 2ml, of which each ml was incubated with array membrane A and array membrane B on a rocking platform overnight at 4°C. The membranes were washed 3 times, for 10 minutes each, with the provided 1X Wash Buffer. The membranes (part A and part B) were then incubated with 20µl of the provided lyophilised biotinylated detection antibody cocktail (A and B accordingly) diluted in 1ml of
Materials and methods

block buffer (Array buffer 2/3) for 2 hours at room temperature. The membranes were then washed 3 times for 10 minutes each and were incubated with Streptavidin-HRP diluted in Array buffer 2/3 at a final volume of 1ml for 30 minutes at room temperature. The washing step was performed again, and the membranes were covered with 2ml of Luminata™ Western HRP solution for 5 minutes at room temperature. Excess Luminata™ solution was then removed and the membrane was wrapped in cling-film and exposed to a medical X-ray film in a dark room. Exposed films were then developed and the relevant spot intensities were quantified using the Storm 840 phosphoimager apparatus. The average density of the duplicate spots was normalised to the positive control spot of each membrane. The normalised density of protein phosphorylation in treated cells was then compared to untreated cells and was expressed as % of change in protein phosphorylation.

2.2.11. ImmunoPrecipitation

Protein A has a high affinity for mouse IgG2b and rabbit IgG and binds to the Fc region of the antibody’s heavy chains resulting in orientating the molecule with antigen-binding sites facing outwards. The antigen is recognised and binds the antigen-binding region (Fab). The antibody that used to ImmunoPrecipitate HDAC2 is mouse IgG2b and the one that recognises p65 is rabbit IgG, hence Protein A agarose beads were used for both IP assays. The principle of ImmunoPrecipitation that was used is also shown in Figure 2.1.
**Figure 2.1. Diagram of a basic ImmunoPrecipitation procedure.** Antigen-containing sample (the cell lysate), antibody and beaded agarose affinity beads (usually Protein A) are allowed to bind. Non-bound sample components are washed away, and then antibody and antigen are eluted with a buffer that disrupts the binding interactions.

Primary monocytes or MonoMac 6 cells were maintained (1×10^6 cells/ml) in RPMI-1540 supplemented and 1% FBS for 24 hours before being subjected to the treatments. Following stimulation, the whole cell protein was extracted using modified RIPA buffer (50 mM Tris HCL pH7.4, 150 mM NaCl, 0.5% NP-40, 0.25% Na-deoxycholate, 1% CHAPS, 1mM EDTA) with freshly added complete protease and phosphatase inhibitor cocktail II. Protein concentration was determined by the BCA assay and 500µg of total protein was used for ImmunoPrecipitation of HDAC2 in primary monocytes and p65 in MonoMac6 cells. The lysates were incubated with agitation with the corresponding antibodies (2µg) overnight at 4°C, at a final volume of 250µl of RIPA buffer. The following day 10µl of the agarose beads/protein A was added to the lysate/antibody mixture and was incubated for 2 hours at
Materials and methods

4°C with agitation. Non-bound proteins and sample components were then washed 3 times with IP wash buffer and centrifuged at 1000 rpm for 20 seconds. 30µl of IP elution buffer was added to the beads/antibody/antigen mixture and left on bench for 1 minute before being neutralised with 3µl of the IP neutralizing buffer. In the case of HDAC2 IP, 20µl of the slurry was kept for the activity assay (as detailed in the following section). The rest of the mixture and the p65 IP sample were then centrifuged at 1000 rpm for 20 seconds and the immunoprecipitated antigen was transferred in a new eppendorf containing NuPAGE® LDS Sample Buffer (4X) and 3.8% β-mercaptoethanol and was boiled for 5 minutes at 95°C. A small aliquot of Beads/antibody mixture was also boiled for a negative control. Normal Western blotting was then carried out for detection of immunoprecipitated p65 and HDAC2.

2.2.12. HDAC activity assay

Immunorecipitated HDAC2 bound to the agarose beads was added in duplicates of 10µl to a Corning® Clear Bottom 96 well plate. For negative control, equal amount of antibody/beads mixture was used without any sample. HDAC activity assay buffer (60µl) and substrate solution (10µM) were added on the wells and were incubated with the immunoprecipitated HDAC2 for 2 hours at 37°C. Trypsin (200µM) was then added to the wells in a final volume of 100µl and incubated with the samples for 30 minutes at 37°C. Fluorescence (excitation 496nm, emission 530nm) was then read in the samples on a Synergy™ HT multi-detection microplate reader from BioTek Instruments, Inc (Winooski, VT, USA). Arbitrary units of HDAC activity were normalised to negative control and were expressed as % of activity in untreated samples.
2.2.13. Co-ImmuoPrecipitation

For the Co-Immunoprecipitation of GRα bound to p65, the IP protocol was performed exactly as described in section 2.2.11. Following Western blotting, the blots were stripped and re-probed with the GRα antibody following the protocols described above. Levels of GRα as analysed using densitometry were then normalised to levels of p65 and the effect of different treatments was expressed as % change of GRα bound to p65 compared to untreated cells.

2.2.14. Statistical analysis

Results (n≥3) are represented as mean ± standard error of the mean (S.E.M.). Results were analysed using one-way ANOVA for repeated measures (Friedman test), followed by Dunnet post-hoc test to determine differences between the control group and each treatment group. The Mann-Whitney non-parametric t-test was also used to compare different treatment groups. The statistical analysis was performed using GraphPad Prism® (GraphPad Software, San Diego, CA). P values of less than 0.05 were considered to be statistically significant.
Chapter III

GSK3β regulates corticosteroid function under conditions of oxidative stress
3.1. Introduction

COPD is characterised by increased number of activated inflammatory cells. These have the capacity to secrete several inflammatory mediators including lipids, cytokines (such as GM-CSF, TNF-α), chemokines (CXCL8) and growth factors (Barnes, Shapiro et al. 2003). Unlike in asthma and other chronic inflammatory airway diseases, COPD patients respond poorly to the anti-inflammatory actions of corticosteroids and this is associated with reduced inhibitory effect of corticosteroids on macrophage release of pro-inflammatory cytokines (Adcock and Barnes 2008; Barnes 2008). The precise molecular mechanisms that regulate corticosteroid unresponsiveness in COPD remain unclear.

Oxidative stress has a profound impact on inflammation. The increased oxidant burden in the lungs of COPD patients, derived from sources including cigarette smoke and the respiratory burst from inflammatory cells, is likely to play a significant role in driving the corticosteroid insensitive chronic inflammation (Adcock, Marwick et al. 2010; Chung and Marwick 2010). Oxidants activate several kinase pathways including (p38 MAPK, ERK1/2 and Akt) and pro-inflammatory transcription factors such as NF-κB in both in vitro and in vivo oxidant models (Chung and Marwick 2010). Many of these pathways are also elevated in the cells and tissue of patients with COPD, leading to an enhanced inflammatory response including elevated cytokine production and inflammatory cell recruitment (Irusen, Matthews et al. 2002; Marwick, Caramori et al. 2010; Chung 2011).

GSK3β is a constitutively active serine/threonine kinase that plays a key role in the regulation of signalling pathways that control metabolism, cell cycle and gene expression (Jope, Yuskaitis et al. 2007). The kinase is inactivated by phosphorylation on its Ser9 residue in response to stimulation of cells by mitogens or growth factors. This creates a
GSK3β regulates corticosteroid function under conditions of OS

pseudosubstrate that occupies its catalytic groove. Aberrant activity of GSK3β has been linked to the progression of several disease states that involve inflammation, including Alzheimer’s disease and diabetes (Henriksen and Dokken 2006; Billadeau 2007).

Recent studies have illustrated that both H₂O₂ and the cellular lipid peroxidation product HNE enhanced basal phosphorylation and activation of the ERK1/2 signalling pathway which also results in Ser9 phosphorylation on GSK3β suggesting the kinase plays a key role in regulating cellular protection in response to oxidant insults (Dozza, Smith et al. 2004). In addition, cigarette smoke exposure induced elevated cytokine release in airway smooth muscle cells in a GSK3β-dependent manner (Baarsma, Meurs et al. 2011). Based on these findings there is an emerging need to investigate the role of GSK3β in mediating oxidant induced activation of the kinase cross-talk and the corticosteroid insensitivity documented in COPD inflammation (Cohen and Goedert 2004; Jope and Johnson 2004; Martin, Rehani et al. 2005). I therefore hypothesised that GSK3β phosphorylation on Ser9 is differentially regulated in peripheral lung macrophages and monocytes from COPD patients and smokers with normal lung function compared to non-smokers. Since phosphorylation on Ser9 suppresses GSK3β activity by disrupting its substrate binding site, I hypothesised that inactivation of GSK3β negatively regulates corticosteroid function.

The aims of this chapter are:

- Assess the levels of the p-Ser9-GSK3β/total GSK3β ratio in peripheral lung macrophages and monocytes from COPD patients, smokers with normal lung function and non-smokers
GSK3β regulates corticosteroid function under conditions of OS

- Investigate whether inhibition of GSK3β activity by pre-treatment with the CT99021 compound, by knocking down GSK3β protein expression using on-target siRNA or by overexpression of the kinase dead mutant K85RGSK3β suppresses dexamethasone function in LPS-stimulated monocytes.

- Assess whether H₂O₂ stimulation suppresses dexamethasone function in LPS-stimulated monocytes is GSK3β dependent by investigating whether overexpression of the constitutively active S9AGSK3β mutant restores dexamethasone function.
3.2. Results

3.2.1. Phosphorylation of GSK3β on Ser9 is increased in peripheral lung macrophages and monocytes in patients with COPD compared to healthy subjects

The levels of p-GSK3β - Ser9, a marker of GSK3β inactivation, were measured in peripheral lung macrophages and peripheral blood monocytes from patients with COPD, smokers with normal lung function and non-smokers. The characteristics of the subjects recruited for the study are illustrated on Table 3.1 and Table 3.2. P-GSK3β-Ser9 staining in the peripheral lung was increased in COPD patients compared to smokers with normal lung function and non-smokers, and was also higher in smokers with normal lung function compared to non-smokers (Figure 3.1). The ratio of phosphorylated to total GSK3β was also increased in peripheral blood monocytes isolated from patients with COPD compared to healthy subjects with no smoking history ($p=0.015$) (Figure 3.2). Exposure of monocytes from healthy subjects to H$_2$O$_2$ (100µM) for 30 minutes, induced a significant 2-fold increase of the Ser9 phosphorylation on GSK3β. An obvious trend for H$_2$O$_2$ – induced p-GSK3β-Ser9 was observed in all patients groups but it did not reach statistical significance. (Figure 3.2).
### Table 3.1. Characteristics of subjects for peripheral lung sections

<table>
<thead>
<tr>
<th>Group</th>
<th>Age</th>
<th>Sex (M/F)</th>
<th>Smoking Status</th>
<th>Pack Years</th>
<th>FEV(_1) (L)</th>
<th>FEV(_1) (% pred)</th>
<th>FEV(_1)/FVC ratio (%)</th>
<th>GOLD Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>COPD</td>
<td>69.1 ± 6.6</td>
<td>18/3</td>
<td>7Current 14Former</td>
<td>40.5 ± 20.1</td>
<td>2.03 ± 0.5</td>
<td>75.3 ± 16.6</td>
<td>56.1 ± 9.1</td>
<td>8 stage 1, 11 stage 2, 2 stage 3</td>
</tr>
<tr>
<td>Smoker</td>
<td>70.0 ± 6.7</td>
<td>18/1</td>
<td>9Current 10Former</td>
<td>49.4 ± 32.3</td>
<td>2.5 ± 0.7</td>
<td>91.8 ± 14.6</td>
<td>75.5 ± 4.6</td>
<td>N/A</td>
</tr>
<tr>
<td>Non-Smoker</td>
<td>67.7 ± 8.1</td>
<td>0/14</td>
<td>N/A</td>
<td>N/A</td>
<td>2.1 ± 0.4</td>
<td>101.5 ± 22.5</td>
<td>76.4 ± 3.5</td>
<td>N/A</td>
</tr>
</tbody>
</table>

FEV\(_1\): Forced expiratory volume in one second; FVC: Force vital capacity; % pred: % predicted; GOLD: Global Initiative for Obstructive Lung Disease. M/F: Male to Female ratio.

### Table 3.2. Characteristics of subjects for isolation of peripheral blood monocytes

<table>
<thead>
<tr>
<th>Group</th>
<th>Age</th>
<th>Sex M/F</th>
<th>Smoking Status</th>
<th>Pack Years</th>
<th>FEV(_1) (L)</th>
<th>FEV(_1) (% Pred)</th>
<th>FEV(_1)/FVC ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COPD</td>
<td>57.8 ± 3.8</td>
<td>4/1</td>
<td>1Current 4Former</td>
<td>35.0 ± 4.5</td>
<td>2.5 ± 0.2</td>
<td>76.9 ± 3.5</td>
<td>59.7 ± 7.6</td>
</tr>
<tr>
<td>Smoker</td>
<td>54.6 ± 1.9</td>
<td>2/1</td>
<td>all current</td>
<td>48.3 ± 5.2</td>
<td>2.9 ± 0.2</td>
<td>93.2 ± 10.3</td>
<td>78.7 ± 3.2</td>
</tr>
<tr>
<td>Non-Smoker</td>
<td>60.3 ± 2.7</td>
<td>1/3</td>
<td>N/A</td>
<td>N/A</td>
<td>2.2 ± 0.3</td>
<td>100.5 ± 7.1</td>
<td>81.1 ± 5.1</td>
</tr>
</tbody>
</table>

FEV\(_1\): Forced expiratory volume in one second; FVC: Force vital capacity; % pred: % predicted; GOLD: Global Initiative for Obstructive Lung Disease. M/F: Male to Female ratio.
GSK3β regulates corticosteroid function under conditions of OS

Figure 3.1. p-GSK3β-Ser9/total GSK3β is increased in COPD peripheral lung macrophages compared to smokers with normal lung function and non-smokers. Phosphorylation levels of Ser9-GSK3β in macrophages in the peripheral lungs of non-smokers, smokers with normal lung function and patients with COPD. Representative images (magnification ×40) of p-Ser9-GSK3β staining in peripheral lung sections from (a) non-smokers, (b) smokers and (c) COPD patients are shown. Percentage of macrophages positively stained for p-Ser9-GSK3β in the peripheral lung sections from non-smokers (n = 14), smokers (n = 19), and patients with COPD (n = 21). NS: Non-smoker. Data represent mean±SEM and one-way ANOVA and Dunnet’s post-hoc test were used to test for statistical significance. **p<0.01, ***p<0.001.
Figure 3.2. p-GSK3β-s9/total GSK3β is increased in COPD peripheral blood monocytes compared to smokers with normal lung function and non-smokers. Ratio of p-Ser9-GSK3β normalised to total GSK3β protein levels in non-stimulated cells and cells exposed to H₂O₂ (100µM) for 30 minutes. Monocytes were isolated from non-smokers with normal lung function (n=4), smokers (n=3) and COPD patients (n=5). Phosphorylation levels of GSK3β were increased in monocytes derived from COPD patients compared to non-smokers. Data represent median with range of p-GSK3β-ser9/total GSK3β. *p<0.05 versus healthy subjects.
GSK3β regulates corticosteroid function under conditions of OS

3.2.2. Inactivation of GSK3β suppresses dexamethasone anti-inflammatory function in human primary monocytes

3.2.2.1. Inhibition of GSK3β activity by treatment with CT99021 had no effect on cell viability and basal inflammatory cytokine release

Levels of the inactive phosphorylated form of GSK3β were increased in both lung macrophages and blood monocytes from COPD patients and smokers with normal lung function compared to non-smokers. To investigate whether GSK3β inactivation has any effect on the inflammatory responses driven by these immune cells, I used monocytes as a cell model to study the effect of treatment with the GSK3β inhibitor CT99021 on cell viability and inflammatory cytokine release. Monocytes were treated with concentrations of CT99021 ranging from $10^{-8}$M to $10^{-5}$M for 16 hours. Cell viability was not affected by treatment with the compound at the highest concentrations lower than $10^{-5}$M (Figure 3.3a). Similarly, basal levels of GM-CSF release were unaffected by exposure to CT99021 at concentrations ranging from $10^{-8}$M to $10^{-6}$M, but at $10^{-5}$M CT99021 reduced levels of basal GM-CSF release (Figure 3.3b). This effect of the compound at high concentration appears to be due to effects on cell viability rather than on inflammatory responses.
3.2.2.2. Treatment of monocytes with CT99021 had no effect on LPS-induced cytokine release

Primary human monocytes were pre-treated with the CT99021 inhibitor (10^{-8}M to 10^{-5}M) and then stimulated with LPS (10ng/ml) to study the effect of GSK3β inhibition on pro-inflammatory GM-CSF release. CT99021 had no significant effect on LPS induced GM-CSF release at concentrations up to 10^{-6}M. However, 10\mu M of the drug had an inhibitory effect on cytokine release (Figure 3.4). These results are in line with the data showing that high concentrations of CT99021 interfere with cell viability (Figure 3.3a) and basal levels of GM-CSF release (Figure 3.3b).
Figure 3.3. The effects of inhibition of GSK3β by treatment with CT99021 on cell viability and basal GM-CSF levels in primary human monocytes. Peripheral blood monocytes were isolated from PBMCs and treated with CT99021 at concentrations ranging from $10^{-8}$ M to $10^{-5}$ M and were incubated overnight. (a) Cell viability was not affected by CT99021 when used at concentrations up to $10^{-6}$ M. (b) Similarly, those concentrations of the compound did not affect levels of GM-CSF release. However, at $10^{-5}$ M, the inhibitor reduced (a) cell viability and (b) the levels of basal GM-CSF release. The data were expressed as % of untreated cells and histograms represent mean±S.E.M. for four independent experiments. *p<0.05 vs untreated cells.
**Figure 3.4.** The effect of inhibition of GSK3β by CT99021 on LPS-induced GM-CSF levels released in primary human monocytes. Monocytes were isolated from PBMCs and pre-treated with CT90021 (10⁻⁸M-10⁻⁵M) for 30 minutes and were then stimulated with LPS (10ng/ml) for 16 hours. The inhibitor had no effect on LPS induced pro-inflammatory cytokine release at concentrations up to 10⁻⁶M. At 10⁻⁵M, CT99021 inhibited LPS-induced GM-CSF release to 56.4±5.7% of levels induced by LPS alone. The data represent mean±S.E.M. of four independent experiments and are expressed as % of LPS induced GM-CSF release. *p<0.05, **p<0.01 vs levels of LPS induced GM-CSF release.
3.2.2.3. Treatment of monocytes with CT99021 reduces dexamethasone function in a concentration-dependent manner

Monocytes isolated from healthy subjects were pre-treated with CT99021 at concentrations that had no effect on cell viability (10⁻⁸M to 10⁻⁶M) for 30 minutes. The cells were then treated with 10⁻⁸M of dexamethasone 30 minutes before being stimulated with LPS (10ng/ml) for 16 hours. Dexamethasone (10⁻⁸M) inhibited LPS-induced GM-CSF release by 69.2±6.6% (Figure 3.5a). Similarly, dexamethasone inhibited LPS-induced TNF-α release to 33.9±7.9% (Figure 3.5b). When cells were pre-treated with CT99021 at 10⁻⁶M, a concentration that had no effect on LPS-induced GM-CSF or TNF-α cytokine release, (Figure 3.4 and 3.5b, respectively) it suppressed dexamethasone’s anti-inflammatory function in a concentration-dependent manner (Figure 3.5). At 10⁻⁶M, CT99021 reversed the anti-inflammatory effects of 10⁻⁸M of dexamethasone by ~50% (69.2±6.6% versus 31.1±7.2%) (Figure 3.5a) and ablated the dexamethasone effect on LPS-induced TNF-α (101.5±3.0% of LPS-induced TNF-α release) (Figure 3.5b). Next I investigated whether GSK3β inhibition exerts similar inhibitory effects in the presence of a higher concentration of dexamethasone. At 10⁻⁶M, dexamethasone suppressed LPS-induced GM-CSF release (Figure 3.6a) and LPS-induced TNF-α (Figure 3.6b) by 74.3±5.9% and by 55.2±9.2%, respectively. The anti-inflammatory effect of dexamethasone was reduced by GSK3β inactivation resulting in 50.8±7.6% and 20.4±5.5% inhibition of LPS-induced GM-CSF (Figure 3.6a) and TNF-α (Figure 3.6b) release, respectively.
**Figure 3.5.** CT99021 reduces dexamethasone (10^{-8}M) anti-inflammatory function in a concentration-dependent manner. (a) Human monocytes were pre-treated with CT99021 (10^{-6}M-10^{-4}M) for 30 minutes followed by treatment with dexamethasone (10^{-8}M) for 30 minutes. The cells were then stimulated with LPS (10ng/ml) for 16 hours and GM-CSF levels were measured in the cell-free supernatants. LPS induced GM-CSF release was significantly inhibited by dexamethasone by 69.2±6.6%. Pre-treatment with CT99021 resulted in a concentration-dependent reduction of dexamethasone-induced suppression of LPS-induced GM-CSF release. However, at 10^{-6}M, the inhibitor suppressed the anti-inflammatory effects of dexamethasone and the levels of GM-CSF were only inhibited by 31.1±7.2%. (b) Similarly, dexamethasone (10^{-8}M)-induced suppression of LPS-induced TNF-α release (33.9±7.9% inhibition) was reversed by CT99021 (10^{-6}M) back to 101.5±3.0% of LPS-induced levels. Data are expressed as % of LPS induced cytokine levels and the bars represent mean±S.E.M of four independent experiments. *p<0.05 vs. LPS-induced GM-CSF, #p<0.05 vs. LPS plus dexamethasone-induced GM-CSF levels.
Figure 3.6. CT99021 inhibits dexamethasone (10^{-6}M) anti-inflammatory function in human peripheral blood monocytes. Inhibition of GSK3β by treatment with the CT99021 (10^{-6}M) reduced the inhibitory function of dexamethasone (10^{-6}M) on LPS stimulated (a) GM-CSF release (74.3±5.9% versus 50.8±7.6% inhibition) and (b) TNF-α release (55.2±9.2% versus 20.4±5.5% inhibition). Histograms represent mean±S.E.M. (n=3). The data are expressed as % of LPS induced cytokine release. *p<0.05 vs LPS-induced cytokine release, #p<0.05 vs LPS plus dexamethasone-induced cytokine release.
3.2.3. Inactivation of GSK3β suppresses dexamethasone function in the human monocytic cell line MonoMac6

3.2.3.1. The anti-inflammatory function of dexamethasone in MonoMac6 cells

The MonoMac6 cells were pre-treated with dexamethasone (10^{-8}M-10^{-6}M) for 30 minutes before being stimulated with LPS (10ng/ml). The inflammatory response to LPS was measured by levels of the pro-inflammatory mediators CXCL8 and TNF-α. Dexamethasone significantly inhibited both LPS-induced CXCL8 (Figure 3.7a) and TNF-α (Figure 3.7b) release in a concentration-dependent manner. The IC_{50} for dexamethasone induced inhibition was 29nM for LPS-induced CXCL8 release (Figure 3.7a) and 8.7nM for LPS-induced TNF-α release (Figure 3.7b).

**Figure 3.7.** Dexamethasone induced a concentration-dependent inhibition of pro-inflammatory cytokine release. The cells were pre-treated with dexamethasone for 30 minutes at the concentrations shown and were then stimulated with LPS (10ng/ml) for 16 hours. (a) CXCL8 and (b) TNF-α levels of release were measured in cell-free supernatants. Dexamethasone inhibited both CXCL8 and TNF-α release in a concentration-dependent manner and had its maximum suppressive effect at 10^{-6} M. Levels of release are expressed as % of LPS induced levels and represent the mean±S.E.M. of four independent experiments. *p<0.05, **p<0.01 vs LPS induced cytokine release, Kruskal Wallis Anova, Dunn’s post t-test.
3.2.3.2. Treatment of MonoMac6 with the GSK3β inhibitor CT99021 had no effect on cell viability or LPS-induced pro-inflammatory cytokine release

MonoMac6 cells were treated with the CT99021 at concentrations ranging from $10^{-8}$M to $10^{-5}$M for 16 hours and cell viability was measured by MTT assay (Figure 3.8a). In line with its effects on primary monocytes, the compound reduced cell viability only at high concentrations of $10^{-5}$M. Next I investigated the effect of CT99021 on LPS-induced CXCL8 at concentrations at which it had no effect on cell viability. CT99021 had no significant effect on LPS-induced CXCL8 release at concentrations ranging from $10^{-8}$M to $10^{-6}$M (Figure 3.8b).

3.2.3.3. Treatment of MonoMac6 with the GSK3β inhibitor CT99021 decreased dexamethasone function in a concentration-dependent manner

MonoMac6 were pre-treated with a concentration range of CT99021 ($10^{-8}$M-$10^{-5}$M) for 30 minutes following treatment with dexamethasone ($10^{-9}$M and $10^{-6}$M) before cells were stimulated with LPS (10ng/ml) for 16 hours. Dexamethasone inhibited LPS-induced CXCL8 release by 71.1±3.5% at $10^{-6}$M (Figure 3.9a). This inhibitory effect of dexamethasone was reversed by treatment with CT99021 ($10^{-6}$M) (71.1±3.5% versus 37.8±7.5% inhibition) (Figure 3.9a). Similarly, when dexamethasone was added at $10^{-9}$M it inhibited LPS-induced cytokine release by 30.6±6.6% (Figure 3.9b). CT99021 ($10^{-6}$M) completely ablated the ability of dexamethasone to suppress LPS-induced CXCL8 release and levels were restored to 104.1±5.0% of LPS-induced levels (Figure 3.9b).
Figure 3.8. Effect of inhibition of GSK3β by CT99021 on cell viability and on LPS-induced CXCL8 levels released in the monocytic cell line MonoMac6. Cells were pre-treated with CT90021 (10^{-8}$M-10^{-5}$M) for 30 minutes and were then stimulated with LPS (10ng/ml) for 16 hours. (a) CT99021 had no effect on cell viability at concentrations up to 10^{-6}M but induced death of 56.5±5.7% of cells at 10^{-5}M. (b) Stimulation of cells with LPS induced a significant increase in CXCL8 release which was unaffected by pre-treatment with the CT99021 (10^{-8}$M-10^{-5}$M). The data represent mean±S.E.M. of four independent experiments and are expressed as % of LPS induced CXCL8 release. *p<0.05 vs untreated cells.
**Figure 3.9.** Inhibition of GSK3β by CT99021 suppresses dexamethasone function in human MonoMac6 cells. MonoMac6 cells were treated with the CT99021 compound for 30 minutes (10⁻⁶M-10⁻⁵M) following treatment with dexamethasone at (a) 10⁻⁶M and (b) 10⁻⁸M. Cells were then stimulated with LPS for 16 hours and levels of CXCL8 release were measured in the cell free supernatants. Dexamethasone significantly inhibited LPS-induced CXCL8 release at both concentrations used. Pre-treatment with CT99021 had a concentration-dependent effect on attenuating dexamethasone function that reached significance at 10⁻⁶.5M and 10⁻⁶M. Data are expressed as % of LPS-induced CXCL8 release. Histograms represent mean±S.E.M. values of four independent experiments. *p<0.05 vs. LPS-induced CXCL8 release, #p<0.05 vs. LPS and dexamethasone-induced CXCL8 release.
3.2.3.4. Treatment of MonoMac6 with on-target GSK3β siRNA inhibited total levels of GSK3β in a concentration- and time-dependent manner

Inhibition of GSK3β by pre-treatment with the CT99021 compound showed a clear suppression of dexamethasone’s function in LPS-stimulated primary monocytes and MonoMac6 cells. To further confirm that this effect is GSK3β-dependent and is not an off-target effect of the compound, I next assessed whether suppression of GSK3β protein expression by siRNA-induced gene silencing leads to similar levels of suppression of dexamethasone’s anti-inflammatory function. MonoMac6 cells were transfected via electroporation with scramble siRNA and on-target GSK3β siRNA at 200nM and 400nM for 24, 48 and 72 hours. Total levels of GSK3β in cells transfected with on-target siRNA were normalised to levels of the kinase when cells were transfected with scramble siRNA. GSK3β siRNA (200nM and 400nM) decreased total protein levels 24 hours post transfection by 34.3±14.1% and 44.1±8.5% accordingly (Figure 3.10a and 3.10d). 48 hours post transfection total levels of GSK3β were inhibited further by 55.3±8.5% with 200nM siRNA and 87.4±4.1% with 400nM siRNA (Figure 3.10b and 3.10d). At 72 hours following transfection, GSK3β protein levels were inhibited only by 33.9±18.24% (200nM siRNA) and 49.4±13.2% (400nM siRNA) compared to control levels, due to de novo protein expression (Figure 3.10c and 3.10d).
GSK3β regulates corticosteroid function under conditions of OS

3.2.3.5. Effect of transfection with GSK3β siRNA on MonoMac6 cell viability over time

MonoMac6 cells were transfected via electroporation with siRNA (scramble and on-target) for 24, 48 and 72 hours. Cell viability was tested before the cells were subjected to further treatments. Subjection of cells to electroporation without addition of siRNAs for 24 (Figure 3.11a), 48 (Figure 3.11b) and 72 (Figure 3.11c) hours affected cell viability and caused death of 50-60% of the total cell number. Transfection of the cells with the scramble siRNA at 200nM and 400nM and on-target GSK3β siRNA at 200nM and 400nM had no additional effect on cell viability (Figure 3.11a, b and c).
GSK3β regulates corticosteroid function under conditions of OS

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`total GSK3β/β-actin % of control-scramble siRNA`

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- `200nM siRNA`
- `400nM siRNA`

Hours

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**GSK3β regulates corticosteroid function under conditions of OS**

**Figure 3.10.** On-target GSK3β siRNA reduced total GSK3β protein levels in a time- and concentration-dependent manner. MonoMac6 cells were transfected with scramble siRNA (blue bars) and on-target GSK3β siRNA (red bars) at 200nM and 400nM for 24, 48 and 72 hours. Levels of total GSK3β protein started decreasing after 24 hours (a, d) of transfection with GSK3β siRNA (200nM and 400nM), were further reduced after 48 hours (b, d) but started returning towards control levels after 72 hours (c, d) of transfection. Figures a, b and c show representative Western blot images that were used for band densitometry. Total GSK3β levels were normalised to levels of β-actin and were plotted as % of total GSK3β protein levels in cells transfected with the control scramble siRNA at the according concentration (d). Histograms represent the mean±S.E.M. of four independent experiments. *p<0.05, vs. Levels of GSK3β in cells transfected with scramble siRNA for 24 hours, #p<0.05, ###p<0.001 vs. Levels of GSK3β in cells transfected with scramble siRNA for 48 hours, &p<0.05 vs. Levels of GSK3β in cells transfected with scramble siRNA for 72 hours.
GSK3β regulates corticosteroid function under conditions of OS

Figure 3.11. Electroporation affected MonoMac6 cell viability but addition of siRNAs had no impact on cell death. Mock electroporation of MonoMac6 cells inhibited cell viability up to 50-60% after (a) 24, (b) 48 and (c) 72 hours. Transfection of cells with scramble or GSK3β siRNA had no additional effect on cell viability. The data are expressed as % of viability of naïve untransfected cells. Histograms represent mean±S.E.M. of three independent experiments.

3.2.3.6. GSK3β protein knock down had no effect on LPS-induced pro-inflammatory cytokine release in MonoMac6 cells

MonoMac6 cells were transfected with scramble and on-target GSK3β siRNA (200nM and 400nM) for 24 and 48 hours. At each time point the transfected cells were stimulated with LPS for an additional 16 hours and release of the both TNF-α and CXCL8 was measured in the supernatants. As previously shown, inhibition of GSK3β by transfection with 200nM and 400nM siRNA for 24 hours resulted in a 34.3±14.1% and 44.1±8.5% loss in total GSK3β levels compared to cells transfected with scramble siRNA (Figure 3.10d). This significant reduction in total GSK3β protein levels had no effect on LPS-induced TNF-α (Figure 3.12a) or CXCL8 release (Figure 3.12b). Following 48 hours of transfection, the total protein levels of GSK3β...
were reduced by 55.3±8.5% (200nM siRNA) and by 87.4±4.1% (400nM siRNA) without any effect on LPS-induced TNF-α (Figure 3.13a) or CXL8 release (Figure 3.13b).

**Figure 3.12.** Reduction of GSK3β protein levels following 24 hours of siRNA transfection had no effect on LPS-induced pro-inflammatory mediators’ release. MonoMac6 cells were transfected with scramble and on-target GSK3β siRNA for 24 hours. Transfected cells were subjected to LPS simulation for 16 hours and the levels of (a) TNF-α and (b) CXCL8 release was measured in the supernatants. Inhibition of GSK3β had no effect on LPS-induced either cytokine release compared to cells transfected with scramble siRNA. Data are expressed as % release of LPS induced cytokine release in cells transfected with scramble siRNA. Histograms represent mean±S.E.M. of three (a) and five (b) independent experiments.
GSK3β regulates corticosteroid function under conditions of OS

Figure 3.13. Reduction of GSK3β protein levels following 48 hours of siRNA transfection had no effect on LPS-induced pro-inflammatory mediators’ release. MonoMac6 cells were transfected with scramble and on-target GSK3β siRNA for 48 hours and were stimulated with LPS for 16 hours. Inhibition of GSK3β had no effect on LPS-induced (a) TNF-α or (b) CXCL8 release compared to cells transfected with scramble siRNA. Data are expressed as % release of LPS induced cytokine release in cells transfected with scramble siRNA. Histograms represent mean±S.E.M. of four (a) and three (b) independent experiments.
GSK3β regulates corticosteroid function under conditions of OS

3.2.3.7. GSK3β protein knock down suppressed dexamethasone anti-inflammatory function in MonoMac6 cells

MonoMac6 cells were transfected with 200nM and 400nM of scramble and on-target GSK3β siRNA for 24 (Figure 3.14) and 48 hours (Figure 3.15). The cells were pre-treated with dexamethasone (10^{-8}M to 10^{-6}M) before being stimulated with LPS (10ng/ml) for 16 hours. Dexamethasone had a significant inhibitory effect on LPS-induced CXCL8 (Figure 3.14a, b) and TNF-α (Figure 3.14c, d) release in cells transfected with scramble siRNA at 200nM (Figure 3.14a, c) and 400nM (Figure 3.14b, d). As previously shown, transfection of cells with 200nM and 400nM on-target siRNA for 24 hours inhibited total protein GSK3β levels (Figure 3.10c). GSK3β knock down for 24 hours had a significant effect on dexamethasone-dependent inhibition of CXCL8 at 200nM of siRNA (p=0.014, Figure 14a) and 400nM siRNA (p=0.0012, Figure 3.14b). Similarly, dexamethasone-induced inhibition of TNF-α release was suppressed by 24 hour transfection with 200nM GSK3β siRNA (p=0.039, Figure 3.14c) and 400nM GSK3β siRNA (p=0.013, Figure 3.14d).

When MonoMac6 cells were transfected with siRNA for 48 hours and then stimulated with dexamethasone and LPS for further 16 hours, the effect of GSK3β knock down on dexamethasone action was reduced. The concentration-dependent inhibitory effect of dexamethasone on LPS-induced CXCL8 release was not significantly affected by 48 hours transfection with 200nM (p=0.383, Figure 3.15a) or 400nM (p=0.0814, Figure 3.15b) of siRNA. The dexamethasone effect on LPS-induced TNF-α release was more sensitive to GSK3β knock down using 200nM of siRNA and its function was significantly suppressed (p=0.035, Figure 3.15b). This effect was blunted when the cells were transfected with 400nM of siRNA (p=0.09, Figure 3.15d).
GSK3β regulates corticosteroid function under conditions of OS

Figure 3.14. Inactivation of GSK3β by siRNA transfection for 24 hours reduced dexamethasone anti-inflammatory function in LPS-stimulated MonoMac6 cells. The concentration-dependent inhibitory function of dexamethasone on LPS-induced CXCL8 (a, b) and TNF-α (c, d) release was significantly suppressed in cells transfected with on-target GSK3β siRNA, 200nM (a, c) and 400nM (b, d) compared to cells transfected with the scramble siRNA for 24 hours (a) p=0.014, (b) p=0.0012, (c) p=0.039 and (d) p=0.013). The IC₅₀ for dexamethasone was increased as shown on individual graphs. Levels of cytokine release are expressed as % of LPS-induced release in cells transfected with scramble siRNA at 200nM and 400nM accordingly. Data represent mean±S.E.M. (n=5 (a), n=6 (b) and n=4 (c, d). The statistical significance of the effect of GSK3β knock down on dexamethasone function was assessed using a two-way ANOVA.
Figure 3.15. Inactivation of GSK3β by siRNA transfection for 48 hours had no overall significant effect on dexamethasone anti-inflammatory function in LPS-stimulated MonoMac6 cells. Dexamethasone-mediated inhibition of LPS-induced CXCL8 (a, b) and TNF-α (d) release was not significantly reduced in cells with on-target GSK3β siRNA, 200nM (a, c) and 400nM (d) compared to cells transfected with the scramble siRNA. However, dexamethasone’s effect on TNF-α release in response to LPS was significantly affected by transfection with 200nM siRNA. The IC$_{50}$ for dexamethasone was changed as shown on individual graphs. Levels of cytokine release are expressed as % of LPS-induced release in cells transfected with scramble siRNA at 200nM and 400nM accordingly. Data represent mean±S.E.M. (n=5 (a), n=6 (b) and n=4 (c, d). The statistical significance of the effect of GSK3β knock down on dexamethasone function was assessed using a two-way ANOVA. (a) $p=0.383; (b) p=0.0814; (c) p=0.035; (d) p=0.09.$
3.2.4. Plasmid overexpression in MonoMac6 cells

3.2.4.1. Transient transfection efficiency and effect on cell viability

MonoMac6 cells were transfected via electroporation with 3 different constructs of plasmid DNA for 18 hours; eGFPpcDNA3.1, the negative control construct pcDNA3.1, the constitutively active S9ApcDNA3.1 and the constitutively inactive K85RpcDNA3.1. The cells were transfected with the plasmid eGFPpcDNA3.1 at concentrations varying from 1µg to 3µg and GFP fluorescence was used as a marker for measuring the transfection efficiency. Figure 3.16 shows the relative EGFP fluorescence in cells transfected with 1µg of eGFPpcDNA3.1 which was significantly higher 18 hours post transfection compared to non-transfected or electroporated cells without the plasmid. Cell viability was decreased by 37.6±13.2% compared to non-transfected cells due to electroporation. Addition of the plasmids had no effect on cell viability 18 hours post transfection (Figure 3.17).

3.2.4.2. Efficiency of the overexpression of the mutant GSK3β mRNA in MonoMac6 cells following plasmid transfection

MonoMac6 cells were transfected with the S9ApcDNA3.1 and K85RpcDNA3.1 constructs and the levels of WT and mutant GSK3β were measured by RT-PCR. The WT GSK3β set of primers recognise the wild type endogenous GSK3β protein as well as the mutant forms. Transfection of the cells for 18 hours with both S9ApcDNA3.1 and K85RpcDNA3.1 significantly increased levels of GSK3β mRNA (Figure 3.18a). To validate that our S9ApcDNA3.1 construct is being overexpressed in the cells, specific set of primers that recognise the mutant sequence were used to amplify the cDNA sequence. As shown in
Figure 3.1b, the S9A mutant isoform is significantly overexpressed in the cells following transfection.

**Figure 3.16.** Transfection efficiency was measured by levels of GFP fluorescence emitted under UV light. Using an excitation wavelength of 490nm and an emission wavelength of 509nm, fluorescence was measured in cells following 18 hours of transfection with the eGFPpcDNA3.1 plasmid (1µg) in the presence or absence of the plasmids of interest, S9ApcDNA3.1 and K85RpcDNA at the concentrations used in the experiments (2µg). GFP emitted fluorescence levels detected are expressed as fold increase compared to non-transfected cells. Data represent mean±S.E.M. of four independent experiments. *p<0.05 vs non-transfected cells. Mock electroporation: cells electroporated without insertion of plasmid DNA.
Figure 3.17. Effect of transient transfection with pcDNA3.1 on cell viability. Cell viability was decreased by 62.4±13.2% 18 hours following mock electroporation of MonoMac6 cells. When cells were transfected with the plasmid constructs, there was no significant additional effect on cell viability. Data are expressed as % of non-transfected cells and represent mean±S.E.M. of four independent experiments. *p<0.05 vs non-transfected cells. Mock electroporation: cells electroporated without insertion of plasmid DNA.
GSK3β regulates corticosteroid function under conditions of OS

Figure 3.18. Overexpression of the mutant GSK3β constructs measured by RT-PCR. Cells transfected with the S9ApDNA3.1 and K85pcDNA3.1 constructs expressed significantly higher levels of GSK3β as recognised by set of primers that bind and amplify both the wild type and mutant GSK3β sequence (a). S9A GSK3β specific primers were used to measure overexpression of the S9ApDNA3.1 (b).
3.2.4.2. Effect of transient plasmid overexpression on basal and LPS-induced pro-inflammatory cytokine release

The cells were subjected to transfection with the control pcDNA3.1 construct, the S9ApcDNA3.1 and the K85RpcDNA3.1 construct in the presence of the eGFPpcDNA3.1 plasmid for 18 hours. Transfected cells were then incubated for further 16 hours in the presence or absence of LPS (10ng/ml) and the levels of GM-CSF release were measured in the cell-free supernatants. All three plasmid constructs induced significant increases in GM-CSF release that reached levels of 100pg/ml to 150pg/ml (Figure 3.19a). These levels are higher than that of LPS-stimulated levels of GM-CSF release in non-transfected but electroporated cells (19.5±2.1pg/ml). Plasmid transfection also increased LPS-induced GM-CSF levels leading to similar amounts of the cytokine released in the supernatants (100pg/ml versus 150pg/ml) (Figure 3.19b). LPS stimulation of the cells following transfection is independent of electroporation and is driven by all three constructs used (Figure 3.19).
GSK3β regulates corticosteroid function under conditions of OS

Figure 3.19. Transfection of MonoMac6 cells with plasmid DNA increases both basal and LPS-induced GM-CSF levels of release. The (a) basal and (b) LPS-induced levels of GM-CSF release in MonoMac6 cells electroporated with no plasmid DNA (mock) (2.4±0.8pg/ml and 19.5±2.1pg/ml accordingly) were both significantly increased by addition of either of the three plasmid constructs to levels reaching 100pg/ml to 150pg/ml. The histograms represent the mean±S.E.M. of GM-CSF concentration (pg/ml) of GM-CSF of four independent experiments. *p<0.05 vs basal and LPS-induced GM-CSF levels in cells subjected to mock electroporation. Mock: cells subjected to electroporation in the absence of plasmid DNA.
3.2.4.3. Measurement of endotoxin levels of the plasmid constructs

Due to the high inflammatory response that the plasmid transfection induced in the MonoMac6 cells, we measured the endotoxin levels in the constructs. The endotoxin assay showed that the constructs have 0.1 to 0.3 EU per µg (Figure 3.20). Next, I washed the endotoxin from the constructs to levels of <0.05EU/µg and repeated the transfection with the purified plasmids. The purified plasmid had a similar effect on both basal and LPS-induced GM-CSF levels, inducing significantly higher levels of cytokine release than that induced by treatment with LPS alone (Figure 3.21). In both unstimulated (Figure 3.21a) and LPS stimulated cells (Figure 3.21b), plasmid transfection resulted in an 8- to 9-fold increase in GM-CSF release compared to LPS which induced a 6-fold increase in cytokine levels (19.8±3.5pg/ml and 127.1±18.8pg/ml accordingly) (Figure 3.21).

Figure 3.20. Endotoxin levels of the plasmid constructs. All three plasmid constructs had endotoxin levels varying from 0.1 EU/µg to 0.4 EU/µg as measured by the endotoxin assay according to the assay standards. The histograms represent mean±S.E.M. endotoxin units for 3 independent experiments. –ve: pcDNA3.1 empty plasmid used as a negative control.
**Figure 3.21.** Effect of plasmid transfection on basal and LPS-induced GM-CSF release following plasmid endotoxin removal in MonoMac6 cells. The plasmids were subjected to endotoxin wash and levels were brought down to <0.05 EU/µg. The cells were transiently transfected with the three constructs for 18 hours and were incubated for further 16 hours in the absence or presence of LPS (10ng/ml). LPS increased GM-CSF levels from 19.8±3.5pg/ml (a) to 129.1±18.8pg/ml (b) in cells that were electroporated without the addition of plasmid DNA. Transient transfection with the any of the three constructs induced an 8- to 9 fold increase of GM-CSF levels in both unstimulated (a) and LPS-stimulated cells (b). Histograms represent mean±S.E.M. of GM-CSF levels (pg/ml) of four independent experiments. *p<0.05, **p<0.001 vs. LPS-induced GM-CSF levels in cells subjected to mock transfection. –ve: pcDNA3.1 empty plasmid used as a negative control.

3.2.4.4. Inhibition of LPS did not reverse plasmid DNA induced pro-inflammatory response in MonoMac6 cells

Non-transfected and transiently transfected MonoMac6 cells were pre-treated with the LPS inhibitor, polymyxin B (50µg/ml) for 45 minutes before being stimulated with LPS (10ng/ml) for 16 hours. The levels of GM-CSF release induced by LPS were significantly suppressed by addition of the inhibitor (Figure 3.22a) in cells subjected to electroporation without addition of plasmid DNA. In cells transfected with the pcDNA3.1 induction of GM-CSF release was unaffected by addition of polymyxin B (Figure 3.21b). As previously shown, LPS had no additional effect on GM-CSF release nor did pre-treatment with Polymyxin (Figure 3.22b).
Figure 3.22. Inhibition of LPS by Polymyxin B did not reverse the pro-inflammatory effect of plasmid transfection in MonoMac6 cells. Pre-treatment of MonoMac6 cells with Polymyxin B for 45 minutes suppressed LPS-induced GM-CSF release in cells that were subjected to electroporation without addition of plasmid DNA construct (a). GM-CSF release was not affected by the LPS inhibitor when cells were transfected with the pcDNA3.1 construct (b). LPS stimulation had no additional effect on the high levels of GM-CSF release and similarly, Polymyxin B (PB) did not inhibit the pro-inflammatory response (b). Data are expressed as % of GM-CSF release in unstimulated non-transfected cells (a) and cells transfected with the pcDNA3.1 plasmid (b). Histograms represent mean±S.E.M. of GM-CSF levels of four independent experiments. *p<0.05, **p<0.001.
3.2.4.5. Transfection of MonoMac6 cells with other plasmid DNA construct induces a pro-inflammatory response

MonoMac6 cells were transfected with a basic pGL3 construct to ensure that the effect is not plasmid specific. The cells were transfected with the DNA for 18 hours and the subjected to treatment with Polymyxin B (50µg/ml for 45 minutes) and LPS (10ng/ml) for 16 hours before GM-CSF release levels were measured in the supernatants. Similar to my previous data with the pcDNA3.1 constructs, transient transfection significantly increased levels of GM-CSF release which were unaffected by addition of the LPS inhibitor. Stimulation of LPS had no additional effect on levels of cytokine release which remained the same even in the presence of Polymyxin B (Figure 3.23).

![Figure 3.23](image)

**Figure 3.23.** Transfection of MonoMac6 cells with a different plasmid DNA construct induced Polymyxin B-independent release of GM-CSF release. Cells transfected with the basic pGL3 plasmid construct produced significantly higher levels of GM-CSF release compared to cells subjected to mock transfection via electroporation. This induction was not suppressed by pre-treatment with the LPS inhibitor Polymyxin B (PB) (50µg/ml). LPS stimulation of the transfected cells had no additional effect on GM-CSG release and cytokine levels were also unaffected by the presence of the LPS inhibitor. Data are expressed as % of GM-CSF release in unstimulated non-transfected cells. Histograms represent mean±S.E.M. of GM-CSF levels of three independent experiments. Mock: cells electroporated in the absence of plasmid DNA; NT: non-treated cells transfected with plasmid DNA.
3.2.4.5. Effect of mutant GSK3β overexpression on dexamethasone function in MonoMac6 cells

In the previous section it was shown that transfection of MonoMac6 cells with the plasmid constructs stimulates an LPS-independent inflammatory response. Since LPS had no additional effect on the release of pro-inflammatory cytokines in transfected cells, I wanted to investigate whether this response is dexamethasone sensitive and whether it is modulated by overexpression of the kinase dead K85R GSK3β mutant. In addition, since H₂O₂ suppresses GSK3β function via Ser9 phosphorylation, I wanted to evaluate its effect on dexamethasone function in cells overexpressing the S9A GSK3β mutant. In order to achieve this, MonoMac6 cells were transfected with the pcDNA3.1 construct, which was used as the negative control, the K85RpcDNA3.1 and the S9ApDNA3.1 construct. Following 18 hours of transfection, the cells were treated with H₂O₂ (100 µM) for 2 hours before being subjected to dexamethasone stimulation (10⁻⁸M) and were then stimulated with LPS (10ng/ml) for 16 hours. The effect of plasmid DNA addition on both basal and LPS-induced GM-CSF release compared to cells electroporated without addition of any plasmid construct (mock) was shown previously in Figure 3.21. Levels of GM-CSF release in response to LPS in cells overexpressing the two mutant GSK3β plasmid constructs were normalised to levels induced in the cells transfected with the negative control pcDNA3.1 plasmid.
GSK3β regulates corticosteroid function under conditions of OS

3.2.4.5.1. Inactive K85R mutant GSK3β overexpression suppresses the anti-inflammatory effect of dexamethasone

Treatment with dexamethasone significantly inhibited LPS-induced GM-CSF release by 26.5±2.5% in the cells transfected with the control pcDNA3.1 plasmid. However, in cells overexpressing the kinase dead K85R GSK3β mutant, dexamethasone did not elicit any suppressive effect on LPS-induced GM-CSF release (122.9±21.1% versus 129.2±27.7% of LPS-induced GM-CSF release in cells transfected with the control pcDNA3.1 vector) (Figure 3.24).

![Figure 3.24. Overexpression of the kinase dead K85R GSK3β mutant suppresses dexamethasone function in LPS stimulated MonoMac6 cells. Dexamethasone (10^{-8}M) significantly reduced LPS-induced GM-CSF release in cells transfected with the control pcDNA3.1. When the inactive K85A GSK3β mutant was expressed in the cells, dexamethasone had no significant inhibitory effect on LPS induced GM-CSF release (122.9±21.1% versus 129.2±27.7%). Data are expressed as % of LPS induced GM-CSF release in the presence of the pcDNA3.1 control plasmid. Histograms represent mean±S.E.M. of four independent experiments. *p<0.05.](image-url)
3.2.4.5.2. Exposure of MonoMac6 cells to H$_2$O$_2$ inhibits dexamethasone-induced suppression of LPS-stimulated GM-CSF release

MonoMac6 cells were pre-treated with H$_2$O$_2$ (100µM) for 2 hours before being subjected to a concentration range of dexamethasone (30 minutes) and LPS (10ng/ml) stimulation for 16 hours. As shown in figure 3.25, pre-treatment of cells with H$_2$O$_2$ suppressed the concentration-dependent anti-inflammatory function of dexamethasone (p=0.0093). At the highest concentration used (10$^{-6}$M), dexamethasone-induced inhibition of GM-CSF release was significantly suppressed and GM-CSF release levels were reversed from 32.8±11.1% to 88.2±12.8% of LPS-induced GM-CSF levels of release.
Figure 3.25. H$_2$O$_2$-induced suppression of dexamethasone anti-inflammatory function in MonoMac6 cells. Dexamethasone ($10^{-9}$-$10^{-6}$M) significantly reduced LPS-induced GM-CSF release in a concentration dependent manner. By contrast, in cells pre-treated with H$_2$O$_2$, dexamethasone failed to suppress GM-CSF release to similar levels ($p=0.0093$). The maximum response to dexamethasone was inhibited by H$_2$O$_2$ and levels of GM-CSF release in response to LPS stimulation were significantly higher (88.2±12.8% vs 32.8±11.1%). Data represent mean±SEM of eight independent experiments. Data were normally distributed as confirmed by the Shapiro-Wilk normality test. Two-way ANOVA was used to test whether H$_2$O$_2$ had a statistically significant effect on dexamethasone’s concentration response curve. % of control: % of LPS induced GM-CSF release.
GSK3β regulates corticosteroid function under conditions of OS

3.2.4.5.3. \( H_2O_2 \)-induced inhibition of dexamethasone function is regulated by Ser9 on GSK3β: overexpression of the S9A mutant kinase restored dexamethasone function

In cells transfected with the control pcDNA3.1, the anti-inflammatory function of dexamethasone was suppressed by pre-treatment with \( H_2O_2 \) (100 µM) for 2 hours and the steroid inhibited GM-CSF levels to 89.7±6.1% of LPS induced levels (Figure 3.26a). However, in cells overexpressing the S9A GSK3β mutant, the inhibitory effect of \( H_2O_2 \) on dexamethasone function was reversed and the steroid significantly suppressed LPS induced GM-CSF levels to 64±5.7% (Figure 3.26b). \( H_2O_2 \) showed no significant additional effect on LPS-induced GM-CSF in the presence or absence of dexamethasone in cells transfected with either the control pcDNA3.1 or the S9ApcDNA3.1 plasmid (Figure 3.26).
Figure 3.26. H$_2$O$_2$-induced suppression of dexamethasone function is reversed in cells expressing the S9A mutant GSK3β kinase. (a) Transfection of monocytes with the control pcDNA3.1 plasmid had no effect on the ability of dexamethasone (10$^{-8}$M) to suppress LPS-induced GM-CSF release by 26.5 ±2.5 %. The anti-inflammatory effect of dexamethasone was attenuated by pre-treatment with H$_2$O$_2$ (100 µM) for 2 hours (p=0.31). (b) In the presence of the active S9A mutant, the inhibitory effect of H$_2$O$_2$ on dexamethasone function was reversed (10.3±6.1% versus 35±5.7%). Data are expressed as % of LPS-induced GM-CSF release in the presence of the pcDNA3.1 control plasmid. Histograms represent mean±S.E.M. of four independent experiments. *p<0.05, **p<0.01.
3.3. Discussion

3.3.1. The inhibitory Ser9 phosphorylation levels of GSK3β are increased in lung macrophages and blood monocytes from patients with COPD and smokers compared to non-smokers

The results demonstrated that the levels of the inactive p-GSK3β-s9 are increased in the peripheral lung macrophages and blood monocytes of COPD patients compared to non-smokers and smokers with normal lung function. Levels of p-GSK3β-s9 were also increased in the lung macrophages and monocytes of smokers compared to age-matched non-smokers. It is evident that lung macrophages and blood monocytes from COPD patients are exposed to reactive oxygen species that derive either exogenously from cigarette smoking and other environmental pollutants or are secreted by inflammatory cells (Marwick and Chung 2010). I therefore investigated whether inactivation of GSK3β is a result of the increased levels of oxidant stress. I exposed healthy monocytes to H$_2$O$_2$ –derived oxidant stress and measured levels of Ser9 phosphorylation on GSK3β. Treatment of peripheral blood monocytes isolated from non-smokers with H$_2$O$_2$ for 30 minutes resulted in a significant increase of p-GSK3β-Ser9 levels, whereas exposure to reactive oxygen species had no additional significant effect on the levels of the inactive GSK3β form in monocytes isolated from COPD patients. However, in all patients groups a trend of increased phosphorylation of GSK3β on Ser9 was observed in the presence of H$_2$O$_2$ and increasing n numbers should assist confirming statistical significance. These results suggest that inactivation of GSK3β in lung macrophages and peripheral blood monocytes, is induced by exposure of the cells to increased levels of reactive oxygen species. My findings are in line
GSK3β regulates corticosteroid function under conditions of OS

with studies showing that oxidative stress-related lipid peroxidation product HNE induces inactivation of GSK3β by increased phosphorylation on its Ser9 residue (Dozza, Smith et al. 2004).

3.3.2. Role of GSK3β inactivation on inflammation

Recently, a number of reports have documented that GSK3β activity is crucial to regulate inflammatory responses either by promoting or inhibiting the expression of pro- or anti-inflammatory cytokines. Inactivation of GSK3β has been shown to have anti-inflammatory effects in LPS-stimulated human monocytes, suggesting the kinase acts as a positive regulator of inflammation (Martin, Rehani et al. 2005). Increasing activity of GSK3β has been also documented to promote inflammatory response in several inflammatory models. A study by Hu, Paik et al. (2006) demonstrated that IFN-γ induces TLR2-dependent production of TNF-α in macrophages via increasing GSK3α/β activity. A second study investigating the mechanisms by which GSK3β positively regulates inflammation showed that inhibition of GSKβ activity suppressed LPS-induced TNF-α production in microglia (Wang, Huang et al. 2010). In addition in a model of colonic inflammation, increased activation of GSK3β led to increased bacterial-induced production of the pro-inflammatory IL-6 and CXCL8 cytokines (Duan, Liao et al. 2007). In addition to the inflammatory models described above suggesting the kinase plays a pro-inflammatory role, other studies have implied that GSK3β has also a role as a negative regulator of inflammation. A study in neonatal mouse cardiomyocytes showed that GSK3β suppresses TNF-α expression in cardiomyocytes following LPS stimulation (Shen, Fan et al. 2008). Similarly, inhibition of the kinase by Ser9 phosphorylation in macrophages augmented the levels of IFNβ in LPS-stimulated macrophages by a MyD88-independent mechanism (Wang, Garcia et al. 2008).
experimental evidence accumulated so far indicate that GSK3β has anti- or pro-inflammatory properties on inflammation depending on the cell type and the stimulus used. The broad array of immune actions affected by GSK3β is attributed partly to the remarkable number of crucial transcription factors and signal transducers that it regulates. It is therefore essential to investigate the role of GSK3β in the inflammatory processes in COPD and evaluate whether its inactivation in COPD immune cells has a direct effect on regulating inflammation. In order to test the above hypothesis, I used monocytes as my cell model since the predominant staining in COPD lung was localised in macrophages, and studied how inactivation of GSK3β affects inflammatory responses and glucocorticoid sensitivity. *In vitro* inactivation of the kinase was performed by pharmacological inhibition using the selective compound CT99021, by gene silencing using small interfering RNA and by transient overexpression of the constitutively active and inactive mutants, S9AGSK3β and K85AGSK3β.

Primary monocytes isolated from healthy volunteers were first subjected to treatment with the small molecule GSK3β inhibitor, CT99021. At concentrations at which the compound had no effect on cell viability (10⁻⁸M to 10⁻⁶M) it also had no effect on basal or LPS-induced pro-inflammatory GM-CSF release. These results do not agree with the ones obtained by Martin *et al.*, 2005. Their work showed that pharmacological inhibition of GSK3β with 10µM of the SB216763 compound, 10mM of LiCl or 200nM of azakenpaullone inhibited LPS-induced pro-inflammatory TNF-α and IL-6 release (Martin, Rehani *et al.* 2005). The anti-inflammatory effects of these drugs may be due to off-target effects of the compounds used. The SB216763 compound has been shown to inhibit other protein kinases including CDK2-cyclin A, ERK8, DYRK1A, PIM3, SRPK1 and HIPK2 (Polychronopoulos, Magiatis *et al.* 2004; Bain, Plater *et al.* 2007). In addition, the second inhibitor they used was LiCl which is
also known to inhibit some non-kinase targets such as inositol monophosphatase and histone deacetylases (Phiel and Klein 2001) which may affect pro-inflammatory gene transcription. The effect on HDACs may be particularly important as these have an evident role in regulating NF-κB induced pro-inflammatory gene transactivation (Adcock, Ito et al. 2005). The third compound they used, azakenpaullone, has been shown to have greater selectivity for GSK over CDK1 and CDK5 but there is lack of investigation of its selectivity against a large panel of protein kinases (Kunick, Lauenroth et al. 2004). In contrast with the compounds mentioned above, CT99021 is the most potent and specific inhibitor of GSK3 up to date (IC$_{50}$=40nM) (Cline, Johnson et al. 2002; Ring, Johnson et al. 2003). Specifically, at concentrations up to 10$^{-6}$M, CT99021 does not inhibit any other protein kinase in the panel used by the study conducted by Cohen and Goedert (2004) and it inhibits CDK2-cyclinA nearly 50-fold less strongly (IC$_{50}$ = 1.4µM). Since CT99021 was used at 10$^{-6}$M in my study it is unlikely to have off-target effects, which was also confirmed when its effects on the activity of a range of kinases were tested as discussed on the following chapter. Similar to the results obtained from primary monocytes, when I repeated these experiments in the monocytic cell line MonoMac6 cells I confirmed that inactivation of GSK3β does not affect pro-inflammatory CXCL8 release.

### 3.3.3. Role of GSK3β inactivation on corticosteroid anti-inflammatory function

Corticosteroids elicit their actions through binding and activation of GRα (Newton and Holden 2007). Recent studies have implicated that GSK3β directly regulates GRα functional effect through phosphorylation of serines 134 and 404 which regulate its nuclear export, promote its association with co-factors and modulate its trans-repressive actions (Chen,
GSK3β regulates corticosteroid function under conditions of OS

Dang et al. 2008; Galliher-Beckley, Williams et al. 2008). However the direct functional impact of GSK3β on the anti-inflammatory actions of corticosteroids has not been assessed. My results showed that pre-treatment of primary monocytes with CT99021 at 10⁻⁶M had no effect on inflammatory cytokine release by itself but significantly suppressed the concentration-dependent anti-inflammatory function of dexamethasone. The levels of LPS-induced TNF-α and GM-CSF cytokine release were not significantly inhibited by dexamethasone in the presence of CT99021. I repeated these experiments in the monocytic cell line MonoMac6 and obtained similar results. LPS-induced CXCL8 release was significantly inhibited by dexamethasone but dexamethasone’s function was attenuated when the cells were pre-treated with CT99021.

In order to validate that GSK3β activity is important for corticosteroid anti-inflammatory function, I knocked down total GSK3β levels by gene silencing using on target siRNA and studied dexamethasone sensitivity upon LPS stimulation. Due to limiting availability of primary monocytes, and since the effect of CT99021 on dexamethasone function was the same in the cell line, these experiments were carried out in the monocytic cell line MonoMac6. Transfection of cells with on-target GSK3β siRNA for 24 hours led to significant inhibition of total kinase levels (50-60%) which were further inhibited following 48 hours of transfection (20-40%) but began to return to control levels due to de novo expression of GSK3β at 72 hours following transfection (60%). I therefore studied the functional effect of GSK3β knock down in cells transfected with siRNA for 24 and 48 hours since this was when levels of the kinase were inhibited the most. Cell viability was significantly affected by electroporation but this method resulted in the highest transfection efficiency compared to other methods that were less toxic to the cells. Although electroporation alone reduced cell
GSK3β regulates corticosteroid function under conditions of OS viability, addition of the scramble and on-target siRNAs had no additional effect on cell death. In addition, transfection of the cells with on-target GSK3β siRNA had no effect on LPS-induced CXCL8 and TNF-α release, independent of the concentration of siRNA used (200nM and 400nM) or the time of transfection (24 and 48 hours). Importantly, and in agreement with the data obtained from pharmacological inhibition of GSK3β, knock down of the protein following 24 hours of transfection with the on-target siRNA, significantly suppressed the concentration dependent anti-inflammatory function of dexamethasone. Interestingly, when cells were transfected with siRNA for 48 hours before being subjected to treatment with dexamethasone and LPS for a further 16 hours, GSK3β knock down did not significantly affect dexamethasone function (CXCL8 and TNF-α release). This result may be due to the fact that the measurements were conducted 64 hours post transfection and initiation of de novo expression of GSK3β may be restoring the kinase's activity in the cells. Further experiments using stable transfections in MonoMac6 cells would help to resolve this observation.

Having shown that both pharmacological and molecular inhibition of GSK3β activity suppresses dexamethasone anti-inflammatory function, I next confirmed my observations by overexpressing the dominant negative K85R GSK3β mutant. When Lys85 on GSK3β is mutated to Arg, conformational changes of the substrate binding groove of kinase occur that inhibit its activity (Jope and Johnson 2004). Cells overexpressing the kinase dead K85R mutant GSK3β did not respond to dexamethasone and LPS-induced GM-CSF release levels were not inhibited by dexamethasone in contrast to cells transfected with the negative control pcDNA3.1 plasmid.
GSK3β regulates corticosteroid function under conditions of OS

My data so far have shown that GSK3β phosphorylation on Ser9, a marker of its inactivation, is increased in COPD and in vitro inactivation of the kinase leads to relative corticosteroid insensitivity. It is evident that the increased levels of reactive oxygen species in the lungs of COPD patients play a significant role on the effectiveness of corticosteroids (Chung and Marwick 2010). Since H₂O₂ induces Ser9 phosphorylation on GSK3β, I next wanted to validate whether this event is involved in driving oxidant stress-induced impaired corticosteroid function. In order to investigate this, I transfected MonoMac6 cells with the S9ApDNA3.1 plasmid construct and evaluated whether substitution of Ser9 with Ala will affect dexamethasone’ anti-inflammatory function following exposure to H₂O₂. In cells expressing the negative control pcDNA3.1, exposure to H₂O₂ suppressed the anti-inflammatory effects of dexamethasone on LPS-induced GM-CSF release. Interestingly, in cells overexpressing the S9AGSK3β mutant, exposure to H₂O₂ no longer affected dexamethasone function. This validates my hypothesis that Ser9 phosphorylation is a key mechanism regulating corticosteroid function, particularly during oxidant-driven inflammation in human monocytes.

Transfection of MonoMac6 cells with the pcDNA3.1 constructs induced an inflammatory response leading to increased levels of GM-CSF release. I first hypothesised that this might be due to endotoxin contamination of the plasmid DNA constructs and I reduced endotoxin levels down to <0.05 EU/µg before repeating the experiments. Interestingly, this did not reverse the pro-inflammatory effect of the DNA constructs plasmids on GM-CSF release. This set of data was conducted on cells that were in a higher passage number which resulted in both higher basal and plasmid DNA-induced GM-CSF levels compared to those measured before the endotoxin removal. I assessed whether the inflammatory effect was
GSK3β regulates corticosteroid function under conditions of OS

LPS-dependent by adding the LPS inhibitor Polymyxin B in combination with the plasmid DNA constructs. Although the drug inhibited LPS-induced GM-CSF release in cells that were electroporated without addition of any DNA construct, it had no effect on GM-CSF release in cells transfected with the control pcDNA3.1 construct. This observation suggests that transfection of MonoMac6 cells with plasmid DNA induces an LPS-independent pro-inflammatory response. The response was induced by both the control pcDNA3.1 plasmid and the S9A GSK3β and K85R GSK3β plasmid constructs suggesting that it is not related to the GSK3β sequence insertion. In addition, when cells were transfected with another plasmid construct, the NF-κB reporter pGL3 construct, a similar inflammatory response was induced, suggesting that MonoMac6 cells are sensitive to both types of plasmid DNA. Both types of vectors contain the amp resistance gene (ampR) which contains two copies of the immunostimulatory sequences (ISS) (palindromic CpG hexamer 5' AACGTT 3') (Klinman, Conover et al. 1998). These CpG sequences have been shown to stimulate an immune response in human monocytes (Sato, Roman et al. 1996) and may be responsible for inducing the inflammatory response observed in MonoMac6 cells. Further investigation is required to confirm whether this is the mechanism driving the plasmid DNA-induced inflammatory response in my cell system. In addition, evaluation of mutant GSK3β overexpression was performed by measuring levels of mRNA expression. Due to the possibility of the primers detecting the according plasmid DNA sequences inserted, Western blotting would be required to further validate the efficiency of my overexpression experiments.

The results obtained in this chapter showed that GSK3β inhibition due to increased phosphorylation of its Ser9 residue is increased in COPD patients and smokers with normal
GSK3β regulates corticosteroid function under conditions of OS

lung function. As I hypothesised, inhibition of GSK3β negatively regulates corticosteroid function in monocytes. As a result I accept that my hypothesis is true in both primary monocytes and MonoMac6 cells. The mechanism(s) by which extracellular reactive oxygen species affect GSK3β function and corticosteroid responsiveness in COPD is unknown. The next chapter describes a series of experiments designed to investigate the pathways involved in oxidant stress-induced GSK3β phosphorylation and the subsequent down-stream effects on GR function.
Chapter IV

The mechanism of GSK3β-regulated corticosteroid function in response to oxidative stress
4.1. Introduction

An increasing number of studies have implicated that exposure of immune cells to reactive oxygen species is a prominent feature of the corticosteroid insensitivity documented in patients with COPD. Oxidative stress leads to activation of the MAPK family, including the p38 MAPK, the ERK1/2 MAPK and the PI3K (Birrell, Wong et al. 2008).

The ERK MAPK pathway is activated by MEK, which is activated by the Raf member of MAP3Ks, in response to activated RTKs (Ramos 2008). The p38 MAPKs (including all p38-α, -β, -γ and -δ isoforms) are activated by MAP2Ks which are similarly activated by MAP3Ks in response to RTKs activation (Cuadrado and Nebreda 2010). Reactive oxygen species can alter protein structure and function by modifying critical amino acid residues of signalling proteins leading to activation of RTKs and the downstream MAPK pathways (Thannickal and Fanburg 2000).

The PI3K cascade is stimulated by phosphorylation of tyrosine kinase receptors, which promotes its direct binding with PI3K or indirect binding through adapter proteins. Activated PI3K phosphorylates (Ser473) and activates Akt (Vanhaesebroeck and Alessi 2000). The PI3K/Akt signalling pathway is redox sensitive. Activation or inhibition of this pathway by the redox system is mainly through oxidative modification of Cys-dependent phosphatases (CDPs) and protein kinases (Lee, Kwon et al. 1998; Yu, Li et al. 2005).

Phosphorylation of the p38 MAPK has been found elevated in COPD macrophages (Renda, Baraldo et al. 2008) and has been associated with corticosteroid insensitivity (Bhavsar, Hew et al. 2008). Recent studies have also identified elevated activation of the PI3K and the downstream target Akt in alveolar macrophages from COPD patients, and have shown that
The effect of CT99021 on global gene expression

activation of the PI3K-δ isoform is a key event mediating in cigarette smoking-induced corticosteroid insensitive emphysema in mice. GSK3β activity is negatively regulated by phosphorylation of its Ser9 residue which can be mediated by ERK1/2, Akt and p38 MAPK (Grimes and Jope 2001). Thus, GSK3β may represent the missing link between oxidant-mediated activation of ERK1/2, Akt and p38 MAPK and the resulting corticosteroid insensitivity documented in COPD.

Both direct and indirect oxidant-mediated disruption of GR-induced functional repression of genes and its ability to bind to its ligand and co-repressors have been implicated to drive corticosteroid insensitivity in COPD (Marwick and Chung 2010). Central to this mechanism is the reduction in activity and expression of co-repressors, HDAC2 which is critical for GR transrepression of several pro-inflammatory genes (Ito, Barnes et al. 2000). HDAC2 is recruited by GR to the site of NF-κB-regulated gene transcription deacetylates the amino terminal tails of the core histone proteins leading to chromatin remodelling and inflammatory gene (Ito, Barnes et al. 2000; Barnes, Ito et al. 2004). Alternatively, HDAC2 can target acetylated p65 and thereby regulate inflammatory gene expression. Reactive oxygen species mediate post-translational modifications (such as phosphorylation and nitration) on HDAC2 that impair the enzyme’s catalytic activity, target it for ubiquitination and degradation by the proteasome (Osoata, Yamamura et al. 2009) and result in increased expression of proinflammatory genes (Ito, Hanazawa et al. 2004). In addition, decreased HDAC2 activity has been shown to impair GR deacetylation, prevent its ability to bind to the NF-κB complex and exert its anti-inflammatory effects. HDAC2 overexpression in BAL macrophages from smokers and COPD patients has been also shown to restore corticosteroid function (Ito, Ito et al. 2005). Interestingly, selective inhibition of the PI3K-δ
The effect of CT99021 on global gene expression

isoform reversed corticosteroid insensitivity in cigarette smoke exposed mice via protection against serine hyper-phosphorylation and enzymatic activity suppression of HDAC2 (Marwick, Ito et al. 2007; Marwick, Caramori et al. 2009). I therefore hypothesised that regulation of GSK3β phosphorylation is mediated by members of the PI3K and MAPK signalling pathway in response to exogenous reactive oxygen species. Since both these redox sensitive pathways are known to regulate GR and HDAC2 activity, I hypothesised that differential regulation of either GR or HDAC2 is involved in mediating the GSK3β-dependent corticosteroid unresponsiveness. To investigate the above hypothesis I set the following aims:

- Investigate which members of the PI3K and MAPK signalling pathways are activated in response to H2O2.

- Evaluate whether inhibition of the key members of these pathways can restore GSK3β phosphorylation on Ser9 in the presence of H2O2.

- Investigate whether the CT99021 compound has any off-target effects on other kinases.

- Investigate how inhibition of GSK3β by CT99021 affects GR nuclear translocation, its binding to p65, p65 DNA binding and HDAC2 activity.
4.2. Results

4.2.1. Mechanism of oxidative stress induced GSK3β inactivation

4.2.1.1. Effect of \( \text{H}_2\text{O}_2 \) exposure on monocyte viability

Monocytes isolated from PBMCs were exposed to \( \text{H}_2\text{O}_2 \) at concentrations ranging from 50\( \mu \text{M} \) to 800\( \mu \text{M} \) for 30 minutes. ROS exposure had no effect on cell viability at concentrations up to 100\( \mu \text{M} \). However, cell death was observed when concentrations were increased from 200\( \mu \text{M} \) to 800\( \mu \text{M} \) of \( \text{H}_2\text{O}_2 \) (Figure 4.1).

4.2.1.2. Exposure to \( \text{H}_2\text{O}_2 \)-induced Ser9 phosphorylation on GSK3β in a time-and concentration-dependent manner

Treatment of monocytes isolated from PBMCs from normal subjects with \( \text{H}_2\text{O}_2 \) (50 \( \mu \text{M}-800 \) \( \mu \text{M} \)) for 30 minutes induced a concentration-dependent increase in GSK3β Ser9 phosphorylation levels (Figure 4.2). The concentration of \( \text{H}_2\text{O}_2 \) that induced optimal phosphorylation of GSK3β and had no effect of cell viability was 100\( \mu \text{M} \) (Figure 4.2), and this concentration was used to study the effects of \( \text{H}_2\text{O}_2 \) exposure over time in the following section.
The effect of CT99021 on global gene expression

Figure 4.1. High concentrations of H$_2$O$_2$ induced reduction of cell viability in primary monocytes. H$_2$O$_2$ had no effect on cell viability at 50µM and 100µM, but resulted in 40-50% cell death at higher concentrations ranging from 200µM to 800µM, *p<0.05 versus untreated cells.

Figure 4.2. H$_2$O$_2$ induced induces phosphorylation of GSK3β (Ser9) in a concentration-dependent manner in primary monocytes. Total levels of GSK3β were unaffected by treatment of monocytes with H$_2$O$_2$. Ser9 phosphorylation of GSK3β was induced at 100µM of H$_2$O$_2$ stimulation and was further increased at higher concentrations. The Western blot is representative of four individual experiments.
4.2.1.3. Effect of H$_2$O$_2$ treatment on kinase phosphorylation over time

Monocytes isolated from PBMCs from healthy subjects were treated with H$_2$O$_2$ (100 µM) for a time period ranging from 5 minutes to 60 minutes. Following 5-10 minutes of exposure, Akt was phosphorylated on Ser473. P-Ser473-Akt is a marker of activation of the upstream PI3K as Akt is its direct substrate (Figure 4.3). Phosphorylation of ERK1/2 (p44/42) on Thr202/Tyr204, a marker of its activation, was also induced by H$_2$O$_2$ (100 µM) time-dependently and was highest after 15 minutes of exposure (Figure 4.4). The phosphorylation status of Ser9 on GSK3β, a marker of its inactivation, was significantly increased following 30 minutes of H$_2$O$_2$ exposure (Figure 4.5a). The levels of phosphorylation on its Tyr216 residue which activates the kinase were also measured following H$_2$O$_2$ treatment. P-Tyr216 levels were increased after 5 minutes of exposure to H$_2$O$_2$ and remained high after 15 minutes. However, at 30 and 60 minutes following treatment with H$_2$O$_2$, the levels decreased back to basal levels (Figure 4.5b).
Figure 4.3. H₂O₂ leads to Akt phosphorylation in primary monocytes. Monocytes from healthy subjects were treated with H₂O₂ (100µM) for a time course ranging from 5 to 60 minutes. Activation of PI3K as measured by the phosphorylation status of its downstream direct target Akt on Ser473 was significantly increased following 5 and 10 minutes of exposure to H₂O₂ (a, b). (a) Western blot is a representative of 4 independent experiments that were used for band densitometry. Phospho-Ser473-Akt levels were normalised to total Akt. (b) Histograms represent the mean±S.E.M. of 4 independent experiments, *p<0.05, **p<0.01.
**Figure 4.4.** \( \text{H}_2\text{O}_2 \) leads to ERK1/2 activation in primary monocytes. Monocytes from healthy subjects were treated with \( \text{H}_2\text{O}_2 \) (100µM) and levels of phospho-p44/42 (ERK1/2) were measured by Western blot analysis following 5 to 60 minutes of treatment. Activation of ERK1/2 isoforms was evident after 5 minutes of treatment and was highest after 15 minutes of \( \text{H}_2\text{O}_2 \) exposure (a, b). (a) Western blot is a representative of 4 independent experiments that were used for band densitometry. (b) Histograms represent the mean±S.E.M. of 4 independent experiments, *\( p<0.05 \), **\( p<0.01 \).
The effect of CT99021 on global gene expression

(a) Total GSK3β

(b) p-Ser9 / total GSK3β

(c) p-Tyr216 / total GSK3β

(min) H₂O₂ (100µM)
Figure 4.5. 

H$_2$O$_2$ exposure leads to GSK3β inactivation in primary monocytes. Monocytes from healthy subjects were treated with H$_2$O$_2$ (100µM) and levels of phospho-Tyr216 and phospho-Ser9 on GSK3β were measured by Western blot analysis following 5 to 60 minutes of treatment. Inactivation of GSK3β was evident at 30 to 60 minutes of exposure to H$_2$O$_2$ as shown by increased levels of Ser9 phosphorylation (a and b). The phosphorylation status of Tyr216 was increased in the first 5 to 10 minutes of H$_2$O$_2$ treatment but returned to basal levels at 30 and 60 minutes following the treatment (a and c). (a) Western blot is a representative of 4 independent experiments that were used for band densitometry. (b, c) Histograms represent the mean±S.E.M. of 4 independent experiments, *p<0.05, **p<0.01 vs untreated cells.

4.2.1.4. Exposure to H$_2$O$_2$ induced Ser9 phosphorylation on GSK3β in an ERK1/2-, Akt-, and p38 MAPK- dependent pathway in monocytes derived from PBMCs

Oxidant-dependent phosphorylation of GSK3β on Ser9 was significantly inhibited by pre-treatment (30 minutes) with the selective MEK inhibitor (U0126) and the selective Akt inhibitor (MK-2206). Inhibition of the p38MAPK (SB239063) prior to H$_2$O$_2$ stimulation also reduced the phosphorylation status of GSK3β but levels were not restored to basal levels. The selective PI3K-δ inhibitor (IC87114) had no significant effect on H$_2$O$_2$ induced p-GSK3β (Figure 4.6).
Figure 4.6. \( \text{H}_2\text{O}_2 \) exposure inhibits GSK3\( \beta \) in peripheral blood monocytes via activation of the AKT, ERK1/2 and p38 MAPK kinases. Monocytes were pre-treated with Akt (MK-2206-Pfizer), MEK (U0126), p38 MAPK (SB239063) and PI3K\( \delta \) (IC87114) inhibitors for 30 minutes. The cells were then subjected to treatment with \( \text{H}_2\text{O}_2 \) (100\( \mu \text{M} \)) for 30 minutes. Exposure to \( \text{H}_2\text{O}_2 \) leads to a significant increase of p-Ser9-GSK3\( \beta \) levels. This \( \text{H}_2\text{O}_2 \) induced effect was inhibited by selective inhibition of Akt, MEK and p38 MAPK. However, oxidant stress induced phosphorylation of GSK3\( \beta \) is independent of the PI3K\( \delta \) isoform activity. (a) Blots are representative of 6 individual experiments. Histograms represent mean±S.E.M. of six independent experiments*\( p<0.05, **p<0.01. \)
4.1.5. Exposure to H$_2$O$_2$ induced activation of ERK1/2 is not mediated by Akt or PI3Kδ in human monocytes

Exposure of primary monocytes to H$_2$O$_2$ for 30 minutes induced a 3-fold increase in the phosphorylation status of Thr202/Tyr204 on p44/42 (ERK1/2) MAPK, which is a marker of kinase activation. Inhibition of MEK by 30 minutes pre-treatment with the compound U0126 (1µM) inhibited ERK1/2 activation and the levels of P-Thr202/Tyr204 on p44/42 MAPK were restored to basal levels. By contrast, inhibition of Akt and PI3Kδ by pre-treatment with the small molecule inhibitors MK-2206 and IC87114 respectively (both at 1µM) for 30 minutes had no effect on H$_2$O$_2$-induced activation of ERK1/2 MAPK suggesting that these kinases are not involved in mediating ERK1/2 activation in response to exogenous H$_2$O$_2$ (Figure 4.7).
The effect of CT99021 on global gene expression

**Figure 4.7.** $\text{H}_2\text{O}_2$ induced activation of ERK1/2 is not mediated by PI3K. Monocytes were pre-treated with Akt (MK-2206-Pfizer), MEK (U0126) and PI3Kδ (IC87114) inhibitors for 30 minutes. The cells were then subjected to treatment with $\text{H}_2\text{O}_2$ (100µM) for 15 minutes. Exposure to $\text{H}_2\text{O}_2$ led to significant increase of phospho-p44/42 MAPK (Erk1/2) on Thr202/Tyr204. This activation of ERK1/2 was only inhibited by pre-treatment with the MEK inhibitor and is not mediated by the PI3K/Akt pathway. (a) Western blots are representative of 4 independent repeated experiments. (b) Histograms represent mean±S.E.M. of 4 independent experiments* $p<0.05$. 
4.2.2. Investigation of the effects of the CT99021 compound and H₂O₂ on kinase activity

In order to confirm that the CT99021 compound does not exert any off-target effects on other kinases known to be involved in regulating inflammation, I investigated its effect on the activity of a range of kinases. I used H₂O₂-stimulated monocytes as a control experiment to confirm the liability of the kinase array as it is known to lead to rapid phosphorylation/activation of a wide range of kinases. Monocytes from healthy subjects were treated with 1µM of CT99021 for 30 minutes, which is the time and concentration with which the compound induced corticosteroid insensitivity as discussed in chapter 3. Whole cell lysates were incubated with a kinase array containing antibodies for a range of phosphorylated kinases as a marker of their activation. The duplicate spots on the membrane, as shown in Figure 4.8a, represent each phosphorylated kinase protein and were used for pixel densitometry. The phosphorylation status of each kinase in the lysates, obtained from cells treated with CT99021, was normalised to the phosphorylation status in the lysates obtained from untreated cells and the values were expressed as fold increase as shown in the histograms in Figure 4.8b. Treatment of monocytes with CT99021 had no off-target effects on the kinases tested. However, there was a significant increase in total levels of beta-catenin, which is the direct downstream effector of GSK3β regulating beta catenin degradation. In addition, as a control experiment, monocytes were treated with H₂O₂ (100µM) for 30 minutes and the results showed a number of kinases being activated by phosphorylation as shown in Figure 4.8c. Activation of ERK1/2 and Stat3 was significantly increased in response to exogenous H₂O₂. Additionally, total beta-catenin protein levels were also increased following treatment with H₂O₂.
The effect of CT99021 on global gene expression

a. NS +ve control
    -ve control

H₂O₂ (100nM) CT99021 (1µM)

b. H₂O₂ (100nM)

c. CT99021 (1µM)
The effect of CT99021 on global gene expression

**Figure 4.8.** Effect of H$_2$O$_2$ exposure and treatment with the CT99021 compound on kinase activation. Monocytes from healthy subjects were treated with the CT99021 (1µM) compound (a and b) or with H$_2$O$_2$ (100µM) for 30 minutes (a and c). The whole cell lysates were used to measure kinase activation by measuring the levels of their phosphorylation as represented by the (a) dark spots (in duplicate) on the kinase array. The spots were used for pixel densitometry and the levels of phosphorylation were expressed as fold increase in phosphorylation of unstimulated cells. (b) H$_2$O$_2$ led to activation of a number of kinases, from which ERK1/2, Stat3 were significantly activated. Total beta-catenin levels were also significantly increased. (c) By contrast, CT99021 did not have any off-target effects and only led to significant increase of levels of beta-catenin. Histograms represent the average signal (pixel density) of the pair of duplicate spots normalized to the equivalent one derived from untreated cells. The average signal is expressed as fold increase compared to the positive control (dark blue bar), represented as a horizontal straight line. The data represent the mean±S.E.M. of 3 independent experiments and *p<0.05, **p<0.01 vs untreated cells.

4.2.3. Mechanism of GSK3β induced impairment of glucorticoid function

4.2.3.1. Effect of GSK3β knock down on GR expression

I investigated the mechanism by which GSK3β modulates dexamethasone function in monocytes. Knock down of GSK3β protein by siRNA (200nM and 400nM) in MonoMac6 cells led to significant decrease of total GSK3β protein levels as also shown in more details in the previous chapter (Chapter 3). Inhibition of total GSK3β protein levels correlated with a 50% decrease of total GRα levels in cells transfected with either 200nM or 400nM of the on-target GSK3β siRNA (Figure 4.9a, b).

4.2.3.2. Effect of GSK3β inactivation on GR nuclear translocation

Next I investigated whether inhibition of GSK3β affects GRα nuclear translocation upon treatment with dexamethasone. MonoMac6 cells were pre-treated with CT99021 (30 minutes) at the concentration that it has a functional effect (10^{-6}M) and were treated with dexamethasone for 2 hours prior to being stimulated with LPS (10ng/ml). LPS did not affect
The effect of CT99021 on global gene expression

GR localisation, whereas treatment with dexamethasone induced nuclear translocation of the receptor. Treatment with the CT99021 compound did not affect GR nuclear translocation in response to dexamethasone either in the presence or the absence of LPS (Figure 4.10a, b).

![Scramble siRNA and on target siRNA](image)

**Figure 4.9.** GSK3β knock down resulted in reduction of total GRα protein levels in MonoMac6 cells. GSK3β protein knock down reduced total protein levels of GSK3β and GRα in MonoMac6 cells. GRα protein levels normalised to β-actin were significantly suppressed in cells transfected with on-target GSK3β siRNA, 200nM (a, b) and 400nM (a, c) for 24 hours. Histograms represent mean±S.E.M. of 3 (b) and 4 (c) independent experiments. KD: knock down of GSK3β protein using on-target GSK3β siRNA.
The effect of CT99021 on global gene expression

a. Cytoplasm

GRα

β-actin

**

b. Nucleus

GRα

Lamin A/C

**

LPS (10ng/ml)
Dex (10⁻⁶ M)
CT99021 (10⁻⁶ M)
The effect of CT99021 on global gene expression

Figure 4.10. GRα nuclear translocation is not affected by GSK3β inactivation. GRα nuclear translocation upon treatment with dexamethasone was not affected by GSK3β inhibition. Levels of cytoplasmic GRα protein levels were decreased upon stimulation with dexamethasone (1µM for 2 hours) (a) which correlated with increased levels of the protein found translocated in the nucleus (b). Pre-treatment with CT99021 for 30 minutes had no effect on GR nuclear localisation (a, b). The Western blot bands were used for densitometry and the values were normalised to β-actin for cytoplasmic levels of GR and Lamin A/C for nuclear levels of GR. Histograms represent mean±S.E.M. (n=3 (a) and 4 (b)). **p<0.01 vs unstimulated cells.

4.2.3.3. Effect of GSK3β inactivation on association of GR with NF-κB/p65

As shown in the previous section GR nuclear translocation was not affected by inhibition of GSK3β activity. Therefore, I next investigated whether binding of GR to NF-κB/p65 in response to dexamethasone is affected by GSK3β inactivation. Monocytes from healthy volunteers were pre-treated with the CT99021 compound for 30 minutes and subsequently stimulated with LPS (10ng/ml) and dexamethasone (1µM) for 2 hours. NF-κB/p65 was ImmunoPrecipitated in the whole cell lysates and the Western blots were re-probed with the GR antibody to detect levels of the receptor that have formed a complex with p65 (Figure 4.11a). In response to LPS and dexamethasone, GR was bound to p65 in the monocytes. In the cells where GSK3β was inhibited by pre-treatment with CT99021 the levels of GR bound to p65 were not affected (Figure 4.11).
The effect of CT99021 on global gene expression

Figure 4.11. Levels of GR bound to NF-κB/p65 are not affected by GSK3β inhibition. GR did not form a complex with p65 under resting conditions and this was unaffected by LPS (2 hours) alone. In the presence of dexamethasone (1µM for 2 hours) a much greater GR/p65 association was seen. In the presence of the GSK3β inhibitor alone, with LPS or with dexamethasone alone, GR did not form a complex with p65. When monocytes were treated with both LPS and dexamethasone, the presence of CT99021 had no effect on the levels of GR bound to p65. The Western blot bands (a) were used for densitometry and the values of GR protein levels were normalised to the levels of ImmunoPrecipitated p65. Histograms represent mean±S.E.M. of three independent experiments with *p<0.05 vs unstimulated cells.
4.2.3.4. NF-κB p65 DNA binding capacity is not regulated by GSK3β kinase activity

I next investigated whether the DNA binding capacity is influenced by GSK3β inactivation in the absence or presence of dexamethasone under LPS stimulation. Monocytes from healthy subjects were pre-treated with the CT99021 (10^{-6}M) inhibitor for 30 minutes before being subjected to treatment with dexamethasone (10^{-6}M) followed by stimulation with LPS (10ng/ml) for 2 hours. Nuclear proteins were extracted and NF-κB/p65 DNA binding capacity was measured. Stimulation of the cells with LPS induced a significant increase in NF-κB DNA binding activity but the ability of the transcription factor to bind to its consensus DNA sequence was not affected by 30 minutes pre-treatment with dexamethasone (10^{-6}M). Inhibition of GSK3β by CT99021 had no effect on NF-κB p65 binding under any stimuli tested (Figure 4.12).
The effect of CT99021 on global gene expression

4.2.3.5. Effect of GSK3β inhibition on HDAC2 mRNA and protein expression

Monocytes from healthy subjects were treated with CT99021 for a time course of 16, 24 and 48 hours. The whole cell lysates were analysed by Western blotting for total protein levels of HDAC2. CT99021 treatment did not affect HDAC2 protein expression after 16 and 24 hours of treatment (1μM) (Figure 4.13a). Monocytes were also treated with CT99021 for a time course of 4, 6, 8 and 16 hours before RNA was collected for gene expression analysis by RT-PCR. HDAC2 mRNA expression was also not affected by treatment with CT99021 (100nM, 500nM and 1μM) for up to 16 hours.

Figure 4.12. NF-κB/p65 DNA binding capacity is not affected by CT99021 in monocytes from PBMC’s. The capacity of the NF-κB p65 transcription factor to bind to its consensus DNA sequences was not affected by inhibition of GSK3β in primary monocytes. LPS stimulation (2 hours) increased nuclear p65 DNA binding capacity. Neither dexamethasone nor CT99021 pre-treatment (30 minutes) had any effect on p65 binding to DNA. Histograms represent mean±S.E.M. of the OD values of four independent experiments, *p<0.05 vs unstimulated cells.
4.2.3.6. Effect of GSK3β inhibition on HDAC2 activity

Inhibition of GSK3β with CT99021 in peripheral blood monocytes for 30 minutes had no significant effect on ImmunoPrecipitated HDAC2 activity at 100nM (10.2±9.2%) but reduced ImmunoPrecipitated HDAC2 activity by 31.3±5.6% (p<0.05) when added at 1µM. Similar levels of reduction in HDAC2 activity (23.3±6.1%) were observed when primary blood monocytes were treated with H₂O₂ (100µM) for 1 hour (Figure 4.14).

4.2.3.7. Inhibition of GSK3β led to post-translational modifications on HDAC2

Inhibition of GSK3β by CT99021 (1µM) for a time course (15, 30, 60 and 120 minutes) led to a two-fold induction of phosphorylation on the Ser-394 residue of HDAC2. This phosphorylation event was only evident following 30 minutes of exposure to CT99021 and p-Ser394-HDAC2 levels were restored to basal levels after 60 minutes (Figure 4.15).
The effect of CT99021 on global gene expression

Inactivation of GSK3β does not affect HDAC2 protein or mRNA expression in peripheral blood monocytes. Inhibition of GSK3β by CT99021 had no effect on HDAC2 mRNA or protein expression. Total protein levels of HDAC2 were unaffected after (a) 16 hours and 24 hours treatment with CT99021 (1µM). (b) HDAC2 mRNA expression levels normalised to 18S were not regulated by CT99021 (100nM, 500nM and 1µM) following 4, 6, 8 and 16 hours of treatment. Blot is representative of n=5. Histograms represent mean±S.E.M. (n=3 and n=4) and data are plotted as fold-increase gene expressions versus non-stimulated cells.
Figure 4.14. Inactivation of GSK3β leads to reduction of HDAC2 enzymatic activity in primary monocytes. Exposure of primary monocytes to CT99021 inhibitor (100nM) for 30 minutes had no effect on the enzymatic activity of ImmunoPrecipitated HDAC2. By contrast, treatment of monocytes with 1µM CT99021 led to significant reduction of HDAC2 activity. Exposure of monocytes to 100µM H₂O₂ for 1 hour also led to inhibition of HDAC2 activity. Histograms represent mean±S.E.M. of four independent experiments. *p<0.05 vs unstimulated cells.
The effect of CT99021 on global gene expression

Figure 4.15. Inactivation of GSK3β leads to increase p-Ser394-HDAC2 levels in primary monocytes.
Treatment of primary monocytes from healthy subjects with CT99021 (1µM) mediated inhibition of GSK3β induced phosphorylation on the Ser394 residue of HDAC2 which peaked following 30 minutes of exposure. Western blots were used for band densitometry and the histograms represent mean±S.E.M. (n= 4). *p<0.05 vs unstimulated cells.
4.3. Discussion

4.3.1. Mechanism of $H_2O_2$ induced GSK3β inactivation

4.3.1.1. Exposure of monocytes to $H_2O_2$ leads to activation of PI3K and ERK1/2 MAPK and inactivation of GSK3β in a time-dependent manner

Monocytes isolated from healthy subjects were treated with $H_2O_2$ as a source of reactive oxygen species. At the concentration where it did not affect cell viability (100µM), treatment with $H_2O_2$ resulted in activation of PI3K which was measured by the phosphorylation levels of the Ser473 residue of its direct downstream target, Akt (Cross, Alessi et al. 1995). Activation of Akt was evident in the first 5 to 10 minutes of exposure to $H_2O_2$ and levels of phospho-Ser473 Akt were then brought back to normal. Activation of PI3K and subsequent phosphorylation of Akt has been previously shown in human monocytic cell lines agreeing with my results (Marwick, Caramori et al. 2009; Mercado, To et al. 2011). By contrast, phosphorylation of p44/42 (ERK1/2) MAPK on Thr202/Tyr204, a marker of its activation, was evident after 5 minutes of exposure, peaked at 15 minutes and remained higher than basal levels up to 30 minutes of treatment with $H_2O_2$. ERK1/2 activation was dependent on MAP/ERK kinase (MEK) activity in my system as inhibition of the kinase blocked $H_2O_2$ induced phosphorylation on ERK1/2. This finding is in line with previous studies showing that oxidative stress leads to phosphorylation of ERK1/2 by MEK in response to activation of RTKs and the downstream effector Raf (Meves, Stock et al. 2001; Aslan and Ozben 2003; Ramos 2008). Phosphorylation of GSK3β on its Ser9 residue, a marker of its inactivation was significantly increased following 30 to 60 minutes of exposure. Another phosphorylation site of GSK3β that regulates its kinase activity is the Tyr216.
The effect of CT99021 on global gene expression

residue. Phosphorylation of this residue was increased the first 5-15 minutes of exposure to H$_2$O$_2$ but levels were decreased at 30 and 60 minutes following treatment. Since H$_2$O$_2$ exposure leads to activation of MEK1 in response to Raf activation in several cell systems (Son, Cheong et al. 2011) and activated MEK1 is known to phosphorylate GSK3β on Tyr216 in airway smooth muscle cells (Takahashi-Yanaga, Shiraishi et al. 2004), this could be a possible mechanism leading to increased Tyr216 phosphorylation on GSK3β in my system. Further investigation is required to identify the exact signalling cascade and kinase responsible for H$_2$O$_2$-induced phosphorylation of the Tyr216 residue on GSK3β. However, since the levels of p-Tyr216-GSK3β were restored to basal levels after 30 and 60 minutes of exposure this confirms that GSK3β activity is inhibited in response to exogenous reactive oxygen at those time points. The use of the kinase dead mutants, as described in previous chapter, may resolve whether prior activation of GSK3β linked to Tyr216 phosphorylation plays a role in the inflammatory or anti-inflammatory response.

4.3.1.2. H$_2$O$_2$ induced inactivation of GSK3β is mediated by p38 MAPK, ERK1/2 MAPK and Akt in primary monocytes

Inactivation of GSK3β in response to exogenous reactive oxygen species in primary monocytes is inhibited by pre-treatment of the cells with the MEK inhibitor, the Akt inhibitor and the p38 MAPK inhibitor. These results show that p-Ser9-GSK3β is regulated by these protein kinases concluding that they are upstream components of this H$_2$O$_2$-activated signalling cascade. As discussed previously, Akt activation follows activation of its upstream regulator PI3K. Since, inactivation of the PI3Kδ isoform had no effect on H$_2$O$_2$-induced inactivation of GSK3β, it is clear that Akt activation and subsequent phosphorylation of its direct target, GSK3β, is not PI3K isoform specific. In addition, inactivation of ERK1/2 MAPK
by inhibition of its upstream regulator, MEK, also inhibited GSK3β phosphorylation on Ser9 in response to H₂O₂ in my cell system. In addition, my results also showed that p38 MAPK is also involved in mediating oxidant stress-induced inactivation of GSK3β. SB239063 exhibits equipotent inhibitory activity against α- and β- p38 isoforms (Underwood, Osborn et al. 2000). The p38α and p38β isoforms are known to be activated in response to reactive oxygen species via oxidative modification of the MAP3K intracellular kinases (Son, Cheong et al. 2011). One suggested mechanism mediating activation of p38 in response to reactive oxygen species is via ASK-1 (apoptosis signal-regulating kinase-1), a member of the MAP3Ks which binds to thioredoxin in resting cells. Upon oxidative stress stimulation, thioredoxin becomes oxidised and dissociates from ASK-1 leading to activation of the p38 MAPK pathway through oligomerisation of ASK-1 (Nagai, Noguchi et al. 2007). The exact nature of the signalling intermediates between p38 MAPK and GSK3β however is not known. My findings are in line with previous work showing that p38 MAPK regulates GSK3β function in the Wnt-beta-catenin signalling cascade and inhibition of p38 blocked Wnt-induced phosphorylation of Ser9 on GSK3β in mouse cancer cells (Bikkavilli, Feigin et al. 2008). Another possible mechanism that has been suggested by other studies is that p38 MAPK is involved in regulating Akt activation in response to intracellular reactive oxygen species leading to regulation of GSK3β activity (Wang, Liu et al. 2011). Further work is required to elucidate the mechanism by which p38 MAPK regulated GSK3β function in my system.
4.3.2. Evaluation of the CT99021 specificity against a range of kinases

My results so far have demonstrated that loss of GSK3β due to the presence of reactive oxygen species leads to corticosteroid insensitivity in healthy monocytes. This was shown by using both siRNA targeting the GSK3β gene and by treatment of monocytes with the CT99021 compound to inhibit its kinase activity. Because my experiments were conducted in primary monocytes, I could not use GSK3β knock down approaches to investigate the mechanism that induces steroid insensitivity due to the limitations of availability of primary monocytes. Therefore, I ought to investigate whether CT99021 has any off target effects or is selective for GSK3β in my cell system. I screened the effects of the CT99021 compound over the activation of a range of kinases. My results showed that CT99021 had no off target effects at the time points tested and it only led to increased levels of total beta-catenin protein levels. In resting cells, beta-catenin is degraded by active GSK3β and the observed increase in its total levels confirms that the target of CT99021 inhibits GSK3β specifically and thus inhibits beta-catenin degradation at the concentration and time points tested. However, to further confirm that the CT99021 compound has no off-target in my model of LPS-stimulated monocytes, another kinase array should be performed to evaluate whether LPS-induced activation of any of the kinases tested may be affected by treatment with the CT99021 compound.

As a positive control for the kinase array I used the same technique to measure the levels of phosphorylated kinases in response to treatment with H₂O₂. This exposure led to significant activation of a number of kinases. The ERK1/2 MAPK was activated confirming my results from Western blotting, possibly due to activation of the upstream kinase Raf MAP3K in response to reactive oxygen species. The signal transducer and activator of transcription 3
(Stat3) was also activated in response to treatment of monocytes with H$_2$O$_2$. Stat3 also has been demonstrated to be sensitive to changes in the redox state several cell types (Simon, Rai et al. 1998; Cao, Tal et al. 2007). The mechanism of ROS induced activation of Stat3 is not known but activation of receptor tyrosine kinases, such as EGFR has been suggested in previous studies (Park, Schaefer et al. 2000). Additionally, H$_2$O$_2$ exposure led to increased total protein levels of beta-catenin. This event confirms that H$_2$O$_2$ inactives GSK3β suppressing its action upon beta-catenin, which would normally lead to degradation of the latter.

### 4.3.3. Mechanism of GSK3β regulated corticosteroid insensitivity in monocytes

Previous studies have documented that reactive oxygen species directly regulate corticosteroid responsiveness via activation of the p38 MAPK and ERK1/2 kinases and subsequent modulation of GR activity and ligand-binding affinity in corticosteroid insensitive diseases such as severe asthma (Irusen, Matthews et al. 2002; Bhavsar, Hew et al. 2008). A recent study identified that stress activated p38 MAPK induces ligand-independent phosphorylation on GR which regulates its co-factor binding and GRE binding (Galliher-Beckley, Williams et al. 2011). Since these pathways were shown to mediate inactivation of GSK3β in my system I ought to investigate how GSK3β inhibition affects GR expression. In the human monocytic cell line MonoMac6, reduction of total GSK3β protein levels via siRNA gene silencing, correlated with a reduction of total GR protein levels. I then investigated how inactivation of GSK3β modulates GRα nuclear translocation. My results clearly showed that despite reduced GRα expression, inhibition of GSK3β by CT99021 had no effect on GRα nuclear translocation upon ligand binding. Therefore I concluded that GSK3β may be involved in regulating GRα protein expression by controlling post-translational modifications.
The effect of CT99021 on global gene expression

and degradation processes that the receptor is subjected to. However, my results agree with previous studies suggesting that reduction of GR protein in COPD is not responsible for the impaired corticosteroid mediated gene transrepression, since even decreased protein levels of the receptor are sufficient for corticosteroid to exert their anti-inflammatory actions upon binding to the receptor in the cytoplasm and translocating into the nucleus (Marwick, Caramori et al. 2009).

Recent studies have identified that GSK3β can lead to in vitro phosphorylation of Ser404 on GR in several cell lines (including human osteosarcoma MG-63, liver hepatocellular Hep-G2 and lung adenocarcinoma epithelial A549 cells) which regulates its ability to bind to co-regulators such as NF-κB (Galliher-Beckley, Williams et al. 2008). Hence I investigated the effect that inactivation of GSK3β may have on GR binding to NF-κB/p65 in response to dexamethasone and LPS. My results demonstrated that the GR-p65 association is not regulated by GSK3β in my system. My observation is in line with previous studies showing that cigarette smoke induced corticosteroid insensitivity is not mediated by altered GR-p65 association in A549 epithelial cells (Ito, Yamamura et al. 2006). However, I cannot exclude the possibility that the reduction of total GR levels due to inhibition of GSK3β has no effect on GR-GRE binding and commercial availability of and anti-p-Ser134-GR or p-Ser404-GR antibody would allow me to investigate whether GSK3β is involved in mediating the p38 MAPK effect on GR. ChIP analysis of GR binding to inflammatory mediator promoters along with analysis of co-factor recruitment by mass spectroscopy would provide useful information.
MAPKs are known to activate NF-κB in airway epithelial cells and macrophages exposed to cigarette smoke (Koch, Giembycz et al. 2004; Birrell, Wong et al. 2008; Cheng, Luo et al. 2009), therefore I investigated whether GSK3β is involved in mediating ROS-induced regulation of NF-κB/p65 DNA binding capacity in peripheral blood monocytes. My data illustrated that NF-κB/p65 ability to bind to its consensus DNA sequences in the nucleus is not affected by inhibition of GSK3β activity. However, further investigation is required to show whether GSK3β inhibition has any effect on post-translational modifications on p65 which do not interfere with its ability to bind to the promoters of inflammatory genes, but regulate the recruitment of co-repressor/activators and may therefore interrupt p65-induced transactivation. In addition, treatment of cells with dexamethasone did not suppress LPS-induced NF-κB/p65 capacity to bind to DNA. This has been previously shown (Ito, Yamamura et al. 2006) and has led to the hypothesis that NF-κB-dependent inflammatory gene transcription is regulated by GR-mediated recruitment of co-repressors, such as HDAC2, which control p65-regulated histone acetylation on the site of gene transcription (Barnes 2009).

Hence, I investigated the possibility that GSK3β modulates GR function via alterations on the function its co-repressor HDAC2. I demonstrated that inhibition of GSK3β had no effect on mRNA and protein expression of HDAC2. However, my results showed that GSK3β inactivation leads to increased Ser394 phosphorylation on HDAC2 which correlates with an approximate 30% reduction in HDAC2 enzymatic activity. A similar reduction in HDAC2 activity was also observed in my system by H₂O₂ agreeing with what has been previously described (Tsai and Seto 2002; Ito, Yamamura et al. 2006; Meja, Rajendrasozhan et al. 2008; Mercado, To et al. 2011). It is unclear how regulation of Ser394 phosphorylation affects the
The effect of CT99021 on global gene expression

enzyme’s activity. Previous studies have proposed that the phosphorylation status of the Ser394 residue on HDAC2 is important for the enzyme’s conformational changes and regulates its binding capacity to other co-regulators (Tsai and Seto 2002). The mechanism by which GSK3β affects HDAC2 phosphorylation and activity has not been elucidated yet. A possible indirect mechanism may involve the actions of casein kinase 2 (CK2). CK2 is known to phosphorylate HDAC2 on Ser394 and is directly inactivated via phosphorylation by GSK3β (Sun, Chen et al. 2002; Tsai and Seto 2002); when the latter is inactive due to reactive oxygen species exposure, CK2 remains active and could then bind to and phosphorylate HDAC2.

Previous studies have suggested that cigarette smoke induced loss of HDAC2 leads to hyperacetylation of GR which in turn disrupts its binding to NF-κB (Ito, Yamamura et al. 2006). This does not seem to be the case in my corticosteroid insensitive system since binding to p65 was not affected by GSK3β inhibition. My mechanism suggests that reduction of HDAC2 activity leads to inhibition of GR function and this may be a result of increased levels of acetylated histones which lead to increased transactivation. Further investigation is required to examine the exact mechanism by which reactive oxygen species-induced inhibition of GSK3β leads to reduction of HDAC2 activity.

Inactive GSK3β is crucial for corticosteroid responsiveness in monocytes as shown in the previous chapter. The results shown in this chapter demonstrated that inactivation of GSK3β takes place in response to intracellular reactive oxygen species, as mimicked by treatment with exogenous H₂O₂ in an Akt, ERK1/2 and p38 MAPK dependent manner. Next, I sought to investigate the downstream mechanism that leads to corticosteroid insensitivity. Inactivation of GSK3β leads to decreased levels of total GR protein but did not affect GR
nuclear translocation or its capacity to bind to its co-regulator NF-κB. The DNA binding capacity of the NF-κB transcription was also unaffected by the activity of GSK3β. Interestingly, I found that inhibition of GSK3β reduced the enzymatic activity of the critical co-repressor of GR, HDAC2. This reduction in activity correlated with an induction of the phosphorylation status of its Ser394 residue. The mechanism by which this phosphorylation regulates the function of the enzyme is not known. My results show that it is a rapid event suggesting that may be priming the enzyme for further post-translational modifications that may alter its deacetylase activity or its binding capacity to co-regulators in human monocytes. Further investigation of the exact role of this phosphorylation event on controlling HDAC2 function under oxidant stress induced inactivation of GSK3β is required.

In this chapter I identified a mechanism involved in regulating GR function in response to oxidant stress in human monocytes. This is mediated via H$_2$O$_2$-induced inactivation of GSK3β which results in a reduction of HDAC2 activity and thus promotes NF-κB-mediated transactivation of pro-inflammatory genes. This oxidant-induced effect on GSK3β function is regulated by activation of the upstream Akt and ERK1/2 MAPK kinase. Based on these findings, I accept my hypothesis. So far, I have investigated the effect of GSK3β inhibition on controlling dexamethasone-induced suppression of GM-CSF, CXCL8 and TNF-α release. In order to investigate how inactivation of GSK3β also affects the mRNA expression of other corticosteroid-regulated genes, I used microarrays and RT-qPCR as discussed in the following chapter.
Chapter V

The effect of CT99021 on global corticosteroid-regulated gene expression
5.1. Introduction

In the previous chapters I discussed how oxidant stress-mediated inhibition of GSK3β induces corticosteroid insensitivity in primary monocytes and MonoMac6 cells. The functional effect of GSK3β inhibition was measured by its inhibitory action on LPS-induced pro-inflammatory cytokines’ and chemokines’ release (GM-CSF, CXCL8 and TNF-α). In order to confirm that the pro-inflammatory effect of GSK3β is regulated on mRNA level of inflammatory mediators, I measured the effects of CT99021 on dexamethasone-induced inhibition of LPS-stimulated gene expression. I used RT-PCR to measure the effects of GSK3β inhibition on corticosteroid-mediated transrepression of IL-8 and CSF2 mRNA expression. Hence, I aimed to investigate what are effects of GSK3β inhibition on global gene expression and which pathways are consequently differentially regulated due to aberrant GSK3β activity. In this chapter, I focus on the effects of CT99021 on LPS-induced and LPS/dexamethasone-induced mRNA expression in MonoMac6 cells. My hypothesis is that GSK3β activity regulates a wide range of corticosteroid-sensitive inflammatory genes’ expression. In order to test this hypothesis I set the following aims:

- Perform microarrays to investigate the effects of CT99021 on LPS/dex-regulated global gene expression
- Identify the key pathways that these genes encode for and assess their role in regulating inflammation
- Perform qPCR for some genes to confirm the data obtained from the microarrays
The effect of CT99021 on global gene expression

5.2. Methods

5.2.1. Culture treatment of MonoMac6 cells and sample preparation

MonoMac6 cells were treated with CT99021 (1µM) for 30 minutes before being stimulated with $10^{-8}$M of dexamethasone (30 minutes) and LPS (10ng/ml) for 16 hours before RNA (0.5µg) was extracted using the RNeasy Mini Kit.

5.2.2. Agilent microarrays

The mRNA expression profile was determined using the Agilent SurePrint G3 Human microarrays v2 following the manufacturer’s instructions. Following normalisation against internal controls, probes with low expression (signal value of < 4.5) were removed.

5.2.3. Data analysis

Raw array data was imported into Partek Genomics Suite (Partek St. Louise, MI) for initial analysis. Principal Component analysis (PCA) was used for array clustering and identification of possible outliers. Since no outliers were identified (Figure 5.1) all data sets (n=4) were included in the differential expression analysis. Differential gene regulation was determined by taking the output from a shrinkage $t$ test (Opgen-Rhein and Strimmer 2007) and then computing the $p$ values and $q$ values (false discovery rate (FDR) with “fdrtool” (Strimmer 2008). The cut-off used for significance is an FDR of less than 0.05, which is a 5% FDR to identify the gene that showed the highest change in expression in cells treated with LPS alone versus LPS with CT99021. When comparing the effect(s) of CT99021 on
The effect of CT99021 on global gene expression

dexamethasone function I used a \( p \) value of <0.05 as a cut-off for significance. In both cases, differences >1.2 fold on mRNA expression were taken into consideration for my analysis.

Gene sets significantly enriched in Partek were transferred to the Database for Annotation, Visualisation and Integrated Discovery (DAVID) version 6.7 (http://david.abcc.ncifcrf.gov/). Pathway analysis was performed by KEGG (Kyoto Encyclopaedia of Genes and Genomes) within DAVID.

Figure 5.1. Principal Component Analysis of microarray data. Clustering of arrays using PCA showed a clear separation of both groups of arrays by the principle component. The repeated treatments \( (n=4) \) were all clustered and no outliers were identified.
5.3. Results

5.3.1. CT99021 regulates the Wnt signalling pathway via up-regulation of *FRAT1* and *FRAT2* gene expression

I investigated the effect of CT99021 on LPS-induced gene expression. Data analysis using Partek identified 17 genes whose expression was differentially regulated (FDR <0.05) by CT99021. Among these genes, *FRAT1* and *FRAT2* were down-regulated by 1.2 and 1.6 fold respectively. In order to identify the pathways that these genes regulate I analysed my data using the KEGG database ([www.genome.ad.jp/kegg](http://www.genome.ad.jp/kegg)). The results showed that CT99021 affected the Wnt/β-catenin signalling pathway (p=0.05) (Figure 5.2) in LPS-stimulated MonoMac6 cells, confirming the specificity of CT99021 action.

5.3.2. Effect of CT99021 on regulation of inflammatory gene expression and the pathways regulated

I next investigated the effect of CT99021 on LPS/dexamethasone-treated MonoMac6 cells. I identified 164 known genes that were differentially expressed due to GSK3β inhibition (p<0.05) (refer to Appendix I for list of genes). I used KEGG analysis within DAVID to identify the key pathways that these genes regulate. As shown in figure 5.3, 14 out of the 164 genes encode for inflammatory chemokines, cytokines and cytokine/chemokine receptors involved in the cytokine-cytokine receptor interaction pathway (p=2.8×10⁻⁶, LPS/dex vs LPS/dex/CT99021). Out of the 164 genes, 10 encode for proteins involved in the chemokine signalling pathway as shown in figure 5.4 (p=1.5×10⁻⁴, LPS/dex vs LPS/dex/CT99021). Among
The effect of CT99021 on global gene expression

down-regulated (2.1 fold change, p=0.0008, LPS/dex vs LPS/dex/CT99021) in response to CT99021 treatment. The gene encoding for the cell division cycle 42 (cdc42) was up-regulated (1.6 fold change, p=9.5×10^{-5} M, LPS/dex vs LPS/dex/CT99021) in response to CT99021. In addition, CT99021 treatment resulted in an up-regulation (LPS/dex vs LPS/dex/CT99021) of mRNA expression of the genes encoding for IL-24 (2.4 fold change, p=0.009), IL-19 (2.5 fold change, p=0.001), IL-10 (1.6 fold change, p=0.001), GM-CSF (encoded by CSF2) (2.1 fold change, p=0.01) and G-CSF (encoded by CSF3) (1.9 fold change, p=0.02). By affecting expression of these genes, CT99021 regulated the activation of the JAK/STAT pathway (p=0.05, LPS/dex vs LPS/dex/CT99021), presumably as a secondary effect (Figure 5.5).
Figure 5.2. Diagrammatic illustration of the effect on CT99021 on regulating expression of the genes involved in regulating the Wnt/β-catenin pathway. FRAT1 and FRAT2 expression was significantly down-regulated (p<0.05) in response to CT99021 in LPS-stimulated MonoMac6 cells. Both genes encode for FRAT1 and FRAT2 (shown as GBP on the diagram), which regulates GSK3β activity and β-catenin degradation in the Wnt signalling pathway. Modified from KEGG (www.genome.ad.jp/kegg).
Figure 5.3. Effect of CT99021 on dexamethasone-dependent suppression of LPS-induced inflammatory gene expression. CT99021 treatment led to up-regulation of 14 genes that encode for chemokines, cytokines and chemokine receptors as illustrated by the red star on the diagram ($p=2.8\times10^{-6}$). Modified from KEGG (www.genome.ad.jp/kegg).
Figure 5.4. Effect of CT99021 on expression of genes involved in regulating the chemokine signalling pathway. 10 genes were identified to be differentially expressed in response to CT99021 in MonoMac6 cells treated with LPS and dexamethasone. CT99021 affected genes encoding for chemokines and chemokine receptors resulting in regulation of the chemokine signalling pathway ($p=1.5 \times 10^{-5}$). Among these genes, PRKACG, which encodes for PKA, showed a 2.1 fold reduction ($p=0.0008$) and CDC42 showed a 1.6 fold increase in mRNA expression in response to CT99021 treatment ($p=9.5 \times 10^{-5}$M). Modified from KEGG (www.genome.ad.jp/kegg).
**Figure 5.5.** Effect of CT99021 on expression of genes that regulate the JAK/STAT signalling pathway. Among the genes whose expression was altered by addition of CT99021 on LPS/dexamethasone-treated MonoMac6 cells, those encoding for IL-10, IL-24, IL-19, GM-CSF and G-CSF were up-regulated. These cytokines are involved in activating the JAK/STAT pathway, which was significantly (p=0.05) affected by treatment with CT99021/LPS/dexamethasone compared to LPS/dexamethasone alone. Modified from KEGG (www.genome.ad.jp/kegg).
5.3.3. Confirmation of the effect of CT99021 on dexamethasone-induced inhibition of \(IL-8\) and \(CSF2\) gene expression in LPS-stimulated MonoMac6 cells

LPS-induced \(IL-8\) mRNA expression was significantly inhibited by \(10^{-8}\)M of dexamethasone (3.5 fold change, \(p=0.02\)). In cells pre-treated with CT99021 (\(10^{-6}\)M) for 30 minutes, dexamethasone’s inhibitory effect was reduced by 1.6 fold. CT99021 had no significant effect on \(IL-8\) expression in response to LPS stimulation (Figure 5.6). LPS-stimulated mRNA expression of \(CSF2\), encoding GM-CSF, was reduced by dexamethasone (\(10^{-8}\)M) by 2.2 fold (\(p=0.05\)). In the presence of CT99021 (\(10^{-6}\)M), dexamethasone’s inhibitory effect was reduced by 1.7 fold. CT99021 treatment led to increased expression of \(CSF2\) mRNA but this effect did not reach statistical significance (Figure 5.7). Although CT99021 had no significant effect on LPS/dex-induced \(IL-8\) or \(CSF2\) expression (Figure 5.6 and 5.7), a trend for reversal of dexamethasone’s inhibitory function on gene expression was observed for both genes.
The effect of CT99021 on global gene expression

Figure 5.6. Effect of CT99021 on dexamethasone function on LPS-induced IL-8 mRNA expression. MonoMac6 cells were pre-treated with CT99021 for 30 minutes before addition of dexamethasone (30 minutes) and LPS stimulation for 16 hours. (Δ)Ct values of IL-8 mRNA expression were normalised to the (Δ)Ct values of 18S mRNA expression levels. Histograms represent mean±S.E.M. of four independent experiments. *p<0.05.
Figure 5.7. Effect of CT99021 on dexamethasone function on LPS-induced CSF2 mRNA expression. MonoMac6 cells were pre-treated with CT99021 for 30 minutes followed by treatment with dexamethasone (30 minutes) and LPS stimulation for 16 hours. (Δ)Ct values of CSF2 mRNA expression were normalised to the (Δ)Ct values of 18S mRNA expression levels. Histograms represent mean±S.E.M. of four independent experiments. *p<0.05.
5.4. Discussion

As shown in the previous chapters, I identified that inhibition of GSK3β does not interfere with the LPS-induced inflammatory response but reverses dexamethasone-induced suppression of inflammatory cytokine release. In order to confirm that these effects are exerted at the mRNA level of these inflammatory mediators and to determine whether the effects of CT99021 on dexamethasone function were widespread, I assessed the effect of CT99021 on global gene expression and the investigated the pathways that are differentially regulated upon GSK3β inhibition in MonoMac6 cells. To confirm the data from the microarrays, I also performed RT-qPCR and measured the gene expression of IL-8 and CSF2 as markers of LPS-induced inflammatory gene transactivation.

5.4.1. Inhibition of GSK3β activity affects the activation of the Wnt signalling pathway via up-regulation of FRAT expression

I investigated the effects of CT99021 on LPS-induced gene expression. Using Partek I investigated how many genes are differentially up-regulated by FDR<0.05 and showed a fold change of >1.5. Among the 17 genes identified, two genes encoding for FRAT1 and FRAT2 proteins were up-regulated in response to GSK3β inhibition. The rest of the genes identified were either unknown or do not regulate any known pathways as analysed by KEGG within DAVID. FRAT1 and FRAT2 are negative regulators of GSK3β activity by competing with Axin for binding to GSK3β and forming the destruction complex that inhibits β-catenin stability (Li, Yuan et al 1999). The mechanisms by which CT99021 down-regulates FRAT mRNA expression are not known. A homeostatic feedback mechanism may be activated in the cells
The effect of CT99021 on global gene expression

that down-regulates FRAT levels to compensate for loss of GSK3β activity upon CT99021 treatment. Nevertheless, my results confirmed that CT99021 is targeting the correct pathways in my system, agreeing with my previous data and hypothesis.

5.4.2. Effect of CT99021 on regulation of inflammatory pathways

Since in my model of corticosteroid insensitivity GSK3β inhibition reduced dexamethasone-mediated suppression of LPS-stimulated release of CXCL8, GM-CSF and TNF-α, I sought to investigate whether this effect of CT99021 was cytokine specific or it affected other inflammatory pathways. Because CT99021 induced a >1.5 fold reduction of dexamethasone function in my system, I used Partek to identify the genes that were differentially expressed (p<0.05) by more than 1.5 fold in response to CT99021 compared to cells treated with LPS and dexamethasone without the drug. Using these parameters, I identified 164 genes whose mRNA expression was differentially regulated by CT99021. I used the KEGG database within DAVID to identify the pathways that these genes regulate. In support of my hypothesis, CT99021 led to up-regulation of the expression of genes that encode for chemokines (CXCL6, CXCL3, CXCL2 and CXCL1) and cytokines (GM-CSF, G-CSF) which are involved in regulating cytokine-cytokine receptor interactions. These regulate the chemokine signalling pathways in neutrophils, lymphocytes and macrophages, suggesting that GSK3β activity is important for regulating these inflammatory pathways and supporting my hypothesis that aberrant GSK3β activity is involved in driving corticosteroid insensitivity in chronic inflammation in COPD. Differential expression of IL-10, IL-24, IL-19, CSF2 (GM-CSF) and CSF3 (G-CSF) controlled by CT99021, affected the activation of the JAK/STAT signalling pathway. Activation of the JAK/STAT pathway in response to IFN-γ has been linked with corticosteroid
The effect of CT99021 on global gene expression

insensitivity in severe asthma and inhibition of this pathway has been suggested to be a novel approach to target steroid-resistant inflammatory lung disease (Clarke, Clifford et al. 2010). In addition, IFN-α-induced activation of the JAK/STAT pathway has been shown to interfere with GR function (Hu, Pace et al. 2009).

Another gene whose expression was down-regulated (2.1 fold change) by inhibition of GSK3β compared to cells treated with dexamethasone and LPS only, was RKACG that encodes for the mammalian PKA. PKA is serine/threonine kinase, known to regulate GSK3β activity via direct phosphorylation of its Ser9 residue, (Fang, Yu et al. 2000; Suzuki, Ozono et al. 2008). PKA has been also shown to phosphorylate FRAT1 (Ser188) and disrupt its activity towards regulation of GSK3β function (Hagen, Cross et al. 2006). Importantly, activation of PKA has been implicated in having a functional effect on corticosteroid action, since it was recently shown to reverse IFN-α-induced reduction of GR activity (Pace, Hu et al. 2011). In addition, PKA activation has been implicated in targeting the phosphorylation status of serine residues on HDAC5 and HDAC8 thereby regulating their enzymatic activity and capacity to bind to their co-repressors (Lee, Rezai-Zadeh et al. 2004; Ha, Kim et al. 2010).

PKA is also known to phosphorylate p65 on Ser276 and up-regulate its transcriptional activity (Yoon, Korade et al. 2008; Arun, Brown et al. 2009; Gao, Hibi et al. 2010) which may play a role in mediating GSK3β-induced corticosteroid unresponsiveness. My findings suggest that PKA mRNA expression is down-regulated in response to CT99021, but assumptions cannot be made on how this affects its protein expression or activity. Since PKA targets and inactivates GSK3β, downregulation of PKA mRNA expression may be another feedback mechanism aiming to restore GSK3β activity in response to CT99021. Further investigation is required to identify the exact role of PKA in regulating corticosteroid
function and the role of GSK3β in mediating this effect. In addition, I identified an increased expression of \textit{CDC42} mRNA in response to CT99021. The protein encoded, cdc42, is known to regulate cell polarity via phosphorylation of GSK3β (Harwood and Braga 2003). Increased mRNA expression of \textit{CDC42} may be also a result of a feedback mechanism turned on to restore GSK3β activity. My findings confirm the on-target effects of CT99021 and also support my hypothesis that GSK3β inhibition is important for regulating inflammatory responses in the presence of corticosteroids. It is particularly important to note that the pathways affected and discussed above, are signalling pathways that involve proteins whose mRNA expression was differentially regulated in response to the CT99021 compound in my system. As a result, no assumptions or conclusions can be withdrawn regarding the activation of these pathways since the activity of their components is regulated either at a post-translational level (via modifications such as phosphorylation, nitration and acetylation) or via ligand-receptor and other protein-protein interactions.

Using PCR to measure the effect of CT99021 on corticosteroid function I confirmed that dexamethasone-induced IL-8 transrepression is negatively regulated by inhibition of GSK3β. A similar reduction on dexamethasone’s repressive function on LPS-induced CSF2 expression was also seen in the presence of CT99021, but none of them reach statistical significance. The effect of CT99021 on dexamethasone function was clearer at the level of CXCL8 and GM-CSF release following 16 hours of treatment, as discussed in chapter III. This may be due to the fact that the mRNA expression was measured also at 16 hours and the effect might have been lost by that time point. In addition, CT99021 showed a pro-inflammatory effect on LPS-induced CSF2 mRNA expression that although it did not reach statistical significance it did not agree with the release data. For that reason, further investigation is required to
measure the effects of CT99021 on dexamethasone-induced gene transrepression looking at the time course of LPS stimulation.

I performed gene microarrays to investigate the global genomic effects of GSK3β inhibition on corticosteroid function. My results showed that several key inflammatory pathways are differentially regulated by GSK3β in monocytes. As a result, I accept the hypothesis that GSK3β activity regulates corticosteroid-sensitive inflammatory cytokines and chemokines. Further investigation of the list of genes affected by CT99021 and their role in driving inflammatory responses/pathways is a future direction required for better understanding of GSK3β-regulated corticosteroid insensitivity in COPD. Since these experiments were conducted at specific time points, I cannot conclude whether the effects of CT99021 on the expression of the genes and the regulation of the pathways described is a primary or a secondary effect. For that reason, a full time course is required to investigate whether these pathways are directly or indirectly regulated by GSK3β. Investigation of the genes whose expression is affected by CT99021 on a dexamethasone full concentration response curve will provide a better understanding and validate my hypothesis. Additionally, promoter analysis of the genes affected upon GSK3β regulation of activity will allow us to investigate whether these effects are mediated by activation of specific transcription factors. The data generated by the microarrays are very important for future analysis and potential hypothesis generation.
Chapter VI

Discussion and future directions
6.1. Rationale of the study

Corticosteroid insensitivity in patients with COPD has been shown to involve the activation of kinase pathways and redox sensitive proteins such as the PI3K/Akt and MAPKs in response to elevated levels of reactive oxygen species that derive from cigarette smoking and other environmental pollutants (Adcock and Barnes 2008; Adcock, Marwick et al. 2010). GSK3β has been implicated to play both pro- and anti-inflammatory roles in several cell and animal models (Cortes-Vieyra, Bravo-Patino et al. 2012). It is also known that GSK3β activity is regulated by the PI3K/Akt and the ERK1/2 MAPK signalling pathways (Grimes and Jope 2001). However, no evidence exists demonstrating whether GSK3β is also modulated by reactive oxidant species in immune cells and how such modulation affects the anti-inflammatory effects of corticosteroids. In this thesis, I sought to investigate how reactive oxygen species regulate GSK3β activity and evaluate whether modulation of its kinase activity interferes with corticosteroid function in COPD. I aimed to identify the key regulators of oxidant stress-induced regulation of GSK3β activity and the downstream effectors of the kinase that control corticosteroid function and the inflammatory response.
6.2. Summary of the study findings

In summary my results demonstrate that oxidative stress induces Ser9 phosphorylation of GSK3β and this inactivation of GSK3β impairs corticosteroid responsiveness. P-GSK3β-Ser9 is elevated in COPD as compared to both healthy smokers and non-smokers. Therefore, the elevation in p-GSK3β-Ser9 may contribute to the relative reduction in corticosteroid responsiveness in COPD. The oxidant-mediated induction of p-GSK3β-Ser9 involved p38 MAPK, ERK1/2 MAPK and PI3K/Akt signalling which are also associated with both COPD and/or reduced corticosteroid function under oxidative stress. Therefore, I postulate that GSK3β may be a downstream effector of the oxidant-dependent induction of p38 MAPK, ERK1/2 MAPK and PI3K/Akt signalling pathways. Activation of this signalling cascade affects GR function by reducing the activity of its critical co-repressor, HDAC2, leading to increased histone acetylation and pro-inflammatory cytokine release that is resistant to corticosteroid function (Figure 6.1)
Figure 6.1. Mechanism of GSK3β-regulated corticosteroid function in the presence of reactive oxygen species in monocytes. (a) In the absence of reactive oxygen species, GRα exerts its anti-inflammatory action by recruiting HDAC2 in the site of NF-κB driven pro-inflammatory gene expression in response to LPS. (b) When monocytes are exposed to reactive oxygen species, the redox sensitive proteins Akt, p38 and ERK1/2 MAPKs are activated and lead to phosphorylation and subsequent inactivation of GSK3β. Inactivation of the kinase induces reduction of HDAC2 activity resulting in decrease of corticosteroid function and increased expression of pro-inflammatory cytokines.
6.3. Discussion

This work shows that p-GSK3β-Ser9 levels, a marker of GSK3β inactivation, are elevated in smokers and further elevated in patients with COPD. p-GSK3β-Ser9 is elevated in response to H$_2$O$_2$ in vitro in a time- and concentration-dependent manner in peripheral blood monocytes. This oxidant-mediated induction of p-GSK3β-Ser9 was abolished by selective inhibition of ERK1/2, Akt and p38 MAPK, suggesting that GSK3β may be an important common redox sensing effector molecule for a number of signalling pathways. These signalling pathways are fundamental in many inflammatory functions including activation of NF-κB, pro-inflammatory cytokine expression and inflammatory cell recruitment in COPD (Thannickal and Fanburg 2000; Yu, Li et al. 2005; Bhavsar, Hew et al. 2008; Birrell, Wong et al. 2008; Matsuzawa and Ichijo 2008; Renda, Baraldo et al. 2008; Chung 2011). In addition to their roles in inflammation, p38 MAPK and PI3K/Akt signalling have also been implicated in the oxidant-mediated corticosteroid responsiveness (Irusen, Matthews et al. 2002; Marwick, Caramori et al. 2010; Marwick and Chung 2010). Certainly, selective inhibition of these pathways improved corticosteroid function in both cells from patients with oxidant associated diseases and in relatively corticosteroid unresponsive in vivo oxidant models such as cigarette smoke exposed mice (Marwick, Caramori et al. 2009; Marwick, Caramori et al. 2010). Previous studies have shown that PI3Kδ is the isoform responsible for the oxidant-induced activation of Akt in monocytes (Marwick, Caramori et al. 2010). However, selective inhibition of PI3Kδ did not affect the oxidant induction on GSK3β-s9. Therefore, other PI3K isoforms or cross-talk from other signalling pathways activated by oxidants, are likely to be responsible for the oxidant-dependent induction of p-GSK3β-s9 through Akt. My data showed that the ERK1/2 and p38 MAPKs are also involved in phosphorylating GSK3β.
Although, there is evidence supporting that ERK1/2 MAPK directly phosphorylates GSK3β (Dozza, Smith et al. 2004), there is not much known about how p38 MAPK affects the kinase activity. Further experiments are required to investigate the involvement of other kinases, such as ASK1 in regulating p38 MAPK-induced phosphorylation of GSK3β. The results also showed that H₂O₂ induced a quick phosphorylation of the Tyr216 residue of GSK3β, known to positively regulate its kinase activity (Cohen and Frame 2001). This may be due to activation of upstream redox sensitive kinases, such as MEK1 (Takahashi-Yanaga, Shiraishi et al. 2004). Measurement of GSK3β kinase activity at several time points of H₂O₂ exposure would provide information on how phosphorylation on Tyr216 affects its substrate binding.

Using pharmacological inhibition, siRNA knock down and kinase dead overexpression, my data shows that abolition of GSK3β signalling reduces the ability of dexamethasone to repress pro-inflammatory cytokine release. Conversely, overexpression of a mutant GSK3β with a Ser9 to alanine mutation restored corticosteroid function in oxidant stress exposed cells. The mechanisms by which oxidative stress imposes a relative corticosteroid unresponsiveness on cells have not been fully resolved but the oxidant-mediated kinase signalling is likely to contribute to several of the mechanisms (Ito, Chung et al. 2006; Adcock and Barnes 2008; Adcock, Marwick et al. 2010). Phosphorylation of Ser211 phosphorylation on GRβ by p38 MAPK promotes GRβ transcriptional activity (Garza, Khan et al. 2010) whilst JNK-mediated phosphorylation of Ser246 inhibits GRβ transcriptional activity (Rogatsky, Logan et al. 1998). The impact of GRβ phosphorylation on its ability to facilitate transcriptional repression is less clear. A reduction in GRβ transcriptional activity may promote inflammation due to a reduction in anti-inflammatory mediators such as SLPI and annexin 1 (Perretti and D’Acquisto 2009) as well as MPK-1, which is a negative regulator of
p38 MAPK (Clark 2003; Clark and Lasa 2003). Increased p38 MAPK is likely to lead to increased NF-κB activation and specific NF-κB-pro-inflammatory transcriptional targeting (Saccani, Pantano et al. 2002). Inhibition of GSK3β did not alter GRα phosphorylation, corticosteroid-induced GRα translocation or its ability to bind to p65 or NF-κB DNA binding activity. However siRNA knock down did reduce the expression of GRα. This result agrees with previous data indicating that since GRα expression in monocytes and macrophages is high, a reduction in the GRα expression levels may not translate into a reduction in corticosteroid function (Marwick, Caramori et al. 2009). ChIP analysis of GR-GRE binding and identification of possible post-translational modifications on GR by mass spectrometry under conditions of oxidative stress-induced GSK3β inhibition would allow better investigation of the effects of GSK3β on GR function and its binding capacity to its co-repressors.

The expression and activity of the important GRα co-repressor HDAC2 maybe also impeded by oxidant and PI3K/Akt-mediated modifications including hyperphosphorylation and nitration (Galasinski, Resing et al. 2002; Ito, Hanazawa et al. 2004; Marwick, Kirkham et al. 2004; Yang, Chida et al. 2006), depriving GRα of a key tool to control gene expression thereby reducing corticosteroid function. Inhibition of GSK3β induced a significant reduction in HDAC2 activity. This reduction in activity was not associated with a change in expression but an increase in HDAC2 phosphorylation at Ser394. CK2 phosphorylates HDAC2 at Ser394 and is a direct target of GSK3β with negatively regulates ifs function (Sun, Chen et al. 2002; Tsai and Seto 2002). Therefore, inactivation of GSK3β may increase CK2 phosphorylation of HDAC2. However, the precise functions of individual HDAC2 phosphorylation sites are unclear and various phosphorylations may promote as well as inhibit its enzymatic function.
(Tsai and Seto 2002). Cigarette smoke exposure in mice reduces HDAC2 activity and is associated with HDAC2 hyperphosphorylation (Marwick, Kirkham et al. 2004; Marwick, Stevenson et al. 2010), whilst certain phosphorylations may be important for conformational changes to allow co-repressor associations (Tsai and Seto 2002). Therefore, it is not clear how the inhibition of GSK3\(\beta\) leading to the induction of Ser394 phosphorylation may alter its activity. Further investigations are required to fully understand the regulation of HDAC2 by phosphorylation. Identification of the exact role of CK2 in mediating GSK3\(\beta\) signalling in my model as well as investigation of additional post-translational modification sites on HDAC2 that may explain the loss of its enzymatic activity or disruption of its binding to co-repressors are key approaches need to be taken next. In addition, in order to validate that the decreased histone deacetylation levels are mediating the reduction of corticosteroid function in response to GSK3\(\beta\) inhibition, promoter analysis and measurement of histone acetylation on the site of NF-\(\kappa\)B-regulated inflammatory gene expression is required.

The microarray data showed that CT99021 treatment affected a number of corticosteroid-regulated genes in response to LPS stimulation in monocytes. Among these, PKA that was significantly upregulated has been shown to regulate post-translational modifications on p65, HDAC5 and HDAC8. Further experiments would be required to validate that GSK3\(\beta\) regulates PKA activity and investigate the possible effects it may exert on p65-induced transactivation as well as site-specific histone acetylation. Investigation of the oxidative stress effects on PKA activity and its levels of expression/activity in monocytes derived from COPD patients would allow clarification of the complex mechanisms that regulate corticosteroid function under oxidative stress in COPD.
The effect of CT99021 on *IL8* and *CSF2* mRNA expression was not as clear as at the cytokine level. This was probably due to the time point chosen to investigate global gene expression in response to CT99021 which was the same as the one used to measure cytokine release and hence the effects might have been lost at the mRNA level. Due to time and expense limitation I was unable to investigate the effects of CT99021 at different time points of LPS stimulation. Further microarray and RT-qPCR experiments also assessing the effects of CT99021 on a full concentration response curve of dexamethasone would confirm the role of GSK3β on corticosteroid function at the mRNA expression level and distinguish the primary effects of CT99021 from secondary effects on the inflammatory response. Nevertheless, the microarray analysis confirmed that CT99021 targeted GSK3β-regulated pathways and that it upregulated several corticosteroid-transrepressed inflammatory genes at the time point used. This observation agrees with my hypothesis and further investigation of the genes found to be differentially regulated will allow better understanding on the mechanisms involved and assist new hypothesis generation.
6.4. Future directions

The following is a summary of future perspectives based on this thesis:

- Perform promoter analysis and measure the level of histone acetylation at the site of NF-κB transactivation upon GSK3β inhibition.

- Investigate whether the pro-inflammatory response observed in the presence of the DNA plasmid constructs is NF-κB driven. Investigate whether these effects are plasmid specific and generate new constructs with different antibiotic resistance genes to validate the results.

- Identify which downstream target of p38 MAPK phosphorylates GSK3β, examining the role of ASK1.

- Investigate the role of CK2 on mediating GSK3β-induced regulation of HDAC2 phosphorylation.

- Identify other post-translational modifications on HDAC2 and assess their functional effect on its enzymatic activity and ability to bind to co-repressors.

- Examine whether GR is subjected to post-translational modifications upon GSK3β inhibition and evaluate the effect that may have on GR function and GR-GRE binding capacity.

- Measure the effects of CT99021 on corticosteroid-mediated gene transrepression using a full time course.
Examine the levels of expression and activity of PKA in primary immune cells from COPD patients compared to smokers with normal lung function and non-smokers.

Investigate the role of PKA in regulating corticosteroid function under conditions of oxidative stress. Examine whether it affects p65 phosphorylation and its transactivation capacity.
7. References


Traves, S. L., S. V. Culpitt, et al. (2002). "Increased levels of the chemokines GROalpha and MCP-1 in sputum samples from patients with COPD." Thorax 57(7): 590-595.


### 8. Appendix I

Table 1. List of genes differentially expressed in response to CT99021 in LPS/dexamethasone treated MonoMac6 cells

<table>
<thead>
<tr>
<th>Probeset ID</th>
<th>Gene symbol</th>
<th>p-value (Dex vs. No Dex)</th>
<th>Fold-Change (Dex vs. No Dex)</th>
<th>Fold-Change (Dex vs. No Dex) (Description)</th>
</tr>
</thead>
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<tr>
<td>NM_207404</td>
<td>ZNF662</td>
<td>0.00674204</td>
<td>-1.81182</td>
<td>Drug down vs No Drug</td>
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<td>ZNF618</td>
<td>0.00861205</td>
<td>-1.60323</td>
<td>Drug down vs No Drug</td>
</tr>
<tr>
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<td>ZNF549</td>
<td>0.00186306</td>
<td>-1.64032</td>
<td>Drug down vs No Drug</td>
</tr>
<tr>
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<td>0.000451584</td>
<td>-1.57979</td>
<td>Drug down vs No Drug</td>
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<tr>
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<td>-1.89155</td>
<td>Drug down vs No Drug</td>
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<tr>
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<td>-1.63206</td>
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<td>1.71353</td>
<td>Drug up vs No Drug</td>
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<td>1.51162</td>
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</tr>
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<tr>
<th>Probeset ID</th>
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