TLR2 Receptor Activation and COX-2 Upregulation in Airway Smooth Muscle Cells: Uncovering the Molecular Mechanisms

A thesis submitted for the degree of
Doctor of Philosophy

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The work described in this thesis was conducted under the supervision of Professor Alaina J. Ammit, Faculty of Pharmacy, The University of Sydney, Australia and under the co-supervision of Professor Philip Hansbro, School of Biomedical Science and Pharmacy, the University of Newcastle, Australia.

I certify that the thesis has been written by me and is not currently being submitted for any other degree. Full acknowledgement has been made where the work of others has been cited or used. Ethical approval was obtained for this project from the University of Sydney Human Ethics Committee.

Nowshin Nowaz Rumzhum, 04.01.16
Publications


5. **Nowshin N. Rumzhum**, Brijeshkumar S. Patel, Pavan Prabhala, Ingrid C. Gelissen, Brian G. Oliver, and Alaina J. Ammit. IL-17A increases TNFα-induced COX-2 protein


9. Hatem Alkhouri, Nowshin N. Rumzhum, Md. Mostafizur Rahman, Meghan FitzPatrick, Monique de Pedro, Brian G. Oliver, Jane E. Bourkeand Alaina J. Ammit. TLR2
activation causes tachyphylaxis to \( \beta_2 \)-agonists \textit{in vitro} and \textit{ex vivo}: modeling bacterial exacerbation. \textit{Allergy} 2014; 69: 1215–1222 (Alkhouri et al., 2014).


Poster Presentations

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B. National

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3. **Md. Mostafizur Rahman**, Hanna Im, Nowshin N. Rumzhum and Alaina J. Ammit. Possible Role of TLR2-Induced Activation of Inflammasome in Airway Smooth Muscle Cytokine Secretion. NewCastle Asthma Meeting-2012 (NAMe8 2012), 11~12 October 2012. Hunter Medical Research Institute. Newcastle, Australia. (Prize money has been awarded for this poster).
1. **Nowshin N. Rumzhum**, Hatem Alkhouri, Md. Mostafizur Rahman, Meaghan FitzPatrick, Monique de Pedro, Brian G. Oliver, Jane E. Bourke and Alaina J. Ammit. TLR2 activation causes tachyphylaxis to β₂-agonists *in vitro* and *ex vivo*: modeling bacterial exacerbation. Faculty Postgraduate Conference-2013, 4 December 2013, Faculty of Pharmacy, The University of University.
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# Table of Contents

Declaration

Publications

Poster Presentation

Oral Presentation

Acknowledgements

Abbreviations

Abstract

## Chapter 1: General Introduction

1.1 Asthma

1.2 Asthma pathogenesis: role of ASM cells

1.3 Asthma management

1.4 Asthma exacerbation

1.5 TLR receptor

1.6 TLR2 receptor activation in ASM cell

1.6.1 Effects on inflammasome activation

1.6.2 Effects on COX-2 upregulation

1.7 COX-2 regulation

1.7.1 Transcriptional regulation
1.7.2 Post-transcriptional regulation ................................................................. 21

1.7.3 Post-translational regulation ................................................................. 22

1.8 Inflammatory mediators .............................................................................. 25

1.8.1 TNFα ........................................................................................................ 25

1.8.2 Pam3CSK4 .............................................................................................. 26

1.8.3 S1P ........................................................................................................... 27

1.8.4 IL-17A ..................................................................................................... 28

1.9 COX-2 upregulation and β2-AR desensitization ........................................... 29

1.10 Targeting COX-2 in airway inflammation: PGE2 receptor signalling .......... 31

1.11 Hypothesis and aim of the study ............................................................... 34

Chapter 2: General Materials and Methods .................................................. 37

2.1 Materials ..................................................................................................... 37

2.2 Methods ..................................................................................................... 37

2.2.1 Cell culture ............................................................................................... 37

2.2.1.1 Solutions ............................................................................................. 37

2.2.1.2 Culture of primary human ASM cells .................................................. 37

2.2.1.3 Passaging ASM cells .......................................................................... 38

2.2.1.4 Plating and starvation ....................................................................... 39
## Table of Contents

2.2.1.5 ASM cell characterization ................................................................. 39

2.2.2 Western blotting ..................................................................................... 39

2.2.2.1 Solutions .............................................................................................. 43

2.2.2.2 Sample preparation and immunoblotting .............................................. 43

2.2.3 RT-PCR ..................................................................................................... 45

2.2.3.1 RNA sample collection and RNA extraction ......................................... 45

2.2.3.2 Reverse transcription and polymerase chain reaction ............................. 46

2.2.4 Enzyme-linked immunosorbent assay (ELISA) ......................................... 48

2.2.5 cAMP assay ............................................................................................ 49

2.2.6 PGE\(_2\) assay ........................................................................................ 50

2.2.7 Statistical analysis .................................................................................. 50

### Chapter 3: NLRP3 inflammasome is not activated in ASM upon TLR2 ligation ..... 52

3.1 Introduction ............................................................................................... 52

3.2 Materials and methods ............................................................................ 54

3.2.1 Cell culture ............................................................................................ 54

3.2.2 Real-Time RT-PCR .............................................................................. 54

3.2.3 ELISAs .................................................................................................. 55

3.2.4 IL-1\(\beta\) neutralization ......................................................................... 55
3.2.5 Statistical analysis ........................................................................................................... 55

3.3 Results ................................................................................................................................... 56

3.3.1 Time course of TNFα-induced IL-1β mRNA expression and augmentation by Pam3CSK4 ........................................................................................................................................... 56

3.3.2 Pam3CSK4 potentiates TNFα-induced IL-6 and IL-8 protein secretion, but IL-1β is not secreted from ASM cells ........................................................................................................................................... 58

3.3.3 Effect of IL-1β neutralizing antibody on IL-6 and IL-8 protein secretion induced by IL-1β recombinant protein ........................................................................................................................................... 60

3.3.4 Neutralization of IL-1β in conditioned media exerts no effect on cytokine secretion . 62

3.3.5 Pam3CSK4 does not affect TNFα-induced NLRP3 and caspase-1 mRNA expression in ASM cells ........................................................................................................................................... 64

3.4 Discussion ................................................................................................................................ 66

Chapter 4 : TLR2 activation causes desensitization of β2-adrenoreceptor via COX-2 production ........................................................................................................................................... 71

4.1 Introduction ................................................................................................................................ 71

4.2 Meterials and methods ............................................................................................................. 73

4.2.1 Cell culture ............................................................................................................................ 73

4.2.2 Chemicals ............................................................................................................................. 73

4.2.3 Real-time RT-PCR ................................................................................................................ 73
Table of Contents

4.2.4 cAMP and PGE₂ assay ........................................................................................................ 74
4.2.5 Statistical analysis ............................................................................................................. 74
4.3 Results .................................................................................................................................. 74
4.3.1 TLR2 ligand engagement upregulates TNFα-induced COX-2 mRNA expression and increases PGE₂ secretion ........................................................................................................ 74
4.3.2 PGE₂ induces heterologous β₂-AR desensitization as measured by inhibition of β₂-agonist-induced cAMP production ........................................................................................................ 77
4.3.3 Celecoxib inhibits PGE₂ secretion but not COX-2 mRNA expression ......................... 79
4.3.4 Pam3CSK4 + TNFα cause tachyphylaxis of β₂-AR agonists mediating MKP-1 gene expression and this can be reversed by celecoxib ................................................................. 81
4.4 Discussion .......................................................................................................................... 83

Chapter 5: Sphingosine 1-phosphate increases COX-2 expression and PGE₂ secretion: effects on β₂-adrenergic receptor desensitization ..................................................................................... 88
5.1 Introduction ......................................................................................................................... 88
5.2 Materials and methods ....................................................................................................... 90
5.2.1 ASM cell culture ................................................................................................................ 90
5.2.2 Chemicals ........................................................................................................................ 90
5.2.3 Real-time RT-PCR ........................................................................................................... 91
5.2.4 PGE₂ assay ..................................................................................................................... 91
5.2.5 Western blotting ........................................................................................................... 91

5.2.6 COX-2 siRNA ............................................................................................................... 92

5.2.7 cAMP assay ................................................................................................................ 92

5.2.8 Statistical analysis .................................................................................................... 93

5.3 Results ........................................................................................................................... 93

5.3.1 S1P upregulates COX-2 mRNA expression and protein upregulation to increase PGE\textsubscript{2} secretion from ASM cells ......................................................................................... 93

5.3.2 Celecoxib has no effect on S1P-induced COX-2 mRNA, but significantly inhibited S1P-induced PGE\textsubscript{2} secretion .................................................................................................. 95

5.3.3 Dexamethasone represses S1P-induced COX-2 mRNA expression and PGE\textsubscript{2} secretion ......................................................................................................................... 96

5.3.4 COX-2 knockdown by siRNA represses S1P-induced COX-2 mRNA expression, protein upregulation and PGE\textsubscript{2} secretion ........................................................................ 98

5.3.5 TNF\textalpha enhances S1P-induced COX-2 mRNA expression and protein upregulation and increases PGE\textsubscript{2} secretion ......................................................................................... 100

5.3.6 S1P induces heterologous $\beta_2$-adrenergic desensitization as measured by inhibition of $\beta_2$-agonist-induced cAMP production; in a manner independent of adenylate cyclase ..... 102

5.4 Discussion ..................................................................................................................... 105
Chapter 6: IL-17A increases TNFα-induced COX-2 protein stability and augments PGE₂ secretion from airway smooth muscle cells ......................................................... 109

6.1 Introduction .................................................................................................................. 109

6.2 Material and methods ................................................................................................. 110

   6.2.1 ASM cell culture .................................................................................................... 110

   6.2.2 Chemicals ............................................................................................................. 111

   6.2.3 PGE₂ assay .......................................................................................................... 111

   6.2.4 Real-time RT-PCR ............................................................................................... 111

   6.2.5 Western blotting .................................................................................................. 111

   6.2.6 cAMP assay ........................................................................................................ 112

   6.2.7 Statistical analysis ............................................................................................... 112

6.3 Results ......................................................................................................................... 113

   6.3.1 IL-17A augments TNFα-induced PGE₂ secretion, but does not increase TNFα-induced COX-2 mRNA expression ................................................................. 113

   6.3.2 IL-17A increases TNFα-induced COX-2 protein upregulation ............................ 115

   6.3.3 Proteasome inhibitors increase TNFα-induced COX-2 protein and PGE₂ secretion. 117

   6.3.4 IL-17A enhances TNFα-induced COX-2 protein stability ...................................... 119
6.3.5 Heterologous β₂-adrenergic desensitization as measured by inhibition of β₂-agonist-induced cAMP production in ASM cells: effects of IL-17A ± TNFα ........................................... 121

6.4 Discussion .......................................................................................................................... 124

Chapter 7: Role of specific PGE₂ receptors ....................................................................... 129

7.1 Introduction ..................................................................................................................... 129

7.2 Material and methods .................................................................................................... 131

7.2.1 ASM cell culture ........................................................................................................ 131

7.2.2 Chemicals .................................................................................................................. 131

7.2.3 Real-time RT-PCR ..................................................................................................... 131

7.2.4 cAMP assay .............................................................................................................. 131

7.2.5 Statistical analysis ....................................................................................................... 132

7.3 Results ........................................................................................................................... 132

7.3.1 PGE₂ induces MKP-1 expression in ASM cells ......................................................... 132

7.3.2 Role of EP receptor antagonists on PGE₂-induced β₂-AR desensitization (short protocol)....................................................................................................................... 134

7.3.3 Role of EP receptor antagonists on PGE₂-induced β₂-AR desensitization (standard protocol)....................................................................................................................... 136

7.4 Discussion ....................................................................................................................... 138
Chapter 8: General Discussion and Conclusion ................................................. 142

8.1 Overview ........................................................................................................ 142

8.2 Our approaches .................................................................................................. 143

8.3 Conclusions and future directions ................................................................. 150

Chapter 9: References ......................................................................................... 157
List of Figures

Figure 1.1 Pathological mechanisms of asthma ................................................................. 5
Figure 1.2 Approaches for asthma management ................................................................. 8
Figure 1.3 TLR receptors and their specific ligands ............................................................ 12
Figure 1.4 TLR2 activated inflammasome and subsequent IL-1β secretion ......................... 15
Figure 1.5 Mechanisms of action of β2-agonist and underlying molecular pathways of β2-AR desensitization ........................................................................................................ 30
Figure 1.6 COX-2 pathways - multiple ways to target COX-2 expression/activity or prostanoid receptors ........................................................................................................................................ 33
Figure 3.1 Time course of TNFα-induced IL-1β mRNA expression and augmentation by Pam3CSK4 (in comparison to IL-6 and IL-8 mRNA temporal kinetics). ............................................ 57
Figure 3.2 Pam3CSK4 potentiates TNFα-induced IL-6 and IL-8 protein secretion, but IL-1β is not secreted from ASM cells ........................................................................................................ 59
Figure 3.3 Effect of IL-1β neutralizing antibody on IL-6 and IL-8 protein secretion induced by IL-1β recombinant protein ........................................................................................................ 61
Figure 3.4 Neutralization of IL-1β in conditioned media has no effect on cytokine secretion.. 63
Figure 3.5 Pam3CSK4 does not affect TNFα-induced NLRP3 and caspase-1 mRNA expression in ASM cells ......................................................................................................................... 65
Figure 4.1 TLR2 ligand engagement upregulates TNFα-induced COX-2 mRNA expression and increases PGE₂ secretion ...................................................................................................... 76
Figure 4.2 PGE₂ induces heterologous β2-AR desensitization as measured by inhibition of β2-agonist-induced cAMP production ....................................................................................... 78
**Figure 4.3** Celecoxib inhibits PGE$_2$ secretion but not COX-2 mRNA expression. .......................... 80

**Figure 4.4** Pam3CSK4 + TNFα cause tachyphylaxis of β$_2$-AR agonists mediating MKP-1 gene expression and this can be reversed by celecoxib. ................................................................. 82

**Figure 5.1** S1P upregulates COX-2 mRNA expression and protein upregulation to increase PGE$_2$ secretion from ASM cells. .................................................................................................................. 94

**Figure 5.2** Celecoxib has no effect on S1P-induced COX-2 mRNA, but significantly inhibited S1P-induced PGE$_2$ secretion. .................................................................................................................. 95

**Figure 5.3** Dexamethasone represses S1P-induced COX-2 mRNA expression and PGE$_2$ secretion. ................................................................................................................................. 97

**Figure 5.4** COX-2 knockdown by siRNA represses S1P-induced COX-2 mRNA expression, protein upregulation and PGE2 secretion. ..................................................................................................... 99

**Figure 5.5** TNFα enhances S1P-induced COX-2 mRNA expression and protein upregulation and increases PGE$_2$ secretion................................................................................................. 101

**Figure 5.6** S1P induces heterologous β$_2$-adrenergic desensitization as measured by inhibition of β$_2$-agonist-induced cAMP production; in a manner independent of adenylate cyclase......... 104

**Figure 6.1** IL-17A augments TNFα-induced PGE$_2$ secretion, but does not increase TNFα-induced COX-2 mRNA expression. .................................................................................................................. 114

**Figure 6.2** IL-17A increases TNFα-induced COX-2 protein upregulation. ................................. 116

**Figure 6.3** Proteasome inhibitors increase TNFα-induced COX-2 protein and PGE$_2$ secretion. ................................................................................................................................................... 118
Figure 6.4 IL-17A increases TNFα-induced COX-2 protein stability. ........................................ 120

Figure 6.5 TNFα induced IκB-α degradation was unaffected by IL-17A. .............................. 121

Figure 6.6 Heterologous β2-adrenergic desensitization as measured by inhibition of β2-agonist-
induced cAMP production in ASM cells .................................................................................. 123

Figure 7.1 PGE2 induces MKP-1 expression and the role of EP2 and EP4 receptors. ............... 133

Figure 7.2 Role of EP receptor antagonists on PGE2-induced β2-AR desensitization (short
protocol) ................................................................................................................................. 135

Figure 7.3 Role of EP receptor antagonists on PGE2-induced β2-AR desensitization (standard
protocol) .................................................................................................................................. 137

Figure 7.4 PGE2 signalling pathways through EP1-4 receptors ................................................. 139

Figure 8.1 Conclusion schematic of this project’s findings ......................................................... 150
List of Tables

Table 1.1 COX-2 inducers and their functional outcomes .......................................................... 17

Table 1.2 Key molecules that induce COX-2 and their impact on signalling cascades .......... 20

Table 2.1 Guidelines for plating of ASM cells for experiments ............................................. 39

Table 2.2 Patient demographics for the cells used in this thesis ........................................... 20

Table 2.3 Primary and secondary antibodies used for immunoblotting ................................. 45

Table 2.4 List of primers and their UniGene ID used in this thesis ........................................ 48
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AC</td>
<td>adenyl cyclase</td>
</tr>
<tr>
<td>AERD</td>
<td>aspirin exacerbated respiratory disease</td>
</tr>
<tr>
<td>AHR</td>
<td>airway hyper-responsiveness</td>
</tr>
<tr>
<td>AIA</td>
<td>aspirin-induced asthma</td>
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<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>AP-1</td>
<td>activator protein-1</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulfate</td>
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<tr>
<td>ARE</td>
<td>adenylate +uridy late-rich elements</td>
</tr>
<tr>
<td>ASA</td>
<td>aspirin sensitive asthma</td>
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<tr>
<td>ASC</td>
<td>apoptosis-associated speck-like protein containing a CARD</td>
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<td>ASM</td>
<td>airway smooth muscle</td>
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<tr>
<td>ASRD</td>
<td>aspirin-sensitive respiratory disease</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>BALF</td>
<td>bronchoalveolar lavage fluid</td>
</tr>
<tr>
<td>BLP</td>
<td>bacterial pathogens produced lipoproteins</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>°C</td>
<td>degree celsius</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<td>CARD</td>
<td>C-terminal caspase-recruitment domain</td>
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<td>CCL-11</td>
<td>C-C motif cytokine 11</td>
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<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<td>Abbreviation</td>
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<tr>
<td>C/EBP</td>
<td>CCAAT/enhancer binding protein</td>
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<td>COPD</td>
<td>chronic obstructive pulmonary disease</td>
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<td>COX</td>
<td>cycloxygenase</td>
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<td>CRE</td>
<td>cAMP response elements</td>
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<td>CREB</td>
<td>cAMP response-element binding protein</td>
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<td>extracellular matrix</td>
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<td>extracellular signal-regulated kinases</td>
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<td>FTY720</td>
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<td>GBD</td>
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<td>GDP</td>
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<td>Gi</td>
<td>inhibitory regulative G-protein</td>
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<tr>
<td>GINA</td>
<td>global initiative for asthma</td>
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<tr>
<td>Abbreviations</td>
<td>Definition</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
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<td>Gs</td>
<td>stimulative regulative G-protein</td>
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<td>GTP</td>
<td>guanosine-5'-triphosphate</td>
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<td>HBEC</td>
<td>human bronchial epithelial cell</td>
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<td>HEPES</td>
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<tr>
<td>NF-κB</td>
<td>nuclear factor kappaB</td>
</tr>
<tr>
<td>NF-IL6</td>
<td>nuclear factor for interleukin-6</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
</tr>
<tr>
<td>NHTBE</td>
<td>normal human tracheobronchial epithelial cells</td>
</tr>
<tr>
<td>NLR</td>
<td>NOD-like receptor</td>
</tr>
<tr>
<td>NLRP3</td>
<td>nucleotide-binding domain and leucine-rich repeat protein–3</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>nM</td>
<td>nanomolar</td>
</tr>
<tr>
<td>NSAID</td>
<td>non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>NSCCa</td>
<td>non small cell carcinoma</td>
</tr>
<tr>
<td>NSCLC</td>
<td>non small cell lung carcinoma</td>
</tr>
<tr>
<td>O/N</td>
<td>overnight</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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</tr>
<tr>
<td>Pam3CSK4</td>
<td>N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2R,S)-propyl]-(R)-cysteinyl-seryl-(lysyl)-3-lysine</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDE4</td>
<td>phosphodiesterase inhibitor 4</td>
</tr>
<tr>
<td>PG</td>
<td>prostaglandin</td>
</tr>
<tr>
<td>PGDH</td>
<td>15-hydroxyprostaglandin dehydrogenase</td>
</tr>
<tr>
<td>PGE₂</td>
<td>prostaglandin E₂</td>
</tr>
<tr>
<td>PGH₂</td>
<td>prostaglandin H₂</td>
</tr>
<tr>
<td>PGI₂</td>
<td>prostaglandin I₂</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PPRE</td>
<td>peroxisome proliferator response element</td>
</tr>
<tr>
<td>PSF</td>
<td>PTB-associated splicing factor</td>
</tr>
<tr>
<td>PTB</td>
<td>polypyrimidine tract-binding protein</td>
</tr>
<tr>
<td>RANTES</td>
<td>regulated on activation, normal T cell expressed and secreted</td>
</tr>
<tr>
<td>RBM3</td>
<td>RNA-binding motif protein 3</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>rpm</td>
<td>rotation per minute</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>S1P</td>
<td>sphingosine-1-phosphate</td>
</tr>
<tr>
<td>SABA</td>
<td>short-acting β₂-agonists</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>sec</td>
<td>second</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>Ser</td>
<td>serine</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SRE</td>
<td>sterol response element</td>
</tr>
<tr>
<td>TBS (-T)</td>
<td>tris-buffered saline solution (with Tween 20)</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N’,N’-tetramethylethylenediamine</td>
</tr>
<tr>
<td>Th17</td>
<td>T-helper 17</td>
</tr>
<tr>
<td>TIA-1</td>
<td>T-cell intracellular antigen 1</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/IL-1 receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumour necrosis factor α</td>
</tr>
<tr>
<td>TNF-R1</td>
<td>TNF receptor 1</td>
</tr>
<tr>
<td>TNF-R2</td>
<td>TNF receptor 2</td>
</tr>
<tr>
<td>TRAF</td>
<td>tumor-necrosis factor receptor-associated factor</td>
</tr>
<tr>
<td>TRIS</td>
<td>tris-(hydroxymethyl)-aminomethane</td>
</tr>
<tr>
<td>TTP</td>
<td>tristetraprolin</td>
</tr>
<tr>
<td>TX</td>
<td>thromboxane</td>
</tr>
<tr>
<td>TXA₂</td>
<td>thromboxane A₂</td>
</tr>
<tr>
<td>USP</td>
<td>ubiquitin-specific processing protease</td>
</tr>
<tr>
<td>3’-UTR</td>
<td>3’-untranslated region</td>
</tr>
<tr>
<td>V</td>
<td>volt</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>μL</td>
<td>microlitre</td>
</tr>
<tr>
<td>μM</td>
<td>micromolar</td>
</tr>
<tr>
<td>β2-AR</td>
<td>β2-adrenergic receptor</td>
</tr>
<tr>
<td>WHO</td>
<td>world health organization</td>
</tr>
</tbody>
</table>
Abstract

Asthma is the most common chronic inflammatory airway disorder globally. Its prevalence is in increase. Accumulated evidence has demonstrated the importance of airway smooth muscle cells (ASM) in asthma pathophysiology. ASM cells participate in asthma pathogenesis through an altered contractile, proliferative and secretory function in asthmatic airways. It has been demonstrated that ASM cells can express functional toll like receptor-2 (TLR2) and activation of TLR2 is believed to be involved in the amplification of airway inflammatory responses during infectious exacerbation. Further, under various conditions of stimulation, ASM cells release several inflammatory mediators; such an example is cyclooxygenase-2 (COX-2). COX-2 can be rapidly and robustly expressed in response to a diverse range of pro-inflammatory cytokines and mediators. Increased levels of COX-2 protein and its prostanoid metabolites serve as key contributors to pathophysiology in respiratory diseases typified by dysregulated inflammation. We, therefore, aim in this thesis to investigate the underlying molecular mechanisms of TLR2 receptor activation and upregulation of COX-2 protein levels in ASM cells in the context of airway inflammation induced by exacerbated bacterial infection.

The inflammasome is a multimeric protein complex thought to be responsible for inflammation associated respiratory diseases, including asthma, via processing and secretion of pro-inflammatory cytokine interleukin-1β (IL-1β). Hence, in Chapter 3 we aim to reveal the molecular mechanisms underlying TLR2-induced activation of the nucleotide-binding domain and leucine-rich repeat protein–3 (NLRP3) inflammasome and investigate associated cytokines secretion in ASM cells in vitro. Since asthma exacerbations are associated with elevated concentrations of secreted IL-1β, we addressed whether the NLRP3 inflammasome is activated under in vitro
Abstract

conditions that mimic infectious (bacterial) exacerbations in asthma. Primary cultures of ASM cells were treated with infectious stimuli (mimicked using the TLR2 agonist Pam3CSK4, a synthetic bacterial lipopeptide) in the presence of an inflammatory stimulus, tumour necrosis factor α (TNFα). The data demonstrate that in ASM cells the Pam3CSK4-mediated augmentation of TNFα-induced IL-6 and IL-8 mRNA and protein concentrations is not mediated by IL-1β secretion via NLRP3 inflammasome activation. The data support that NLRP3 inflammasome is not activated in ASM upon TLR2 ligation.

Life-threatening asthma exacerbation is the most common and very serious concern for those asthma patients who suffer further respiratory tract infections. β2-adrenergic receptor (β2-AR) agonists are the first line therapy in the treatment of asthma. Recent studies, however, have revealed that β2-agonists lose bronchodilatory efficacy because the receptor-mediated molecular pathways responsible for their beneficial actions are desensitized by infection. In Chapter 4, we determine whether infectious stimuli interact to further enhance ASM cell inflammatory responses and examine the role of COX-2 in this context. In an in vitro model of bacterial infection in ASM cells, we show that activation of TLR2 by Pam3CSK4 in the presence TNFα, leads to the secretion of significant amounts of COX-2 mRNA and protein. Subsequently, the level of COX-2 product, PGE2 is increased. β2-agonists exert their bronchodilatory effects upon binding with β2-AR receptors that result in the secretion of cyclic adenosine monophosphate (cAMP). They also show anti-inflammatory effects via releasing critical anti-inflammatory protein mitogen activated protein kinase phosphatase-1 (MKP-1). We found that an increased level of TLR2 agonist-induced PGE2 reduces cAMP production as well as anti-inflammatory protein, MKP-1, secretion in response to short-acting (salbutamol) and long-acting (formoterol)
Abstract

β2-agonists. Taken together, these results show that, like viruses, bacteria induce prostanoid-dependent β2-AR desensitization on ASM cells.

Elevated COX-2 protein secretion was suggested to play a role in asthma exacerbation that also corroborate with our previous study (Chapter 4), we were therefore interested in exploring the effects of COX-2 upregulation by some other inflammatory mediators. Chapter 5 investigate the effects of sphingosine-1-phosphate (S1P), a bioactive sphingolipid that plays an important role in pathophysiology of asthma, on COX-2 upregulation alone and in combination with TNFα in bronchial ASM cells and provide the first demonstration of β2-AR desensitization by S1P in vitro. ASM cells were pretreated with S1P for up to 24 h and the temporal kinetics of COX-2 mRNA expression and PGE2 secretion were measured. It was found that S1P significantly enhanced COX-2 expression and PGE2 secretion and this was repressed by the selective COX-2 inhibitor, celecoxib, the corticosteroid dexamethasone, or siRNA knockdown of COX-2 expression. We also found that TNFα upregulates S1P-induced COX-2 protein expression and augments PGE2 in an additive manner. Further experiments investigated the effects of S1P-induced COX-2 upregulation and PGE2 secretion on heterologous β2-AR desensitization as measured by inhibition of cAMP production in response to the short-acting β2-agonist, salbutamol, and the long-acting β2-agonist, formoterol. The data suggest that S1P has the potential to repress β2-adrenergic activity via increasing COX-2 mediated PGE2 production in ASM cells.

Evidence suggests that pivotal cytokine, IL-17A, is found in elevated levels in respiratory diseases, including severe asthma, and directs pulmonary immunity and inflammation. In Chapter 6 we examine the impacts of IL-17A on COX-2 mRNA expression, protein upregulation and subsequent PGE2 secretion from ASM cells. Using an in vitro inflammatory model with
TNFα we show that IL-17A has no effect when added alone but it substantially augments TNFα-mediated responses in ASM cells. Interestingly, we found that IL-7A had no effect on COX-2 mRNA expression; rather it enhances TNFα-induced COX-2 protein stability. Moreover, in this study we reveal that TNFα-induced COX-2 is subject to proteasomal degradation, the first evidence demonstrating post-translational regulation of COX-2 protein stability and confirmed by blocking TNFα-induced COX-2 proteasomal degradation by using two inhibitors of the proteasome - bortezomib and MG-132. Therefore, we have shown that IL-17A acts to increase TNFα-induced PGE2 by enhancing COX-2 protein stability, but not COX-2 gene expression. This study also examined the impact of IL-17A on β2-AR desensitization on ASM cells and found that IL-17A did not show any effect on β2-AR desensitization when treated alone or in combination with TNFα that differs from our previous studies.

PGE2 is a multifunctional protein that exerts its effects via four receptor subtypes, EP1-4. In Chapter 7, the aim was to investigate the role of specific EP receptors (EP2 and EP4) in the context of airway inflammation. To explore the underlying molecular mechanisms of PGE2-induced β2-AR desensitization we used two different protocols, short (serum starvation after 48 h of cells plating) and standard (serum starvation after one week of cells plating). In the short protocol, it appears that the EP2 receptor may be involved in β2-AR receptor desensitization, whereas in the standard protocol it does not. However, the preliminary data of this chapter warrants further work to clarify which class of receptor is involved in β2-AR receptor desensitization.

In conclusion, studies conducted in this thesis provide greater understanding of molecular mechanisms underlying TLR2 receptor activation and COX-2 protein upregulation in ASM cells.
Our increased understanding about these pathways can be exploited as a source of therapeutic target for respiratory diseases in future drug development.
Chapter 1
General Introduction
Chapter 1
General Introduction

1.1 Asthma

Asthma is a common and chronic lung disease characterized by airway inflammation, remodelling, and hyperresponsiveness to contractile stimuli that promote airway constriction and wheezing. According to the World Health Organization (WHO), asthma is of major public health importance as it is one of the most common and increasingly prevalent chronic diseases in the world. An estimated 334 million people worldwide suffer from asthma, with 250,000 annual deaths attributed to the disease. The most alarming statistic is that the number of people with asthma will grow by more than 100 million by 2025 (Masoli et al., 2004; Asher and Pearce, 2014). Its social and economic impact is therefore substantial. Despite significant understanding of the fundamental mechanisms of asthma pathogenesis and other inflammatory airway diseases, therapeutic approaches to control the disease still remain a significant unmet need. Hence, it is vitally important to conduct extensive research on asthma control and mechanisms.

Asthma is considered to be a significant cause of morbidity and mortality as it affects 5% of adults and 10% of children; these numbers are expected to increase further (Woolcock and Peat, 1997). Even though it is considered that asthma is most commonly an early childhood disease, it can develop at any stage in life, including adulthood. According to The Global Asthma Report 2014, asthma was estimated as the 14th most important disorder in terms of global years lived with disability. Disability from chronic diseases, including chronic respiratory diseases, is becoming an increasingly important issue for all health systems. It is therefore important to
continue the monitoring of asthma prevalence and severity globally, and actions should be taken to reduce the burden of asthma accordingly.

Asthma has significant impact on the quality of life in terms of restriction of activities, interrupted sleep, disturbed routines, increased stress and poor school performance in children (O'Connell, 2004). It is also a costly health problem and the economic burden of asthma is enormous, creating very large expenses for the patient, family and society both nationally and internationally. It is estimated that for children and adults with asthma, the annual cost is US$14 billion in direct costs (those associated with medical treatment for the illness) and indirect costs (those associated with nonmedical output losses resulting from the consequences of the illness) (O'Connell, 2004). Promoting cost-effective management in order to reduce asthma-associated morbidity and mortality via optimal treatment is now a challenging issue worldwide.

1.2 Asthma pathogenesis: role of ASM cells

Asthma is a complex and chronic inflammatory disease that results from overactive inflammatory signalling pathways. Inflammation plays the pivotal role in the pathophysiology of asthma, causing airway muscles to be tighten and swollen. These changes cause the airway to become narrow, making it difficult for air to pass through. In asthma the airway tubes become smaller or narrower mostly due to underlying inflammation or swelling. This leaves less space inside the airway tubes. Increased sticky mucus production blocks the airways, and contraction of muscles around the airways, or bronchospasm, makes the airway tighter (Figure 1.1). Asthma symptoms vary from person to person and can be minor to severe and may also change over time. Typical asthma symptoms include loss or shortness of breath, frequent coughing especially at night, wheezing, chest tightness, pain and pressure. Acute and chronic asthma is characterized
by airway inflammation, airway hyperresponsiveness (AHR) and airway remodelling (Im and Ammit, 2014). Possible factors that may lead to the development of asthma include diet, climate, community health care standards, genetic factors, and occupational exposure to certain dusts or chemicals (Moffatt et al., 2007; Postma et al., 2011). The most common characteristic features of asthma are described below:

**Bronchoconstriction:** One of the most prominent physiological events that trigger clinical symptoms leading to narrowing of the airway and ultimately limiting airflow is bronchoconstriction. In severe asthma exacerbation, bronchial smooth muscle contraction (bronchoconstriction) occurs quickly to narrow the airways in response to a range of stimuli, including allergens or irritants (Ishmael, 2011).

**Airway Inflammation:** Airway inflammation is an important feature of asthma which is further exaggerated by bronchoconstriction. The extent of clinical severity of asthma depends on airway inflammation that eventually leads to airway hyperresponsiveness. Inflammation is also a major cause of airway swelling that further interferes in airflow. Airway inflammation appears to be central to the pathogenesis of all of these clinical manifestations of asthma that trigger the activation of a number of inflammatory cells including mast cells, eosinophils, neutrophils, T-lymphocytes, epithelial cells and macrophages (O'Byrne, 1996).

**Airway remodelling:** In addition to the inflammatory features mentioned earlier, the airway wall of patients with asthma is usually characterised by a number of structural changes that are grouped together under the common term “airway remodelling” (Beckett and Howarth, 2003). Airway remodelling causes activation of a wide range of structural cells with subsequent permanent changes in the airway. This ultimately leads to airflow limitation and airway sensitivity and, most importantly, patients become less responsive to therapy. Asthma severity
depends on the structural changes of the airway, which include, airway wall thickening, subepithelial fibrosis, increase in myocite muscle mass, airway smooth muscle hypertrophy and hyperplasia, blood vessel proliferation and dilation, mucus hypersecretion, epithelial hypertrophy and mucus metaplasia.

Figure 1.1 Pathological mechanisms of asthma (Doeing and Solway, 2013).

Cell biology studies suggest that asthma is a complex multicellular disease, involving atypical responses of many different types of lung cell (Locksley, 2010). Among the many cells of the lung, epithelial cells and airway smooth muscle (ASM) cells are considered as two major cell types that are ultimately responsible for the most common symptomatic pathology in asthma. Epithelial cells act as the first line barrier against external stimuli and initiate airway
inflammation and participate in mucus production, whereas ASM cells cause symptomatic airway narrowing by rapid and extensive contraction (Erle and Sheppard, 2014). Moreover, in asthmatic conditions it is found that ASM cell mass is increased which can potentiate asthma exacerbation (Khan, 2013).

ASM cells are considered to play a key role in the pathophysiology of asthma. Several studies suggest that ASM cells are responsible for secreting a number of inflammatory mediators including chemokines, cytokines, extra cellular matrix (ECM) proteins and a wide range of growth factors (Maruoka et al., 2000; Ammit et al., 2001; Hirst, 2003). The inflammatory mediators secreted by ASM cells lead to an early response consisting of bronchoconstriction, plasma exudation, mucus secretion, neural effects and attraction and activation of inflammatory cells, as well as long-lasting effects such as structural changes of airways or airway remodelling. Previous reports also support that ASM cells are not only involved in airway narrowing, but also play a role in the remodelling and inflammation of the airways observed in asthma (Hirst, 2003; Lazaar and Panettieri, 2005; Zuyderduyn et al., 2008). Moreover, during infectious exacerbations in asthma ASM cells participate in the amplification of inflammatory responses via augmented cytokine production. Hence, targeting ASM cells to understand the cellular and molecular mechanisms associated with asthma exacerbation may offer new and important therapeutic targets in treating this common lung disease.

1.3 Asthma management

Asthma is a chronic disease that has no cure. Though asthma cannot be cured, with good management, people with asthma can lead normal, active lives. Asthma symptoms can be improved by adapting a proper management plan that includes both non-pharmacological and
pharmacological approaches. Non-pharmacological approaches include avoiding asthma triggers where possible and following lifestyle advices, such as, not smoking, eating a healthy diet, keeping up-to-date with immunization, responding to worsening symptoms, regular health reviews or monitoring with doctors and, most importantly, seeking emergency care when needed.

Asthma medicines are used to stop, control or prevent asthma symptoms in order to reduce the number and severity of asthmatic episodes and to improve airflow. Pharmacologically, asthma is treated largely via long term control (‘preventer’) and quick relief medicine (‘reliever’) (Martinez and Vercelli; Peters et al., 2006). Steroidal anti-inflammatory drugs are considered as key preventer lifesaving medications that prevent asthma attacks by reducing swelling and mucus production in the airways. Bronchodilators or β2-agonists are the standard relievers that relieve symptoms of asthma by relaxing the airway muscles to help to open up the airways. Two types of β2-agonists have been characterized. Short-acting β2-agonists (SABAs) - provide quick relief of asthma symptoms but the effects last only 4-6 h and long acting β2-agonists (LABAs) - last about 12 h and are considered to be maintenance drugs. However, asthma can be well controlled with combination therapies that contain both a corticosteroid and a bronchodilator, particularly a long acting β2-agonist (Currie et al., 2005; Selroos, 2007). Some other asthma medications also available are listed in Figure 1.2.
Long term use of asthma medicines often leads to unwanted side effects including nausea, headache, skeletal muscle tremor, skin and muscle atrophy, disturbed wound healing, osteoporosis, growth failure, steroid psychoses, depression, peptic ulcers, upper gastrointestinal bleeding, pancreatitis and severe immunosuppressant effects (Lulich et al., 1986; Schacke et al., 2002). Further, the levels of asthma control vary with drug dose over time and environmental factors. Initial asthma treatment depends on the severity of asthma and follow-up treatment depends on how well the asthma action plan is controlling the symptoms and preventing asthma attacks (Reddel et al., 2015). Asthma treatment for certain groups of people, such as children,
pregnant women, or those for whom exercise brings on asthma symptoms are adjusted to meet their special needs.

It is apparent that in spite of extensive social awareness and asthma education, the management of acute severe asthma is variable and often unsuccessful, as the current pharmacological approach to combat asthma is commonly associated with a number of side effects. Treatments may even lose their effect or become insensitive under certain conditions. One such condition is asthma exacerbation (Oliver et al., 2014). It is therefore crucial to re-evaluate the drugs currently available, both in terms of their clinical use and our understanding of their modes of action, along with the development of new potent prophylactic agents.

1.4 Asthma exacerbation

Most people with asthma exhibit various degrees of inflammation in their airways and their airways become much more sensitive to certain irritants; commonly known as ‘triggers’. Triggers worsen the condition of already inflamed airways, thus provoking an asthma attack commonly known as ‘asthma exacerbation’. Each individual tends to have different asthma triggers. Common asthma triggers include, pollens, dust, animals, air pollution, food additives, strong perfumes, exercise, cigarette smoke, some medicines e.g.: aspirin, changes in temperature and humidity, psychological influences e.g. extremes of emotion, workplace irritants e.g.: paint and varnish fumes, flour, wood dust and, most importantly, respiratory viral or bacterial infections (Wark and Gibson, 2006). Asthma exacerbation is associated with accelerated loss of lung function (Dougherty and Fahy, 2009). During acute exacerbation the signalling pathways involved in airway inflammation and remodelling are activated more easily resulting in progressive loss of lung function, including shortness of breath, coughing, wheezing and chest
tightness. Severe asthma exacerbation can even cause death. Hence, patients experiencing asthma exacerbation need immediate treatment, close observation and frequent assessment of lung function.

Viral and bacterial infections are the most common causes of respiratory infection that eventually lead to asthma exacerbation. Respiratory viral or bacterial infections work in an additive or synergistic manner that cause normal asthmatic symptoms to become exacerbated. There is a strong association between respiratory infections and the risk and pathogenesis of asthma exacerbation and it is believed that recognition of respiratory microorganisms by pattern recognition receptors can inadvertently initiate or exacerbate an unrelated inflammatory condition (Edwards et al., 2012). In a recent review article Oliver et al. explored the relationship between viral infections and asthma exacerbation and explained why current therapeutics do not fully inhibit virus-induced exacerbations, for example, β₂-adrenergic desensitisation and corticosteroid insensitivity as well as which aspects of virus-induced inflammation are likely to be attenuated by current therapy (Oliver et al., 2014).

Exacerbations can range from mild to life-threatening, and they contribute substantially to urgent healthcare utilization. Although the frequency of exacerbations can be reduced by good medical management and asthma action plans, infectious exacerbations cannot be completely eliminated. It is more likely that when lung function worsens asthmatic patients require greater amounts of their asthma medicines, to achieve asthma control. Treatment options are currently limited, and thus, further research into the molecular basis of infectious exacerbation is urgently required. During infection pathogens interact with airway cells mostly via Toll-like receptors (TLRs), a family of pattern-recognition receptors that recognize pathogen-associated molecular patterns (PAMPs) (Koziol-White and Panettieri, 2011; Manetsch et al., 2012b). Therefore, investigation
of the molecular mechanisms downstream of TLR signalling may identify new approaches to restrain infectious exacerbations in chronic airways disease.

1.5 TLR receptors

TLRs are a class of pathogen-recognition receptors that can recognize specific molecular structures found in microbes. They are important for the innate and adaptive immune response to pathogen and damage associated molecular patterns (PAMPs, DAMPs). Respiratory tract infection by various pathogens, including bacteria, viruses and fungi, are a frequent cause of inflammatory airway disease exacerbation. Thereby, activation of TLRs in the airways can play a key role contributing to infectious asthma exacerbations. Interestingly, in addition to their ability to detect external pathogens, TLRs are also apparently able to mediate responses to a variety of endogenous host molecules including reactive oxygen species (ROS), high mobility group box (HMGB) protein and surfactant protein A via a number of signalling pathways (Frantz et al., 2001; Biragyn et al., 2002; Park et al., 2004). The capability of TLRs to respond to an extraordinarily diverse range of exogenous and endogenous stimuli makes them exclusively potential targets in infective and non-infective inflammatory responses in the airway diseases.

TLR proteins belongs to a family of type I transmembrane receptors characterized by an NH2-terminal extracellular leucine-rich repeat domain (LRR) and a COOH-terminal intracellular tail containing a conserved region called the Toll/IL-1 receptor (TIR) homology domain (Basu and Fenton, 2004). The extracellular domain contains a varying number of LRR, which are believed to be primarily responsible for ligand binding. Additionally, these domains may also play a key role in TLR dimerization, whereas the intracellular TIR domain involves protein-protein interactions between TLR proteins and their downstream signal transduction components (Basu
and Fenton, 2004). Upon ligand binding, TLRs activate several downstream signalling pathways, including activator protein-1 (AP-1), nuclear factor kappaB (NF-κB) or mitogen-activated protein kinases (MAPKs), resulting in the production of pro-inflammatory cytokines, chemokines and other inflammatory mediators (Akira and Takeda, 2004).

![Figure 1.3 TLR receptors and their specific ligands (Medzhitov, 2001).](image)

The TLR family consists of 11 members (TLR1-TLR11) that recognize distinct PAMPs found in bacteria, fungi, viruses, helminths and protozoa (Sukkar et al., 2006). TLRs recognize a variety of PAMPs (Figure 1.3) and show distinct function in innate immune recognition. The amino acid sequences of the human TLRs reveal that members of the TLR family can be divided into five subfamilies: the TLR3, TLR4, TLR5, TLR2 and TLR9 subfamilies (Takeda et al., 2003). Our target of interest, TLR2, belongs to the TLR family that includes TLR1, TLR6, TLR10, TLR14 and possibly the avian TLR15 (Roach et al., 2005). It is located at the cell surface and upon binding with its ligands, dimerises with TLRs of the same family. Heterodimerization capacity of TLR2 receptor with other TLRs of the same family enabled TLR2 to detect a broader range of
bacterial PAMPs (Zähringer et al., 2008). Most cells of the human innate and adaptive immune system express TLR2 and its heterodimers (Flo et al., 2001; Dasari et al., 2005; Bekeredjian-Ding and Jego, 2009; Chang, 2010; Meyer-Bahlburg and Rawlings, 2012). Human ASM cells express mRNA of a number of TLRs and this expression was shown to be increased upon stimulation with pro-inflammatory stimuli (Morris et al., 2005; Sukkar et al., 2006; Lee et al., 2010). Considering the fact that TLR2 has been implicated in a number of lung-associated immune responses, understanding and targeting these pathways may allow regulation of the excessive proinflammatory responses during infectious exacerbation.

1.6 TLR2 receptor activation in ASM cells

TLR2 receptors are expressed on ASM cells and their activation leads to a variety of cellular responses, including the production of the neutrophil chemoattractant chemokine IL-8 (Sukkar et al., 2006) and IL-6 (Chiou and Lin, 2009), key cytokines responsible for airway inflammation. In ASM cells activation of TLR2 receptors are involved in the intensification of airway inflammatory responses during infectious exacerbations in asthma via increased cytokine production (Manetsch et al., 2012b). Some other effects of TLR2 activation are described below:

1.6.1 Effects on inflammasome activation

Respiratory infections are key factors underlying asthma exacerbation. Inflammasomes are multiprotein complex signalling molecules that are activated by a range of substances produced during infections, tissue damage or metabolic imbalances. Inflammasomes are typically composed of three components; NOD-like receptor (NLR) proteins, the adaptor molecule apoptosis-associated speck-like protein containing a CARD (ASC) domain and caspase-1
[reviewed in (Im and Ammit, 2014)]. Upon activation caspase-1 promotes the maturation and secretion of the proinflammatory cytokines interleukin (IL)-1β and IL-18 (Schroder and Tschopp, 2010). A growing body of evidence has suggested that the nucleotide-binding domain and leucine-rich repeat protein–3 (NLRP3) inflammasome is important in chronic airway diseases such as asthma and chronic obstructive pulmonary disease (COPD) (Birrell and Eltom, 2011; dos Santos et al., 2012).

TLR ligation associated proinflammatory signalling pathways are found important for both inflammasome activity and pro-IL-1β availability (Figure 1.4). Microbial sensing through TLRs to activate the NLRP3 inflammasome to release IL-1β has been demonstrated in various cells (Kanneganti et al., 2006; Netea et al., 2009; Segovia et al., 2012; Lim et al., 2013). Interestingly, evidence suggests that TLR activated regulation of NALP3 inflammasome-mediated IL-1β production in mononuclear phagocytes appears to be cell-type specific. For example, TLR stimulation alone is sufficient to induce inflammasome-mediated IL-1β production in monocytes (Netea et al., 2009; Carta et al., 2011), the same as in both bone marrow–derived and splenic dendritic cells that secrete substantial amounts of inflammasome-mediated IL-1β upon TLR activation in absence of secondary signals (Nguyen et al., 2007; He et al., 2013). However, in many cellular models the activation of the inflammasome requires a secondary signal in order to release active IL-1β. In in vitro culture system adenosine triphosphate (ATP) often added in order to stimulate this activation step (Latz et al., 2013). Tissue macrophages require both primary and secondary signals for inflammasome-mediated IL-1β secretion (Netea et al., 2009).
Figure 1.4 TLR2 activated inflammasome and subsequent IL-1β secretion. Ligation of TLR2 ligand with the receptor activates inflammaosmes (NLRP3, Caspase-1 and ASC) that further promotes maturation and secretion of IL-1β from cytoplasm to outside the cell.

To date, however, most NLRP3 inflammasome studies have been limited to airway epithelium and macrophages. The ability of ASM cells to produce and secrete pro-inflammatory mediators, including cytokines and chemokines, has been established by many studies. Hence, exploring the possible involvement of the NLRP3 inflammasome and IL-1β activation in the enhanced synthetic function of ASM cells in response to invading pathogens via TLR2 receptor stimulation would be very worthwhile.

1.6.2 Effects on COX-2 upregulation

Cyclooxygenase (COX), also known as prostaglandin H/G synthase, is the rate-limiting enzyme responsible for the production of prostanoids from arachidonic acid, hydrolyzed from cell membrane phospholipids catalyzed by phospholipase A₂. Two isoforms of COX have been
identified: COX-1 and COX-2, each encoded by different genes. COX-3 also exists but the enzyme is encoded by the same gene of COX-1 so may be considered a variant rather than a true isoform. COX-1 is believed to be expressed constitutively as a “housekeeping” enzyme, whereas COX-2 can be either inducible or constitutive, depending on the tissue. As an inflammation-associated enzyme, COX-2 has long been regarded as playing a pivotal role in the pathogenesis of airway inflammation in respiratory diseases such as asthma and COPD. Table 1.1 summarises the role of COX-2 in different airway cells. A diverse range of mediators present in the airway microenvironment have been demonstrated to induce COX-2 and increase COX-2-mediated prostanoid products in cells implicated in airway inflammation. Stimulation of TLR4 by lipopolysaccharide (LPS) is the prototypical pathogen mimic approach that has been long recognised to induce COX-2 in lung models in vitro and in vivo (Swierkosz et al., 1995). More recently, rhinoviral infection of epithelial cells in vitro has been shown to increase viral genetic material and activation of TLRs on ASM cells. This cross-talk activates TLR3 and TLR 7/8-induced COX-2 expression and results in prostanoid production by ASM cells (Van Ly et al., 2013). Stimulation of TLRs can also augment COX-2 expression; in this way infectious stimuli can greatly enhance COX-2 upregulation induced by inflammatory mediators. Evidence suggests that prostanoids produced via COX-2 upregulation show diverse actions: these can be either beneficial or deleterious and their impact on respiratory disease can be dictated by local concentration and specific interaction with individual receptors. Understanding the regulation of COX-2 expression and associated receptor-mediated functional outcomes may reveal a number of critical steps amenable to pharmacological intervention. These may prove invaluable in our quest towards future development of novel anti-inflammatory pharmacotherapeutic strategies for the treatment of airway diseases.
## Table 1.1 COX-2 inducers and their functional outcomes

<table>
<thead>
<tr>
<th>Inducer(s)</th>
<th>Cell Type(s)/ Model(s)</th>
<th>Secreted Prostanoid(s)</th>
<th>Functional Outcome(s)</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR ligand</td>
<td>human ASM HBEC</td>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>β&lt;sub&gt;2&lt;/sub&gt;-AR desensitization</td>
<td>(Alkhouri et al., 2014) (Van Ly et al., 2013)</td>
</tr>
<tr>
<td>S1P</td>
<td>human ASM HTSMCs</td>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>β&lt;sub&gt;2&lt;/sub&gt;-AR desensitization IL-6 secretion</td>
<td>(Rumzhum et al., 2015b) (Hsu et al., 2015)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>human ASM NHTBE human ASM</td>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>β&lt;sub&gt;2&lt;/sub&gt;-AR desensitization mucus secretion IL-6 secretion</td>
<td>(Pang and Knox, 1997; Laporte et al., 1998; Pascual et al., 2001) (Kim et al., 2002) (Lahiri et al., 2001)</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>human ASM</td>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>β&lt;sub&gt;2&lt;/sub&gt;-AR desensitization VEGF mediated angiogenesis</td>
<td>(Pang and Knox, 1998) (Knox et al., 2001)</td>
</tr>
<tr>
<td>TNF, PAF, LPS</td>
<td>NHTBE</td>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>mucus secretion</td>
<td>(Gray et al., 2004)</td>
</tr>
<tr>
<td>CSE</td>
<td>HTSMCs human sputum</td>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>IL-6 secretion airway remodelling</td>
<td>(Lin et al., 2010) (Chen et al., 2008)</td>
</tr>
<tr>
<td>Capsaicin</td>
<td>human BALF guinea pig BALF</td>
<td>TXA&lt;sub&gt;2&lt;/sub&gt;, PGE&lt;sub&gt;2&lt;/sub&gt;, PGF&lt;sub&gt;2α&lt;/sub&gt;,PGI&lt;sub&gt;2&lt;/sub&gt;</td>
<td>cough reflex sensitivity</td>
<td>(Stone et al., 1992; Fujimura et al., 1995; Ishiura et al., 2009; Maher et al., 2009) (Kamei et al., 2004)</td>
</tr>
<tr>
<td>Leptin, Cytomix</td>
<td>human ASM</td>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>anti-proliferative effect</td>
<td>(Nair et al., 2008) (Belvisi et al., 1998)</td>
</tr>
<tr>
<td>Inhaled allergen</td>
<td>mouse BALF</td>
<td>PGI&lt;sub&gt;2&lt;/sub&gt;</td>
<td>immunomodulatory function</td>
<td>(Jaffar et al., 2002)</td>
</tr>
<tr>
<td>Viral Infection</td>
<td>mouse and rat BALF</td>
<td>PGD&lt;sub&gt;2&lt;/sub&gt;</td>
<td>eosinophilic inflammation and bronchial hyperresponsiveness</td>
<td>(Shiraishi et al., 2008)</td>
</tr>
<tr>
<td>Cytokine</td>
<td>human ASM</td>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>reduce RANTES release</td>
<td>(Lazzeri et al., 2001)</td>
</tr>
</tbody>
</table>
1.7 COX-2 regulation

COX-2 expression can be regulated at different levels including transcriptional, post-transcriptional or post-translational. In order to better understand the role and function of COX-2 upregulation in respiratory disease, it is necessary to first explore the regulatory mechanisms and molecules that control COX-2 expression more generally. In the following text further details on COX-2 regulation are described. Table 1.2 includes reference to inducers signalling pathways that have been reported.

1.7.1 Transcriptional regulation

The human COX-2 gene is located on chromosome 1, which contains 10 exons and 9 introns and is approximately 8.3 kb long. Structural analysis of human COX-2 gene reveals the presence of a 5'-flanking region, a 10 exon encoding site, the exon-intron boundaries and the 3'-untranslated region (UTR) in both strands of DNA (Appleby et al., 1994). The 5'-flanking region consists of approximately 800 bp of nucleotides and this 5'-UTR region contains distinct combinations of \textit{cis}-regulatory elements including, peroxisome proliferator response element (PPRE), two cyclic AMP response elements (CRE), a sterol response element (SRE), two NF-κB sites, a specificity protein-1 (SP1) site, a CAAT enhancer binding protein (C/EBP), or nuclear factor for interleukin-6 (NF-IL6) expression motif, two activator protein-1 (AP-1) sites, an E-box, and a TATA box (Appleby et al., 1994; Harper and Tyson-Capper, 2008); all are the binding sites for a number of \textit{trans}-regulatory transcription factors. The 10 exon region encodes the entire open reading frame of the COX-2 gene which is unusually large as it includes not only the final 410 bp of the coding region but also 2550 bp of 3'-UTR (Appleby et al., 1994). The three potential polyadenylation sequences of 3'-UTR are approximately 280 bp apart from each other. The 23 copies
of the ATTTA RNA instability element of the 3′-UTR of the human COX-2 gene are responsible for the post-transcriptional regulation of COX-2 expression (Sawaoka et al., 2003). This 3′-UTR is characteristic of many other important genes that need to be rapidly “turned on” and then “turned off”. Thus, in a manner similar to expression mechanisms shared by proto-oncogene and cytokines (Caput et al., 1986; Xu et al., 1997), transcriptional activation of COX-2 gene expression occurs very promptly, but transiently, due to instability determinants in the 3′-UTRs. Specific binding of RNA-binding proteins to the COX-2 ARE regulates the stability of COX-2 mRNA. Thereby the levels of COX-2 mRNA expression are determined by post-transcriptional regulation along with transcriptional mechanisms (Dixon et al., 2000). Further information on post-transcriptional control of COX-2 gene expression will be given in the following sections.
Table 1.2 Key molecules that induce COX-2 and their impact on signalling cascades

<table>
<thead>
<tr>
<th>Inducer(s)</th>
<th>Cell Type(s)</th>
<th>Involved Pathway(s)</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-β</td>
<td>human epithelial cell line (A549)</td>
<td>NF-κB and post-transcriptional regulation</td>
<td>(Newton et al., 1997a; Newton et al., 1997b; Newton et al., 1997c)</td>
</tr>
<tr>
<td></td>
<td>human ASM</td>
<td>ERK and p38 MAPK pathways</td>
<td>(Laporte et al., 1999; Laporte et al., 2000; Nie et al., 2003)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>different regulatory elements, transcription factors, and histone H4 acetylation</td>
<td></td>
</tr>
<tr>
<td>Bradykinin</td>
<td>ASM</td>
<td>CREB-1 binding to the CRE in the COX-2 promoter</td>
<td>(Nie et al., 2003)</td>
</tr>
<tr>
<td></td>
<td>pulmonary artery smooth muscle cells</td>
<td>activation of phospholipase A₂ to promote release of the COX-2 substrate - arachidonic acid</td>
<td>(Bradbury et al., 2004)</td>
</tr>
<tr>
<td>Oxidative stress</td>
<td>human lung fibroblasts</td>
<td>ERK and NF-κB</td>
<td>(Martey et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>ASM</td>
<td>TLR4/MyD88/TRAFl6/c-Src/NADPH oxidase signalling that leads to the activation of MAPKs and NF-κB</td>
<td>(Lin et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>human epithelial cell line (A549)</td>
<td>P2 purinoceptors/PKCs/NADPH oxidase-mediated activation of Jak2 and STAT3 pathway</td>
<td>(Cheng et al., 2013a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cyclic GMP-mediated pathways</td>
<td>(Watkins et al., 1997)</td>
</tr>
<tr>
<td>S1P</td>
<td>rat vascular smooth muscle</td>
<td>Ca²⁺-dependent PKC and Src activation via S1P3 receptor coupled to pertussis toxin-sensitive and -insensitive G proteins</td>
<td>(Nodai et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>ASM</td>
<td>S1PR1/3/c-Src/PYK2/p42/p44 MAPK- or JNK1/2- and S1PR1/3/c-Src/p38 MAPK-dependent AP-1 activation</td>
<td>(Hsu et al., 2015)</td>
</tr>
<tr>
<td>trypsin</td>
<td>ASM</td>
<td>PAR-2-independent mechanism</td>
<td>(Chambers et al., 2003)</td>
</tr>
</tbody>
</table>
1.7.2 Post-transcriptional regulation

COX-2 is known to be regulated at the post-transcriptional level by RNA-binding proteins and microRNAs (miRNAs). The 3′-UTR of COX-2 mRNA contains multiple copies of adenylate and uridylate rich (AU-rich) elements (AREs), and much has been learned over the past decade or so in regards to the cis-acting motifs within this region and the RNA-binding proteins that interact with AREs to dictate mRNA stability. Mukhopadhyay et al. identified a novel RNA binding protein, CUGBP2 (CUG triplet repeat, RNA-binding protein 2), which stabilizes COX-2 mRNA but inhibits its translation (Mukhopadhyay et al., 2003). Augmented binding of the stabilizing Human Antigen R (HuR) to the ARE of the COX-2 3′-UTR is responsible for COX-2 mRNA stability (Erkinheimo et al., 2003; Subbaramaiah et al., 2003; Doller et al., 2009) in different cells. Studies by Hall-Pogar et al. suggest that alternative polyadenylation of the COX-2 gene is an important post-transcriptional regulatory event and some RNA-binding proteins, U1A, PTB (polypyrimidine tract-binding protein), p54nrb and PSF (PTB-associated splicing factor) can regulate the polyadenylation signal upon binding with upstream sequence elements (Hall-Pogar et al., 2005; Hall-Pogar et al., 2007). Time-dependent modulation of transcriptional and post-transcriptional phases of the COX-2 biosynthetic pathway can be achieved by anti-inflammatory corticosteroids and confirmed by actinomycin D analysis (Raz et al., 1989). Tristetraprolin (TTP), a mRNA-binding protein that promotes message instability, has been shown to bind with COX-2 mRNA in the region of the 3′-UTR, and thus participate in post-transcriptional regulation of COX-2 in cancer cell lines (Sawaoka et al., 2003). Other cytoplasmic proteins with RNA-binding properties can also regulate COX-2 gene expression including: T-cell intracellular antigen-1 (TIA-1) (Dixon et al., 2003; Phillips et al., 2004), RNA-binding motif protein 3 (RBM3) (Cok and Morrison, 2001; Sureban et al., 2008) and Apobec-1 (Anant et al., 2004). The
stress-inducible 70-kDa heat shock protein (Hsp70) has also been recently identified as a novel post-transcriptional regulator of COX-2 gene that binds and stabilizes COX-2 mRNA (Kishor et al., 2013). Notably, the p38 MAPK pathway activated by various inflammatory mediators plays an important role in post-transcriptional regulation of COX-2 by influencing mRNA message stability as well as its abundance (Ridley et al., 1998; Dean et al., 1999; Jang et al., 2000; Zhang et al., 2000; Lasa et al., 2001).

In addition to regulation by RNA-binding proteins, COX-2 has more recently been found to be a target of various microRNAs. Small non-coding RNAs called microRNAs (miRNAs), composing approximately 21–24 nucleotides, are considered to be vital post-transcriptional gene regulators through their ability to control mRNA stability and translation by imperfect base-pairing to the 3′-UTR of their target mRNAs. Binding of two miRNAs, mmu-miR-199a and mmu-miR-101, results in post-transcriptional regulation of the COX-2 gene by affecting mRNA stability and translation (Chakrabarty et al., 2007). Another microRNA, miR-16, is known to silence COX-2 gene expression by two mechanisms: directly, by binding to the microRNA response element motif in the COX-2 3′-UTR and indirectly, by reducing the levels of HuR (Agra Andrieu et al., 2012). MicroRNA-320a is found to be regulating COX-2 expression through extracellular signal-regulated kinases (ERK)/NF-κB pathways, hence, effecting pro-inflammatory cytokine production (Cheng et al., 2013b).

### 1.7.3 Post-translational regulation

Although most of the research on COX-2 regulation has been focused at the transcriptional and post-transcriptional levels, post-translational control is increasingly recognized as an important contributor to overall COX-2 protein production. The COX-2 protein that is immediately
translated from stable mRNA undergoes several post-translational regulatory modifications to control the abundance of active protein. COX-2 protein is found localized in the endoplasmic reticulum (ER) and the nuclear envelope (Reiger et al., 1993). This spatial regulation of COX-2 protein is regulated by N-glycosylation. Among five potential N-glycosylation sites of COX-2 protein sequences, three are always glycosylated, one (Asn\textsuperscript{580} in human and mouse) is glycosylated more than 50% of the time, and one is never glycosylated (Otto et al., 1993). The variability of glycosylation at Asn\textsuperscript{580} results in the formation of two distinct glycoforms, unmodified and modified, with molecular weights 72 and 74 kDa, respectively. Characterization by mass spectrometry reveals that the carbohydrate moieties at each site are composed of high-mannose oligosaccharide (Percival et al., 1997; Nemeth et al., 2001). Glycosylation at Asn\textsuperscript{580} plays a significant role in the COX-2 protein turnover (Sevigny et al., 2006). The mature monomer of COX-2 consists of three folding domains including an epidermal growth factor-like domain, a membrane binding domain, and a catalytic domain in sequence (Yuan and Smith, 2015). A number of reports have identified the mechanistic cascades required for the maturation of COX-2 in the ER lumen including cleavage of the N-terminal signal sequence, N-glycosylation at multiple sites, disulfide bond formation, membrane insertion, and dimer formation (Otto et al., 1993; Xiao et al., 1998; Spencer et al., 1999; Yuan et al., 2006).

Protein degradation is also an important factor in post-translational regulatory control and properly folded COX-2 has been reported to be degraded via two independent pathways (Mbonye et al., 2008; Wada et al., 2009). The first degradative pathway is called substrate-induced COX-2 degradation and it is considered to be proteasome independent (Mbonye et al., 2008; Mbonye and Song, 2009). Arachidonic acid is the endogenous fatty acid substrate of COX-2 which is subject to oxygenation as a consequence of suicide inactivation of COX-2.
catalytic activity (Mevkh et al., 1993; Song et al., 2001). Substrate-dependent degradation of COX-2 occurs as a result of structural damage to the enzyme that occurs by fatty acid substrate oxygenation.

The second pathway involves ER-associated degradation (ERAD), a process by which ER-associated N-glycoproteins are selectively removed for degradation and subsequently undergo proteolysis by 26 S proteasome. Because COX-2 is a luminal integral membrane protein, it needs to be transported out of the ER for degradation; this characteristic feature makes COX-2 an exclusive ERAD substrate (Mbonye and Song, 2009). Several studies have described the ERAD process for COX-2 protein in different cells (Mbonye et al., 2006; Mbonye and Song, 2009; Yuan and Smith, 2015). This process occurs at a relatively constant rate and it is believed that glycosylation of Asn$^{594}$ of COX-2 is critical for initiating the entry of the enzyme into the ERAD system and, most importantly, any mutation of this site will prevent glycosylation as well as make COX-2 more stable (Mbonye et al., 2006; Mbonye et al., 2008). COX-2 protein ubiquitination and degradation through the 26 S proteasome in the cytoplasm is also shown in several other reports (Rockwell et al., 2000; Figueiredo-Pereira et al., 2002; Neuss et al., 2007; Chen et al., 2013) and Sing et al. revealed the importance of the ER-mannosidase I enzyme in facilitating the ERAD-mediated ubiquitin-proteasomal degradation of COX-2 in human uterine stromal cells (Singh et al., 2012). The membrane protein caveolin-1 has been implicated in the post-translational regulation of COX-2 via ERAD-mediated ubiquitination and proteasome degradation pathways (Chen et al., 2010; Chen et al., 2013). Further investigations are warranted to fully understand this contribution.
1.8 Inflammatory mediators

Numerous mediators are capable of eliciting an inflammatory response from airway cells [reviewed in (Barnes et al., 1998)]. The cross-talk between cells and the mediators is the principle driving-force for the inflammatory response observed in respiratory diseases like asthma. In this project we have chosen TNFα, Pam3CSK4, S1P and IL-17A for their potential role in airway inflammation in the context of TLR2 activation and COX-2 upregulation. In the following sections we have given brief discussion about them.

1.8.1 TNFα

TNFα is a multifunctional pro-inflammatory cytokine that belongs to the TNF superfamily. It is a crucial cytokine mainly secreted by activated macrophages but also by other immune cells like mast cells, lymphocytes, endothelial cells and, importantly for this study, also by structural cells such as fibroblasts, epithelial cells and smooth muscle cells (Wajant et al., 2003; Brightling et al., 2008). Though TNFα is detectable in healthy individuals, elevated serum and tissue levels are found in inflammatory and infectious conditions. Literature suggests that large amounts of TNFα are released in response to LPS and other bacterial products (Wajant et al., 2003). Two forms of TNFα with distinct biological activities have been identified, membrane-associated and soluble TNFs. TNFα exert their action by binding with one of two distinct receptors, TNFR1 and TNFR2, which are differentially regulated on various cell types in normal and diseased tissue (Al-Lamki et al., 2001). This cytokine is involved in the regulation of a wide spectrum of biological processes including, cell growth promotion & inhibition, angiogenesis, immunomodulation and inflammation. Thereby, it plays an important role in modulating a wide range of inflammatory conditions such as rheumatoid arthritis (Sarzi-Puttini et al., 2005),
cardiovascular diseases (Sack, 2002), psoriasis (Kleyn and Griffiths, 2006) and cancer (Li et al., 2015). The role of TNFα has been implicated in many aspects of the airway pathology in respiratory diseases (Berry et al., 2007; Brightling et al., 2008; Matera et al., 2010) and this project also highlighted the inflammatory role of TNFα in asthmatic condition. By stimulating a number of inflammatory pathways and releasing associated pro-inflammatory modulators, TNFα plays an important role in increasing airway hyperresponsiveness of ASM cells and airway remodelling (Amrani et al., 2001; Ammit et al., 2002). Our earlier studies also demonstrated TNFα as a model inflammatory mediator that secretes a significant amount of pro-inflammatory cytokines in ASM cells and confirmed its role in the pathology of inflammatory lung diseases like asthma.

1.8.2 Pam3CSK4

It is well described that the cells of the innate immune system identify molecules synthesized exclusively by microbes and coordinate the inflammatory response to pathogens via TLRs signal transduction pathways. Most common microbial products include LPS, peptidoglycans, lipoteichoic acids and bacterial pathogens produced lipoproteins (BLPs) (Henderson et al., 1996; Aliprantis et al., 1999). BLPs are bacterial cell wall components found both in gram-negative and gram-positive bacteria that possess pro-inflammatory cytokine-inducing ability (Kostyal et al., 1994). The immunomodulatory effects of BLP are mediated through their amino acid terminus and N-palmitoyl-S-[2,3-bis-(palmitoyloxy)-(2R,S)-propyl]-(R)-cysteinyl-seryl-(lysyl)3-lysine (Pam3CSK4) is a well-established synthetic tripalmitoylated lipopeptide that mimics the acetylated amino terminus of bacterial lipoproteins (Manukyan et al., 2005). Aliprantis et al. first described Pam3CSK4 as a potent TLR2 activator that induces the signalling cascade leading to
the activation of NF-κB (Aliprantis et al., 1999). It is believed that synthetic lipopeptides provide an important tool for studying innate immune recognition mechanisms as they are not only more easily obtained than the lipopeptides directly obtained from organisms but also are free of other contaminating bacterial components (Manukyan et al., 2005). Several reports demonstrate that TLR engagement with synthetic ligands or bacterial products enhance TLR2 function and induce proinflammatory cytokine secretion from human ASM cells, including eotaxin and IL-6, IL-8 (Sukkar et al., 2006; Manetsch et al., 2012b). Manetsch et al. suggests that Pam3CSK4 robustly amplifies TNFα-induced IL-6 and IL-8 expression in ASM cells and enhances neutrophil chemotaxis and also demonstrates that the effect of Pam3CSK4 occurs via phosphorylation of CREB protein at Ser^{133} and CRE-mediated transcription, rather than via enhanced NF-κB- or MAPK-mediated signalling (Manetsch et al., 2012b). In our studies, we have used Pam3CSK4 as a potent TLR2 agonist (Pam3CSK4 also targets TLR1, however, in this thesis we focused only TLR2) and suggested that ligation of TLR2 with Pam3CSK4 may enhance the ASM inflammatory response during infectious exacerbation.

1.8.3 S1P

S1P is a bioactive end product of sphingolipid metabolism and has been identified as a unique molecule capable of mediating diverse biological responses. S1P level is found to be increased in multiple cell types in response to numerous stimuli, including antigen, cytokines and both receptor tyrosine kinase and G protein-coupled receptor (GPCR) agonists (Pyne and Pyne, 2000). It has been anticipated that S1P can act both as an extracellular mediator via a family of plasma membrane GPCRs, and as an intracellular second messenger for growth factors, by altering the activity of specific intracellular target proteins (Pyne and Pyne, 2000). It has been shown to
regulate cell growth, proliferation, differentiation and chemotaxis of various cell types, and the well-recognized role for S1P has been proposed in disease states such as atherosclerosis (Wang et al., 1999b) and certain cancers (Hong et al., 1999; Wang et al., 1999a). Ammit et al. first showed that S1P levels were elevated in the airways of asthmatic subjects following antigen challenge and explored the association of S1P with airway inflammation (Ammit et al., 2001). In addition, S1P was identified as an effective regulator of ASM growth, contraction and synthetic functions. Several further studies indicate S1P mediates the activation of a number of signalling pathways leading to physiological and also pathophysiological effects via COX-2 protein upregulation (Nodai et al., 2007; Fuerst et al., 2014; Hsu et al., 2015). Therefore, we believe that a better understanding of signalling mechanisms underlying S1P-induced COX-2 gene regulation will create opportunities for the development of anti-inflammatory therapeutic strategies for asthma.

1.8.4 IL-17A

In our study we have also used cytokine IL-17A as an inflammatory mediator in asthmatic conditions, confirming that, over the last decade, this cytokine has become of increasing interest in airway disease. Among six members in the interleukin 17 cytokine family (IL-17A-F), the biological function and regulation of IL-17A and IL-17F are best understood (Jin and Dong, 2013). IL-17A is an approximately 15 kDa glycoprotein, originally identified as the human homologue, mostly secreted by activated T lymphocytes cells and also by several other innate immune cell types, such as lymphoid tissue inducer cells, natural killer and natural killer T cells and macrophages. It thereby plays key regulatory roles in host defence and inflammatory diseases (Henness et al., 2004; Jin and Dong, 2013). IL-17 family cytokines exert their biological
functions by binding with surface receptors on target cells. Five IL-17 receptor families have been identified including IL-17RA, IL-17RB, IL-17RC, IL-17RD and IL-17RE (Moseley et al., 2003). Upon receptor-ligand interaction IL-17A promotes the secretion of various pro-inflammatory cytokines and chemokines via stimulating a number of downstream signalling pathways including NF-κB and MAPK activation, tumor-necrosis factor receptor-associated factor (TRAF6), IκB kinase I (IKKi), ubiquitin-specific processing protease 25 (USP25) signal transduction pathways (Jin and Dong, 2013). Several reports suggest the association of high concentrations of IL-17A with autoimmunity and inflammatory diseases such as rheumatoid arthritis, psoriasis, multiple sclerosis, inflammatory bowel disease (IBD) and cancer (Zhang et al., 2013; Kirkham et al., 2014; Huang et al., 2015; Omrane et al., 2015). Moreover, an increased level of IL-17A protein is found in human airways during severe inflammatory conditions (Molet et al., 2001; Laan et al., 2002; Chesne et al., 2014). Previous studies in ASM cells also confirm the involvement of IL-17A in the production of a large spectrum of inflammatory cytokines (IL-6, IL-1β, and IL-11), chemokines (Eotaxin/CCL-11 and CXCL8) and augmentation of ASM cell proliferation and migration, thus enhancing the inflammatory response (Dragon et al., 2007; Chang et al., 2012). Hence, the functional role of IL-17A in developing airway diseases led to further exploration mechanisms at the cellular level to develop a better understanding of the involvement of IL-17A in severe asthma.

1.9 COX-2 upregulation and β2-AR desensitization

β2-agonists are the first line therapy to relieve acute bronchoconstriction in people with asthma or COPD. β2-agonists exert their effects by binding with the β2-AR, a seven transmembrane GPCR. Binding of β2-agonists with β2-AR results in activation of adenylyl cyclase (AC) through a
stimulated Gs protein coupled mechanism that leads to the formation of second messenger cyclic adenosine monophosphate (cAMP), which in turn activates protein kinase A (PKA) (Figure 1.5). PKA phosphorylates a number of target proteins resulting in smooth muscle relaxation or bronchodilation. However, heterologous and homologous β₂-AR desensitization can occur in a diverse range of clinically-relevant contexts, including β₂-agonist overuse, inflammation and bacterial and viral infectious exacerbation [reviewed in (Shore and Moore, 2003)]. Homologous desensitization occurred when the receptor lost its responsiveness upon binding with its agonist itself (Hall et al., 1993), whilst heterologous desensitization is attributed to receptor downregulation by other mediators present in the microenvironment around asthmatic airway smooth muscle (Pang and Knox, 1997; Laporte et al., 1998; Pang et al., 1998b).

Figure 1.5 Mechanisms of action of β₂-agonists and underlying molecular pathways of β₂-AR desensitization.
COX-2 immunoreactivity is found to be increased in asthmatic airways (Nasser et al., 1996; Demoly et al., 1997) and levels of PGE$_2$ are increased in the lungs of people with COPD (Montuschi et al., 2003) in a manner correlated with airflow obstruction severity (Montuschi et al., 2003; Chen et al., 2008). PGE$_2$ exerts multiple physiological and pathophysiological effects in airways. It is well-recognized that the β$_2$-AR on human ASM cells is subject to homologous desensitization induced by PGE$_2$. This was first demonstrated in 1993 when Hall et al. showed that pretreatment of cells with PGE$_2$ induced a dose-dependent reduction in cAMP release in response to isoprenaline (Hall et al., 1993). Further mechanistic insights into the underlying mechanisms and direct links to COX-2-mediated prostanoid production followed (Laporte et al., 1998; Pang et al., 1998b; Penn et al., 1998; Pascual et al., 2001; Shore and Moore, 2003); with many of these studies demonstrating that PGE$_2$ directly induces β$_2$-AR desensitization and thus tachyphylaxis to the first line bronchodilators in respiratory diseases - β$_2$-agonists (Shore and Moore, 2003; Billington et al., 2013). Rhinoviral infection has also been shown to desensitize the β$_2$-AR and reduces cAMP generation in response to β$_2$-agonists (Trian et al., 2010) in a prostanoid-dependent manner (Van Ly et al., 2013). Thus, the clinical consequence of COX-2-mediated upregulation of PGE$_2$ secretion in the context of β$_2$-AR desensitization on ASM cells may prove detrimental and perhaps particularly so in infectious exacerbation.

1.10 Targeting COX-2 in airway inflammation: PGE$_2$ receptor signalling

Increased levels of COX-2 protein and prostanoid metabolites serve as key contributors to pathobiology in respiratory diseases typified by dysregulated inflammation. However, COX-2 products may not be all bad: prostanoids can exert anti-inflammatory/bronchoprotective
functions in airways in addition to their pro-inflammatory actions. There are multiple ways to target COX-2, including: inhibiting enzymatic activity with non-selective (e.g. NSAIDs) or selective inhibitors (such as coxibs), repressing COX-2 mRNA expression by targeting transcriptional or post-transcriptional mechanisms (RNA-binding proteins or miRNAs) and enhancing COX-2 protein stability by affecting N-glycosylation or ERAD-mediated proteasomal degradation. These molecular mechanisms represent feasible options for targeting COX-2 as they are amenable to manipulation and some are controlled by spatiotemporal regulation so that beneficial effects within the context of respiratory disease remain a distinct possibility.

Traditional NSAIDs, such as aspirin, can inhibit both COX-1 and COX-2. However, their regular use is associated with side effects, such as gastric bleeding due to their inhibitory effects on COX-1. Most importantly, in asthmatic patients the use of aspirin may lead to aspirin-sensitive respiratory disease (ASRD), or aspirin exacerbated respiratory disease (AERD), or aspirin-induced asthma (AIA), or aspirin sensitive asthma (ASA) where the expression of COX-2 is critically low. A number of reports suggest that downregulation of PGE2 production associated with EP2 receptor defects is identified in AERD pathogenesis (Szczechlik et al., 1975; Szczechlik, 1990; Sestini et al., 1996; Celik et al., 2001; Szczechlik and Stevenson, 2003; Jinnai et al., 2004; Ying et al., 2006; Corrigan et al., 2012; Liu et al., 2013; Steinke and Borish, 2015). Moreover, modern NSAIDs that inhibit COX-2 selectively are associated with a lower incidence of upper gastro-intestinal disease (Chang and Howden, 2004). Hence, at this stage the greatest potential is offered by our advancing knowledge of the prostanoid receptor family to control the direct effects of prostanoids leaving the in general effects of COX-2. Prostanoids exert their diverse functions by binding with several receptor subtypes, including: the PGD receptor (DP1 and DP2), four subtypes of the PGE receptor (EP1, EP2, EP3, and EP4), the PGF receptor (FP), the PGI
receptor (IP) and the TxA receptor (TP) (Narumiya and FitzGerald, 2001) (Figure 1.6). Development of selective agonists and antagonists are valuable pharmacological tools and exciting lead compounds for future drug discovery in respiratory conditions (Buckley et al., 2011; Birrell et al., 2013; Maher et al., 2015). Whether further studies will reveal that receptor redundancy exists, and that being more specific by targeting individual prostanoid receptors do not offer any added benefit over global approaches will emerge. However, coupled with mouse models where individual prostanoid receptors are knocked-out, these approaches offer an elegant means to address unanswered questions regarding the impact of COX-2 products in clinically relevant in vivo models of respiratory disease. This was recently shown by Birrell et al. where the EP₄ receptor was shown to be responsible for anti-inflammatory effects of PGE₂ in the lung (Birrell et al., 2015).

**Figure 1.6 COX-2 pathways - multiple ways to target COX-2 expression/activity or prostanoid receptors.**
1.11 Hypothesis and aim of the study

In this thesis, we largely aim to explore the underlying molecular mechanisms of TLR2 receptor activation, COX-2 protein upregulation and β2-AR desensitization in ASM cells. We have addressed two basic hypotheses:

1. Activation of TLR2 receptor in ASM cells may upregulate some key inflammatory regulators including NLRP3 inflammasome and COX-2.

2. In ASM cells, COX-2, induced by not only TLR2 activation but also by some other inflammatory mediators (S1P & IL-17A), may be responsible for β2-AR desensitization via producing PGE2.

The aims of this thesis are as follows:

I. Role of TLR2 in the activation NLRP3 inflammasome in ASM cells

A growing body of evidence suggests that the NLRP3 inflammasome is important in chronic airway diseases such as asthma and COPD [reviewed in (Im and Ammit, 2014)]. Therefore, in this study the aim was to determine the possible involvement of the NLRP3 inflammasome and IL-1β activation in ASM cells in response to Pam3CSK4-induced TLR2 activation.

II. TLR2 activation induced desensitization of β2-AR via COX-2 production

Research has revealed that β2-AR agonists lose bronchodilatory efficacy because the receptor-mediated molecular pathways responsible for their beneficial actions are desensitized by infection (Trian et al., 2010). To date, most studies have focused on viral infection, leaving the impact of bacterial infection on β2-AR desensitization relatively under-investigated. We address this in this study.
III. COX-2 expression and PGE₂ secretion by S1P: effects on β₂-AR desensitization

Cellular studies in ASM have shown that inflammatory mediators and infectious stimuli reduce β₂-adrenergic responsiveness in a COX-2-mediated, PGE₂-dependant manner (Van Ly et al., 2013). In this study, we show that S1P, which plays an important role in the pathophysiology of asthma, also induces β₂-AR desensitization in bronchial ASM cells and exerts hyporesponsiveness to β₂-agonists.

IV. TNFα-induced COX-2 protein stability and PGE₂ secretion by IL17A

Recent evidence has implicated IL-17A as a key driver of disease exacerbation in severe asthma (Brandt et al., 2013) and in vivo models of infectious exacerbation (Essilfie et al., 2011; Lunding et al., 2015; Roos et al., 2015). We address this herein and examine the impact of IL-17A on COX-2 mRNA expression, protein upregulation and subsequent PGE₂ secretion from ASM cells. In this study we also examined the impact of IL-17A on β₂-AR desensitization on ASM cells.

V. Identify the role of specific PGE₂ receptors

Understanding the effect of PGE₂ is very complicated, as their biological activities are mediated via four distinct EP receptor subtypes, EP₁-₄, that are coupled to different intracellular signal transduction pathways (Chung, 2005). In this study, we attempt to find the role of specific PGE₂ receptors, in particular, EP₂ and EP₄ receptors, in the context of airway inflammation.
Chapter 2
General Materials and Methods
Chapter 2
General Materials and Methods

2.1 Materials

Unless otherwise specified, all chemicals and reagents used in this study were purchased from Sigma-Aldrich (St.Louis, MO).

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Solutions

Cell culture/feeding media for the culture of ASM cells is composed of Dulbecco’s Modified Eagle’s Medium (DMEM) with phenol red, enriched with 200 mM L-glutamine, 1 M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), antibiotic-antimycotic liquid (containing 10,000 units/mL pencillin, 10,000 µg/mL streptomycin and 25 µg/mL amphotericin B) (Invitrogen, Carlsbad, CA) and 10% heat-inactivated (56 °C for 30 min) foetal bovine serum (FBS) (Interpath Services, Heidelberg West, VIC, Australia). Starving media used in cell culture consists of 0.1% (v/v) BSA (30% w/v solution, sterile) in DMEM (no other supplements are added).

2.2.1.2 Culture of primary human ASM cells

Human bronchi from the large bronchial airways were obtained from patients undergoing surgical resection for carcinoma or lung transplant donors in accordance with procedures approved by the Central Sydney Area Health Service and the Human Ethics Committee of the...
University of Sydney. Primary ASM cells were dissected, purified and cultured as previously described by Johnson *et al.* (Johnson et al., 1995b). Specific to our settings, after dissection of the bronchi from the surrounding tissue and removing of the epithelium layer individual ASM tissue bundles were plucked out and placed into 25cm² tissue culture flask (Sarstedt, Nümbrecht Germany) with DMEM containing phenol red and supplemented with 2 mM L-Glutamine, 20 mM HEPES, 10% heat inactivated FBS and 2% Antibiotic-Antimycotic mix (200 U/L Penicillin G, 0.5 µg/mL Amphotericin B and 200 µg/mL Streptomycin sulphate). The flasks were then placed in a 5% CO₂ humidified incubator at 37°C. Cells grew to confluence over 14-21 days and were passaged when confluent.

### 2.2.1.3 Passaging ASM cells

Before trypsinizing the cells (2 min at 37°C) the media was aspirated and the cells were washed two times with sterile PBS (Ca²⁺/Mg²⁺-free) to remove all traces of FBS. Cell passaging was completed using a trypsin solution (0.5 g/l porcine trypsin and 0.2 g/l EDTA in Hank's balanced salt solution with phenol red). The cell attachment was checked microscopically (Olympus CKX31, Olympus, Center Valley, PA) and if necessary the detachment was facilitated by agitation. After detachment, feeding media was added to inhibit further trypsic activity. Cell numbers were assessed on a haemocytometer using trypan blue solution. Cells were plated at a density of 1x10⁶ cells/T175 flask (Sarstedt, Nümbrecht, Germany) placed at 5% CO₂ in air at 37°C, media change every week and passage into further T175 flasks (split 1:3) every 2 weeks, depending on cell need. For all experiments in this thesis, smooth muscle cells were used between passage 4 and 8. Primary ASM cells typically use maximum passage number 8 to 10.
2.2.1.4 Plating and starvation

Cells were plated at a density of $1.0 \times 10^4 \text{ cells/cm}^2$ according to the guidelines in Table 2.1 and commonly grown for one week (unless otherwise specified). Feeding media was changed approximately 72 h after plating. Before performing the experiments, the cells were growth arrested generally to synchronise all the cells into a particular growth cycle phase (Davis et al., 2001). The feeding media was aspirated, the cells were washed twice with sterile PBS, and then starving media was added for 48 h. In most of our experiments we used 6-well plates (unless otherwise specified).

**Table 2.1 Guidelines for plating of ASM cells for experiments**

<table>
<thead>
<tr>
<th>TYPE OF PLATE</th>
<th>PLATING DENSITY</th>
<th>MEDIA PER WELL</th>
</tr>
</thead>
<tbody>
<tr>
<td>96-well</td>
<td>3,200 cells/well</td>
<td>0.1 mL</td>
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<tr>
<td>6-well</td>
<td>96,000 cells/well</td>
<td>2 mL</td>
</tr>
<tr>
<td>24-well</td>
<td>18,000 cells/well</td>
<td>1 mL</td>
</tr>
<tr>
<td>100 mm</td>
<td>785,000 cells/well</td>
<td>10 mL</td>
</tr>
</tbody>
</table>

2.2.1.5 ASM cell characterization

To characterize primary ASM cells, after the first passage, the Respiratory Research Group performs an immunoassay for the expression of the muscle cell markers for α-smooth muscle actin and calponin as previously described by Johnson *et al.* (Johnson *et al.*, 1995a). In following passages the cells were visually examined and kept showing the, for primary ASM cells, typical hill-and-valley morphology, were elongated, thin, spindle-shaped and showed concentric, oval nuclei. The following table details the patient demographics for the cells used in this thesis.
Table 2.2: Patient demographics for the cells used in this thesis

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Age</th>
<th>Gender</th>
<th>Diagnosis</th>
<th>FEV1 (L) and/or % predicted</th>
<th>FVC (L) and/or % predicted</th>
<th>FEV1: FVC (%)</th>
<th>Smoking History</th>
<th>Height (cm)</th>
</tr>
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<tbody>
<tr>
<td>2821</td>
<td>66</td>
<td>M</td>
<td>NSCCa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2839</td>
<td>65</td>
<td>M</td>
<td>Malignant metastatic melanoma</td>
<td>2.86</td>
<td>3.58</td>
<td></td>
<td></td>
<td>175</td>
</tr>
<tr>
<td>3476</td>
<td>80</td>
<td>F</td>
<td>NSCCa</td>
<td></td>
<td></td>
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<tr>
<td>3706</td>
<td>66</td>
<td>M</td>
<td>NSCCa</td>
<td>3.44 102%</td>
<td>4.79 106%</td>
<td>72</td>
<td>smoker, 62.5 pack years</td>
<td>180</td>
</tr>
<tr>
<td>3717</td>
<td>70</td>
<td>M</td>
<td>Carcinoma (Ca)</td>
<td>2.14</td>
<td>3.84</td>
<td></td>
<td>ex-smoker 100 pack years stopped 1 year prior</td>
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</tr>
<tr>
<td>3729</td>
<td>59</td>
<td>M</td>
<td>Lesion (incidental finding) +COPD</td>
<td>2.6 69%</td>
<td>3.5 71%</td>
<td></td>
<td>ex-smoker 25/day x 30 years stopped 1 week prior</td>
<td>178</td>
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<tr>
<td>3731</td>
<td>64</td>
<td>M</td>
<td>Ca</td>
<td>2.4</td>
<td>4.15</td>
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<td>ex-smoker 50/day x 50 years stopped 6 weeks prior</td>
<td>167</td>
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<tr>
<td>3737</td>
<td>71</td>
<td>M</td>
<td>NSCCa</td>
<td>2.77 93%</td>
<td>3.49 89%</td>
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<td>no</td>
<td>175</td>
</tr>
<tr>
<td>3764</td>
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<td>M</td>
<td>Ca/COPD</td>
<td>1.4</td>
<td>3.7</td>
<td></td>
<td>ex-smoker</td>
<td>180</td>
</tr>
<tr>
<td>3777</td>
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<td>F</td>
<td>NSCCa</td>
<td>2.42 92%</td>
<td>2.9 94%</td>
<td>83</td>
<td>ex-smoker, quit 1/8/2010 (operation 31/08/2010)</td>
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<tr>
<td>3817</td>
<td>66</td>
<td>M</td>
<td>Squamous cell Ca</td>
<td>1.74</td>
<td>2.9</td>
<td></td>
<td>ex-smoker 25/day x 40 years stopped 11 years prior</td>
<td>174</td>
</tr>
<tr>
<td>3832</td>
<td>59</td>
<td>F</td>
<td>Ca (adeno)</td>
<td>111</td>
<td>106</td>
<td></td>
<td>no</td>
<td>151</td>
</tr>
<tr>
<td>3839</td>
<td>62</td>
<td>F</td>
<td>Squamous cell Ca</td>
<td>0.78 38%</td>
<td>1.47 55%</td>
<td></td>
<td>ex-smoker 20/day x 30 years, stopped 4 weeks prior</td>
<td>150</td>
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<tr>
<td>3879</td>
<td>68</td>
<td>F</td>
<td>Ca</td>
<td>1.31 48%</td>
<td>2.56</td>
<td></td>
<td>ex-smoker (25/day x 45 years, quit 6 years prior)</td>
<td>173</td>
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<td>ID</td>
<td>Age</td>
<td>Gender</td>
<td>Diagnosis</td>
<td>Sex Hist</td>
<td>Expiratory Function</td>
<td>Smoking History</td>
<td>Weight</td>
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<tr>
<td>3891</td>
<td>73</td>
<td>M</td>
<td>NSCCa</td>
<td>1.21</td>
<td>38%</td>
<td>ex-smoker, quit 1999</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>3892</td>
<td>58</td>
<td>F</td>
<td>NSCCa</td>
<td>2.22</td>
<td>96%</td>
<td>non-smoker</td>
<td>88</td>
<td></td>
</tr>
<tr>
<td>3897</td>
<td>67</td>
<td>F</td>
<td>squamous cell carcinoma, carcinoid tumour</td>
<td>1.81</td>
<td>93%</td>
<td>non-smoker</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3902</td>
<td>51</td>
<td>M</td>
<td>IPF</td>
<td>0.95</td>
<td>1.3</td>
<td>non-smoker</td>
<td>176</td>
<td></td>
</tr>
<tr>
<td>3903</td>
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<td>M</td>
<td>IPF</td>
<td>1.06</td>
<td>1.42</td>
<td>ex-smoker, 10 pack years</td>
<td>177</td>
<td></td>
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<tr>
<td>3907</td>
<td>63</td>
<td>M</td>
<td>NSCLCa</td>
<td>2.32</td>
<td>71%</td>
<td>ex-smoker, 125pk yrs, 6/12 prior (quit 6 months prior)</td>
<td>173</td>
<td></td>
</tr>
<tr>
<td>3910</td>
<td>68</td>
<td>F</td>
<td>Ca</td>
<td>2.21</td>
<td>94%</td>
<td>heavy smoker currently 10-15/day, approx 30-40 pack years, stopped 2/52 prior</td>
<td>171</td>
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</tr>
<tr>
<td>3912</td>
<td>67</td>
<td>M</td>
<td>NSCLCa</td>
<td>2.22</td>
<td>3.1</td>
<td>ex-smoker, 25 packs/day x 40 years, quit 2 weeks prior (2/52)</td>
<td>168</td>
<td></td>
</tr>
<tr>
<td>3920</td>
<td>71</td>
<td>M</td>
<td>NSCCa</td>
<td>1.3</td>
<td>51%</td>
<td>ex-smoker, 60/day, quit 16 yrs prior</td>
<td>165</td>
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<td>3923</td>
<td>66</td>
<td>M</td>
<td>NSCCa</td>
<td>2.38</td>
<td>71%</td>
<td>ex-smoker, quit 2001</td>
<td>180</td>
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<tr>
<td>3926</td>
<td>62</td>
<td>F</td>
<td>Emphysema</td>
<td>0.41</td>
<td>1.96</td>
<td>ex-smoker, 60 pack years</td>
<td>153</td>
<td></td>
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<tr>
<td>3932</td>
<td>73</td>
<td>F</td>
<td>NSCCa</td>
<td>2.4</td>
<td>100%</td>
<td>ex-smoker, 28 pack yrs, quit 30 yrs prior</td>
<td>173</td>
<td></td>
</tr>
<tr>
<td>3935</td>
<td>57</td>
<td>F</td>
<td>COPD</td>
<td>0.55</td>
<td>1.64</td>
<td>ex-smoker, 30 pack yrs</td>
<td>153</td>
<td></td>
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<tr>
<td>3938</td>
<td>56</td>
<td>F</td>
<td>Adenocarcinoma, malignant neoplasm</td>
<td>2.2</td>
<td>66%</td>
<td>ex-smoker 10/day, 20/day for 20 years, ceased 1/1/1997</td>
<td>168</td>
<td></td>
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<tr>
<td>3944</td>
<td>78</td>
<td>F</td>
<td>Secondary malignant carcinoma</td>
<td>1.82</td>
<td>88%</td>
<td>ex-smoker</td>
<td>158</td>
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<tr>
<td>ID</td>
<td>Age</td>
<td>Sex</td>
<td>Diagnosis</td>
<td>NYHOM</td>
<td>NYH05</td>
<td>Description</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>3946</td>
<td>51</td>
<td>F</td>
<td>Emphysema</td>
<td>0.47</td>
<td>1.84</td>
<td>ex-smoker, 35 pack/years</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3948</td>
<td>59</td>
<td>F</td>
<td>COPD</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>3953</td>
<td>75</td>
<td>M</td>
<td>squamous cell carcinoma, mucinous adenocarcinoma</td>
<td>2.15</td>
<td>0.47</td>
<td>ex-smoker, 20-30/day, quit 2002</td>
<td></td>
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<tr>
<td>3971</td>
<td>60</td>
<td>F</td>
<td>Alpha1 anti-trypsin deficiency</td>
<td>0.46</td>
<td>1.91</td>
<td>10 pack/ys</td>
<td></td>
<td></td>
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<tr>
<td>3994</td>
<td>58</td>
<td>M</td>
<td>COPD</td>
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<td>3.11</td>
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<tr>
<td>4005</td>
<td>72</td>
<td>F</td>
<td>Adenocarcinoma, NSCCa</td>
<td>1.49</td>
<td>1.24</td>
<td>ex-smoker quit 1990, 35/day</td>
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<tr>
<td>4036</td>
<td>71</td>
<td>M</td>
<td>NSCCa</td>
<td>2.02</td>
<td>2.87</td>
<td>ex-smoker quit 1997</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4037</td>
<td>61</td>
<td>M</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>4039</td>
<td>47</td>
<td>M</td>
<td>Acute interstitial pneumonia</td>
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<td>68</td>
<td>M</td>
<td>NSCCa</td>
<td>2.97</td>
<td>3.6</td>
<td>ex-smoker quit 1972</td>
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<td>4073</td>
<td>46</td>
<td>M</td>
<td>Emphysema</td>
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<td>4077</td>
<td>61</td>
<td>F</td>
<td>NSCCa</td>
<td>2.08</td>
<td>2.72</td>
<td>ex-smoker quit 1998, 15 pack yrs</td>
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<td>4083</td>
<td>25</td>
<td>F</td>
<td>Cystic fibrosis</td>
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<td></td>
</tr>
<tr>
<td>4085</td>
<td>54</td>
<td>M</td>
<td>Pulmonary fibrosis</td>
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<td></td>
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<tr>
<td>4098</td>
<td>41</td>
<td>F</td>
<td>Adenocarcinoma NOS, paraneoplastic neuropathy</td>
<td>2.25</td>
<td>2.48</td>
<td>smoker, quit 3wks ago, 60 pack yrs</td>
<td></td>
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<tr>
<td>4100</td>
<td>64</td>
<td>F</td>
<td>NSCLC, metastatic adenocarcinoma</td>
<td>1.22</td>
<td>1.44</td>
<td>heavy smoker, quit 2 weeks ago</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4159</td>
<td>38</td>
<td>F</td>
<td>LUL tumour inflammatory histopathology, no malignancy. Chondrodermatitis isnodularishelicis, CT spiculated 1.5cm subpleural mass LUL, no malignant cells</td>
<td>3.13</td>
<td>4.01</td>
<td>15 cigs day for 20 years</td>
<td></td>
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</tbody>
</table>
2.2.2 Western blotting

2.2.2.1 Solutions

5x Cell lysis/sample buffer is composed of TrisHCl 250 mM, DTT 500 mM, SDS 10% (w/v), bromophenol blue 0.5% (w/v), glycerol 50%, pH 6.8 and was used to collect cell lysates. The separating gel (8% or 12%) consisted of a 30% acrylamide- 0.8% bisacrylamide- solution, TRIS (pH 8.8).

1.5 M, SDS 10%, N,N,N’,N’ tetramethylethylenediamine (TEMED) and a 30% (w/v) ammonium persulfate (APS) solution. The stacking gel (5%) consisted of a 30% acrylamide-0.8% bisacrylamide- solution TrisHCl (pH 6.8) 1 M, SDS 10%, TEMED and a 30% APS solution. The electrophoresis buffer (pH 8.3) contained TRIS 25 mM, glycine 250 mM, and SDS 0.5 %. The transfer buffer (pH 8.5) used for electro transfer consisted of TRIS 25 mM, glycine 192 mM and 20% (v/v) methanol. The transfer buffer used for diffusion transfer is composed of NaCl 50mM, EDTA 2mM, Tris-HCl pH 7.5 10 mM and DTT. Tris- buffered saline solution with tween (TBS-T) pH 7.6 is composed of Tris 20 mM, NaCl 137 mM, and 0.1% (v/v) Tween 20. Blocking buffer contained 5% (w/v) skim milk powder in TBS-T (pH 7.6).

2.2.2.2 Sample preparation and immunoblotting

According to cell needs and experimental plans, ASM cells were plated in petri dishes, 6-well plates (BD Bioscience, Franklin Lakes, NJ) and cultured until they reached confluence. After growth arresting for 48 hours, cells were treated according to the experimental plans as indicated in the respective Chapters. Cells were then washed two times with ice-cold PBS, 1x sample
buffer was added and after scraping, the lysates were collected in eppendorf tubes and boiled for 5 min. To fractionize the samples by size and to detect specific proteins of interest, a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed. The SDS-PAGE was run at 200 V constant using the Power Pac 200 (Bio-Rad, Hercules, CA) until the bromophenol blue in the sample buffer completely reached the end of the gel. Proteins were then transferred onto a nitrocellulose membrane (Pall Corporation, Port Washington, NY) by electrotransfer (100 V constant on the Power Pac 200, 1 h). To check the uniformity and the overall effectiveness of the protein the membranes were stained with Ponceau. After blocking the membranes for 1 h at room temperature with blocking buffer on the rocking platform mixer (Ratek Instruments, Boronia, VIC, Australia) and subsequent washing with TBS-T the membranes were incubated with the primary antibody specific to the target protein (see Table 2.2). After 3 other wash steps, primary antibodies were detected with goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (see Table 2.2) and visualized by enhanced chemiluminescence (PerkinElmer, Wellesley, MA). The blots were exposed to a medical film (Fujifilm, Tokyo, Japan) and then developed with an x-ray processor (SRX-101A, Konica, Tokyo, Japan). Densitometry analysis was performed using ImageJ.
Table 2.3 Primary and secondary antibodies used for immunoblotting

<table>
<thead>
<tr>
<th>Antibody Type</th>
<th>Buffer</th>
<th>Incubation</th>
<th>Company</th>
<th>C/N</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary antibody</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Mouse monoclonal COX-2</td>
<td>Blocking buffer</td>
<td>Overnight, 4°C</td>
<td>Santa Cruz Biotechnology</td>
<td>SC-19999</td>
<td>1:500</td>
</tr>
<tr>
<td>Rabbit polyclonal IκB-α</td>
<td>Blocking buffer</td>
<td>Overnight, 4°C</td>
<td>Santa Cruz Biotechnology</td>
<td>SC-371</td>
<td>1:500</td>
</tr>
<tr>
<td>Mouse monoclonal α-Tubulin</td>
<td>Blocking buffer</td>
<td>30 min, RT</td>
<td>Santa Cruz Biotechnology</td>
<td>SC-32293</td>
<td>1:10000</td>
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<tr>
<td><strong>Secondary antibody</strong></td>
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<tr>
<td>Anti-mouse IgG</td>
<td>Blocking buffer</td>
<td>1 h, RT</td>
<td>Cell signaling</td>
<td>7076</td>
<td>1:10000</td>
</tr>
<tr>
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<td>Blocking buffer</td>
<td>1 h, RT</td>
<td>Cell signaling</td>
<td>7074</td>
<td>1:10000</td>
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</tbody>
</table>

2.2.3 RT-PCR

2.2.3.1 RNA sample collection and RNA extraction

ASM cells were plated in 6-well plates at a density of $10^4$ cells/cm$^2$, cultured for one week and growth-arrested for 48 h prior to the experiment. On the day of the experiment the cells were treated according to the experimental protocol. For the extraction of total RNA an adapted protocol from Qiagen protocol: RNeasy® Mini Handbook (Qiagen, Valencia, CA) was used. After completely aspirating the cell-culture medium and washing the wells 2 times with PBS, RLT lysis buffer, containing guanidine thiocyanate and β- mercaptoethanol was added to the wells, to lyse the cells and to immediately inactivate RNases to ensure purification of intact RNA. The lysates were collected with a rubber scraper (Sarstedt, Nümbrecht, Germany) and
pipetted into a 1.5 mL microcentrifuge tube. A syringe and a needle were used to fully lyse and homogenize the cells. To shear the DNA the lysates were passed through a 23-gauge needle attached to a sterile plastic syringe (needle and syringe both from: Livingstone International, Rosebery, NSW, Australia) at least 5-10 times. To provide appropriate binding conditions for the RNA to the RNeasy membrane, 70% ethanol was added to the homogenized lysates and mixed well by pipetting up and down. After transferring the samples to a provided RNeasy spin column placed in a 2 mL collection tube, the samples were centrifuge for 15 sec >10,000 rpm in a microcentrifuge (Centrifuge 5415R, Eppendorf AG, Hamburg Germany). In this step the total RNA binds to the membrane and any contaminants are efficiently washed away. After 3 more wash steps the total RNA was eluted by placing the RNeasy spin column in a new 1.5 mL collection tube, adding RNase-free water directly to the spin column membrane and after centrifugation for 1 min at >10,000 rpm the high-quality RNA was collected. To check the integrity of the RNA 5µl of each sample (5 µL of extracted RNA + 1 µL of RNA loading buffer) were run on a 1% agarose/TAE gel in 1x TAE (Tris-acetate-EDTA) at 100 V for about 30 min. The RNA integrity was assessed using the denaturing agarose gel electrophoresis method followed by ethidium bromide staining. Assessing the gels on the transilluminator, both 18S and 28S rRNA appeared as sharp, distinct bands after electrophoresis of total eukaryotic RNA and therefore the RNA was considered to be intact.

2.2.3.2 Reverse transcription and polymerase chain reaction

After this control step reverse transcription was performed to prepare single-stranded DNA from the RNA samples using the Fermentas RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Thermo Scientific, Burlington, Canada). To avoid any contamination and to ensure
the preparation of a high-quality complementary DNA (cDNA) all tubes used were autoclaved and only filter tips were used for the preparation of the samples. All the following reactions were prepared on ice. The water/primer master mix, containing Random Hexamer Primer and bottled water (Livingstone International, Rosebery, NSW, Australia), was prepared and aliquotted to the appropriate number of autoclaved, thin-walled 200 µL PCR tubes. Then the extracted RNA samples were added to each PCR tube. The tubes were placed into the PCR machine (MJ Research, PTC-200 Thermal Cycler, GMI inc, Ramsey, Mn) and heated at 70°C for 5 min for denaturation and to provide the right annealing conditions for the primers. After that step the tubes were placed on ice and reaction buffer, RNase inhibitor and dNTPs were added to the samples and the tubes were heated at 25°C for 5 min in the PCR machine. After adding the reverse transcriptase (RevertAid™ M-MuLV Reverse Transcriptase, Fermentas, Thermo Scientific, Burlington, Canada) to each tube the rest of the program was run to obtain the cDNA. The conditions were the following: 25°C for 10 min then 42°C for 60 min, 70°C for 10 min, and then the samples were cooled to 4°C and the cDNA was collected. Subsequent mRNA expression of the target genes was quantified by real-time RT-PCR using an ABI Prism 7500 real-time PCR machine (Applied Biosystems, Foster City, CA) and the appropriate primer set (Assays on Demand; Applied Biosystems, Foster City, CA). The samples were multiplexed with a eukaryotic 18S rRNA endogenous control probe (Applied Biosystems, Foster City, CA) and subjected to the following cycle parameters: in TaqMan method- 50°C for 2 min, 1 cycle; 95°C for 10 min, 1 cycle; 95°C for 15 sec, 60°C for 1 min, 40 cycles and in SYBER green method- 95°C for 10 min, 1 cycle; 95°C for 15 sec, 60°C for 1 min, 40 cycles. Fold increase of mRNA expression was quantified by delta delta Ct calculations.
Table 2.4 List of primers and their UniGene ID used in this thesis

<table>
<thead>
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<th>Gene Name</th>
<th>UniGene ID</th>
<th>Assay ID</th>
<th>Assay Type</th>
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<tbody>
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<td>Hs01555410_m1</td>
<td>TaqMan</td>
</tr>
<tr>
<td>IL-6</td>
<td>Hs.654458</td>
<td>Hs00174131_m1</td>
<td>TaqMan</td>
</tr>
<tr>
<td>IL-8</td>
<td>Hs.624</td>
<td>Hs00174103_m1</td>
<td>TaqMan</td>
</tr>
<tr>
<td>NLRP3</td>
<td>Hs.159483</td>
<td>Hs00918082_m1</td>
<td>TaqMan</td>
</tr>
<tr>
<td>Caspase-1</td>
<td>Hs.2490</td>
<td>PPH00105B</td>
<td>SYBER Green</td>
</tr>
<tr>
<td>18S</td>
<td>Not known</td>
<td>PPH0566E</td>
<td>SYBER Green</td>
</tr>
<tr>
<td>COX-2</td>
<td>Hs.196384</td>
<td>Hs0015133_m1</td>
<td>TaqMan</td>
</tr>
<tr>
<td>MKP-1</td>
<td>Hs.171695</td>
<td>Hs00610256_g1</td>
<td>TaqMan</td>
</tr>
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<td>18S</td>
<td>HSRRN18S</td>
<td>Hs03003631_g1</td>
<td>TaqMan</td>
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</table>

2.2.4 Enzyme-linked immunosorbent assay (ELISA)

For all ELISAs conducted during this study a protocol adapted from BD Biosciences protocol: Human IL-6/ IL-8 ELISA was used and capture antibody, detection antibody, protein standards and streptavidin-HRP were all provided by BD Bioscience Systems Human IL-6/ IL-8 ELISA Set (BD Bioscience, Franklin Lakes, NJ).

To perform an ELISA a 96-well ELISA plate (NUNC Maxisorp Immuno Plate F96; Noble Park North, VIC, Australia) was coated with a capture antibody diluted in coating buffer (0.1 M sodium carbonate, pH 9.5) specific for the target protein. For all recommended antibody dilutions, the manufacturer’s lot-specific Instruction/Analysis Certificate was consulted. The plate was sealed and incubated overnight at 4°C. The wells were washed 3 times with wash buffer (PBS with 0.05% Tween-20) to wash off possible unbound capture antibody. Subsequently the plate was blocked with assay diluent (PBS with 10% FBS, pH 7.0) and incubated for 1 h at RT to block all unbound sites and therefore to prevent false
positive results. After 3 other wash steps the standards and cell supernatant dilutions (prepared in 0.1% BSA in DMEM) were added as set out in the experimental protocol, the plate was sealed and incubated for 2 h at RT. During this incubation the protein of interest binds to the capture antibody. Following 5 other wash steps a biotinylated detection antibody together with a streptavidin-horseradish peroxidase conjugates (SAv- HRP) in assay diluent was added, in one-step incubation, to the wells. The plate was again sealed and incubated for 1 h at RT. Subsequent 7 wash steps were performed to wash off unbound Biotin/Streptavidin reagent and the plate was incubated with the substrate solution (TMB 2-Component Microwell peroxidase substrate system; KPL, Inc., Gaithersburg, MD) for 30 min at room temperature in the dark. During this incubation the enzyme streptavidin-horse radish peroxydase (HRP), bound to the detection antibody, converts the added substrate in to a coloured product and the colour is proportional to the amount of bound protein. To stop this process an acidic stop solution was added to the wells and the colour was measured using a spectrometer at 450 nm wave length (Microplate Reader, Model 680, Bio-rad, Hercules, CA).

2.2.5 cAMP assay

Cell lysates preparation and measurement of intracellular cAMP concentration were performed using a specific enzyme immunoassay (EIA) kit according to the manufacturer’s instruction (Cayman Chemical Co. Ann Arbor, Michigan, USA). In brief, reactions were terminated and cells were lysed by the addition of 0.1 M HCL. The contents were then cleared of debris by centrifugation at 1000 x g for 10 min. The aliquots were diluted in EIA buffer and incubated with reconstituted cAMP monoclonal antibody and cAMP AChE tracer in 96-well plates for 18 h at 4°C. After washing with wash buffer the plates were incubated with reconstituted Ellman’s reagent
for 1 h with gentle shaking in dark. The absorbance was measured at 415 nm and quantities of cAMP were determined using manufacturer’s analysis tools (excel workbooks) comparing with cAMP standards concentrations ranging from 750 – 0.3 pmol/mL. The results were represented as pmol cAMP/mL.

### 2.2.6 PGE$_2$ assay

The levels of PGE$_2$ in culture supernatants were measured using enzyme immunoassay (EIA) kits according to the manufacturer’s instruction (Cayman Chemical Company, Ann Arbor, Michigan, USA). Briefly, different dilutions (straight, 1/5, 1/10, 1/50, 1/100) of each sample and standards, concentration range 7.8-1000 pg/mL were incubated with reconstituted PGE$_2$ monoclonal antibody and PGE$_2$ AChE tracer for 18 h at 4°C. After 5 times washing with wash buffer the plate (96-well plate supplied with EIA kits) was incubated with immediate reconstituted Ellman’s reagent (200 μL/well) for 1 h with gentle shaking in dark. Absorbance was taken at 415 nm and results were calculated using manufacturer’s analysis tools (excel workbooks). The results were represented as pg PGE$_2$/mL supernatant.

### 2.2.7 Statistical analysis

Statistical analysis was performed using either the Student's unpaired t test, one-way ANOVA or two-way ANOVA followed by Bonferroni’s post-test. P values <0.05 were sufficient to reject the null hypothesis for all analyses.
Chapter 3

NLRP3 inflammasome is not activated in ASM upon TLR2 ligation

Chapter 3
NLRP3 inflammasome is not activated in ASM upon TLR2 ligation

3.1 Introduction
Inflammasomes represent a multiprotein high molecular weight complex that control innate immunity and inflammation. Several evidences support the key role of inflammasome activation in lung diseases such as asthma and COPD by regulating the processing and release of proinflammatory cytokines (predominantly IL-1β) (Birrell and Eltom, 2011; dos Santos et al., 2012). The NLRP3 inflammasome has been best characterized, and both in vivo and in vitro models of allergic asthma and asthmatic inflammation have demonstrated an important role for the NLRP3 inflammasome in the amplification and potentiation of inflammation during airway diseases (Ather et al., 2011; Besnard et al., 2011; Bauer et al., 2012; Im and Ammit, 2014).

Elevated IL-1β protein secretion that is stimulated via active inflammasome components was suggested to play a role in asthma exacerbation (Bochkov et al., 2010; Bauer et al., 2012), we were interested in exploring whether the NLRP3 inflammasome is activated under in vitro conditions in ASM cells that mimic infectious exacerbations in asthma.

The NLRP3 inflammasome is a multimeric intracellular protein complex consisting of three domains: NLRP3, the apoptosis associated speck-like protein containing the caspase activation and recruitment domain, and caspase-1 (Cassel et al., 2009). NLRP3 expression is inducible, and mRNA regulation can take place at the transcriptional and posttranscriptional levels (Bauernfeind et al., 2009; Qiao et al., 2012). Recognition of invading pathogens by the NLRP3 domain results in the assembly of three components to form a stable inflammasome, which in
turn activates pro-caspase-1 into active caspase-1 autocatalytically (Birrell and Eltom, 2011). Caspase-1 is a cysteine protease that cleaves inactive precursors of IL-1β into bioactive cytokine, which is then competent to be secreted out of the cell.

During the past decade, the ability of ASM cells to produce and secrete proinflammatory mediators, including cytokines and chemokines, has been established by many studies, as reviewed elsewhere (Damera et al., 2009; Ozier et al., 2011). Furthermore, accumulating evidence has shown that the treatment of ASM cells with IL-1β in vitro augments the production of numerous proinflammatory mediators, including IL-6, IL-8 (Hedges et al., 2000), IL-17A (Henness et al., 2004), PGE₂ (Laporte et al., 1998), and matrix metalloproteinase–9 (Liang et al., 2007), thus serving to amplify airway inflammation. The enhanced synthetic function of ASM may play a pivotal role, leading to acute exacerbations of asthma, by provoking and amplifying airway inflammatory responses (Damera and Panettieri, 2011; Manetsch et al., 2012b). The possible involvement of the NLRP3 inflammasome and IL-1β activation in the enhanced synthetic function of ASM cells in response to invading pathogens remains largely unexplored.

When human body is infected by bacteria or virus, cells of the innate immune system recognize PAMPs and the activation of TLR2 become crucial for the induction of immune response against the pathogen or for an effective presentation of antigens to the adaptive system. TLR2 receptors are mainly responsible for the recognition of various bacterial cell wall components (Chaudhuri et al., 2005). In this study, we aim to show the engagement of TLR2 with the TLR2 agonist Pam3CSK4 exerted any effect on NLRP3 expression in ASM cells in vitro. Pam3CSK4 is a well-recognised synthetic lipopeptide (LP) that mimics bacterial lipoproteins and therefore activates TLR2 (Aliprantis et al., 1999). We explored the role of the inflammasome and the secretion of IL-1β from ASM cells in response to Pam3CSK4 activation and also measured the
downstream IL-6 and IL-8 cytokines secretion. We found that the cytokines are upregulated upon TLR2 ligation though this upregulation of cytokines is IL-1β-independent. Further we found that the engagement of TLR2 with the TLR2 agonist Pam3CSK4 exerted no effect on NLRP3 and caspase-1 expression. These results suggest that because of the absence of key components, NLRP3 inflammasome activation will not be possible, and consequently IL-1β will not be processed for secretion. Our data support this assertion, because we show that although TLR2 ligation increased TNFα-induced IL-1β mRNA expression, IL-1β protein is not secreted from ASM cells. Thus, the NLRP3 inflammasome is not activated in ASM upon TLR2 ligation.

3.2 Materials and methods

3.2.1 Cell culture

For detailed description, see Chapter 2.2.1.

Unless otherwise specified, all chemicals used in this study were purchased from Sigma-Aldrich (St.Louis, MO).

3.2.2 Real-Time RT-PCR

Total RNA was extracted using the RNeasyMini Kit (Qiagen, Doncaster, Victoria, Australia), and reverse-transcribed using the RevertAid First Strand cDNA Synthesis Kit (Fermentas Life Sciences, Hanover, MD). Real-time RT-PCR was performed on an ABI Prism 7500 (Applied Biosystems, Foster City, CA) with IL-1β (TaqMan ID: Hs01555410_m1), IL-6 (Hs00174131_m1), IL-8 (Hs00174103_m1), NLRP3 (Hs00918082_m1), and 18S ribosomal RNA measured with TaqMan probes (Applied Biosystems) or caspase-1 (PPH00105B) and 18S (PPH0566E) measured by SYBR Green RT² qPCR Primer Assays (Qiagen), thermal cycle- 95°C
for 10 min, 1 cycle; 95°C for 15 sec, 60°C for 1 min, 40 cycles. For detailed description, see Chapter 2.2.3.

3.2.3 ELISAs

IL-1β (DuoSet DY201; R&D Systems, Minneapolis, MN), IL-6 (BD OptEIA 555220; BD Biosciences, San Diego, CA), and IL-8 (BD OptEIA 555244; BD Biosciences) ELISAs were performed according to the manufacturers’ instructions. For detailed description, see Chapter 2.2.4.

3.2.4 IL-1β neutralization

To neutralize IL-1β, conditioned media from treated ASM cells or IL-1β recombinant protein (R&D Systems) were incubated for 1 h at 37°C with 2 mg/mL of either vehicle, normal mouse IgG1 (Santa Cruz Biotechnology, Santa Cruz, CA) as an isotype control, or a monoclonal mouse IgG1 antibody to human recombinant IL-1β (clone 8516; R&D Systems).

3.2.5 Statistical analysis

Statistical analysis was performed using either the Student’s unpaired t test or two-way ANOVA, followed by the Bonferroni post hoc test. P<0.05 was considered sufficient to reject the null hypothesis for all analyses. Data represent means ± SEMs.
3.3 Results

3.3.1 Time course of TNFα-induced IL-1β mRNA expression and augmentation by Pam3CSK4

We measured the temporal kinetics of TNFα-induced IL-1β mRNA expression in ASM cells, and examined whether the TLR2 agonist, Pam3CSK4, up-regulated IL-1β concentrations. As shown in Figure 3.1A, TNFα significantly enhanced IL-1β mRNA expression over time, with significant expression observed as early as 1 h, and a peak of 90.4- ± 18.4-fold at 2 h, before subsiding to concentrations that were not significantly different from those of vehicle controls by 8 h (P<0.05). Pam3CSK4 alone also significantly enhanced IL-1β mRNA expression, with a significant 14.8- ± 8.4-fold increase observed at 4 h (P<0.05). Importantly, TNFα-induced IL-1β upregulation was significantly potentiated by Pam3CSK4 (P<0.05). As shown in Figure 3.1A, the effect of Pam3CSK4 on TNFα-induced IL-1β mRNA was first observed at 2 h (although not significantly), and this effect increased to significant levels at 4 h, and by 24 h was measured at 337.0- ± 48.0-fold, compared with 23.0- ± 14.9-fold using TNFα alone (P<0.05). Interestingly, when we compared the temporal kinetics of IL-1β mRNA expression with those of IL-6 (Figure 3.1B) and IL-8 (Figure 3.1C), we also observed a later significant upregulation of TNFα-induced cytokine secretion at 24 h (P<0.05). This raises the intriguing possibility that the early phase of IL-1β mRNA may undergo translation, to result in IL-1β protein secretion that can then act back on ASM cells in an autocrine manner to stimulate the cytokine mRNA expression observed at 24 h. Thus, we hypothesize that the TLR2 agonist, Pam3CSK4, activates the inflammasome to result in IL-1β secretion from ASM cells and the potentiation of cytokine secretion from ASM cells.
Figure 3.1 Time course of TNFα-induced IL-1β mRNA expression and augmentation by Pam3CSK4 (in comparison to IL-6 and IL-8 mRNA temporal kinetics). Growth-arrested ASM cells were pretreated with vehicle or Pam3CSK4 (1 μg/mL) for 1 h, followed by treatment with vehicle or TNFα (10 ng/mL) for 0, 1, 2, 4, 8, and 24 h. (A) IL-1β, (B) IL-6 and (C) IL-8 mRNA expression was quantified by real-time RT-PCR and results expressed as fold increase compared to vehicle-treated cells at 0 h (mean±SEM values from n=7 primary ASM cell cultures). Statistical analysis was performed using two-way ANOVA then Bonferroni’s post hoc test (where * denotes a significant effect of treatment on mRNA expression, compared to vehicle-treated cells, and § indicates a significant effect of Pam3CSK4 on TNFα-induced mRNA expression at the same time point (P<0.05)).
3.3.2 Pam3CSK4 potentiates TNFα-induced IL-6 and IL-8 protein secretion, but IL-1β is not secreted from ASM cells

To address this hypothesis, we measured concentrations of IL-1β, IL-6, and IL-8, as secreted from ASM cells over 0 to 24 h. As shown in Figures 3.2B and 3.2C, respectively, Pam3CSK4 pretreatment significantly enhanced TNFα-induced IL-6 and IL-8 at 24 h (Figures 3.2A and 3.2B; P<0.05), in confirmation of our earlier report (Manetsch et al., 2012b). We then attempted to measure IL-1β in the same supernatants, using an ELISA that had been confirmed able to measure secreted IL-1β that resulted from inflammasome activation (Rajan et al., 2010). Importantly, IL-1β was not detected (Figure 3.2A), suggesting that even though IL-1β mRNA was up-regulated, the inflammasome was not activated and IL-1β was not secreted from ASM cells.
Figure 3.2 Pam3CSK4 potentiates TNFα-induced IL-6 and IL-8 protein secretion, but IL-1β is not secreted from ASM cells. Growth-arrested ASM cells were pretreated with vehicle or Pam3CSK4 (1 μg/mL) for 1 h, followed by treatment with vehicle or TNFα (10 ng/ml) for 0, 1, 2, 4, 8, and 24 h. ELISA were used to measure (A) IL-1β and (B) IL-8 and (C) IL-6 protein secretion. Please note that IL-1β secretion from ASM cells (A) was not detected. Statistical analysis was performed using two-way ANOVA then Bonferroni’s post-test (where * denotes a significant effect of treatment on protein secretion, compared to vehicle-treated cells, and § indicates a significant effect of Pam3CSK4 on TNFα-induced protein secretion at the same time point (P<0.05)). Data represent mean±SEM values from n=4 primary ASM cell cultures.
3.3.3 Effect of IL-1β neutralizing antibody on IL-6 and IL-8 protein secretion induced by IL-1β recombinant protein

To confirm that IL-1β was not secreted from ASM cells, we used an IL-1β–neutralizing antibody. We initially validated IL-1β neutralization by incubating a range of concentrations of IL-1β recombinant protein (1–500 pg/mL) with IL-1β–neutralizing antibody, and measured its inhibitory effect on IL-1β–induced IL-6 and IL-8 protein secretion. As shown in Figure 3.3, IL-1β induced a concentration-dependent augmentation of IL-6 and IL-8 protein secretion in vehicle-treated or isotype control–treated ASM cells, with no significant difference between cells treated with vehicle or the isotype control. Importantly, the neutralization of IL-1β reduced IL-6 protein secretion from ASM cells in response to a range of IL-1β concentrations (1–500 pg/mL; Figure 3.3A), with only 79.5 ± 39.8 pg/mL IL-6 secreted in response to 500 ng/mL IL-1β preincubated with the IL-1β neutralizing antibody, as opposed to approximately 150-fold greater amounts of IL-6 secretion after preincubation with the isotype control (P<0.05). Similarly, this down-regulation by IL-1β neutralization was also observed in IL-8 protein secretion, as depicted in Figure 3.3B.
Figure 3.3 Effect of IL-1β neutralizing antibody on IL-6 and IL-8 protein secretion induced by IL-1β recombinant protein. To validate the effect of neutralization antibody on IL-6 (A) and IL-8 (B) protein secretion, vehicle or increasing concentrations of IL-1β recombinant protein (1-500 pg/mL) were incubated for 1 h at 37°C with 2 μg/mL of either vehicle, isotype control or IL-1β neutralizing antibody, and then added to growth-arrested ASM cells for 24 h. IL-6 and IL-8 protein secretion was measured by ELISA. Statistical analysis was performed using two-way ANOVA then Bonferroni’s post-test (where * denotes a significant effect of IL-1β neutralizing antibody on protein secretion, compared with isotype control (P<0.05)). Data represent mean±SEM values from n=3 primary ASM cell cultures.
3.3.4 Neutralization of IL-1β in conditioned media exerts no effect on cytokine secretion

We next examined whether the neutralization of IL-1β in conditioned media exerted any effect on IL-6 and IL-8 protein secretion by ASM cells. As shown in Figure 3.4A, the removal of IL-1β by the neutralizing antibody exerted no effect on the amount of IL-6 secreted in response to conditioned media from cells stimulated with TNFα alone or Pam3CSK4 + TNFα. Similarly, IL-8 secretion in response to conditioned media preincubated with IL-1β neutralizing antibody was not significantly different from that preincubated with the isotype control (Figure 3.4B). As a positive control, we confirmed IL-1β neutralization with ASM cells treated with 100 ng/mL IL-1β recombinant proteins in parallel experiments. As shown in Figures 3.4C and 3.4D, IL-1β neutralization significantly inhibited IL-6 and IL-8 secretion from ASM cells, compared with the isotype control (P<0.05).
Figure 3.4 Neutralization of IL-1β in conditioned media has no effect on cytokine secretion. Conditioned media was prepared by removing supernatants from ASM cells treated with TNFα or Pam3CSK4 + TNFα for 1, 2, 4, 8, and 24 h. Conditioned media was incubated at 37°C for 1 h with 2 μg/mL of either IL-1β neutralizing antibody or isotype control then incubated with ASM cells for 24 h and the resultant (A) IL-6 and (B) IL-8 protein in the supernatants measured by ELISA. Results were expressed as a percentage of TNFα – isotype control at 1 h. To confirm IL-1β neutralization, parallel studies were conducted with vehicle or IL-1β recombinant protein (100 pg/mL) treated under identical conditions and (C) IL-6 and (D) IL-8 measured by ELISA. Statistical analysis was performed using the Student's unpaired t test (where * denotes a significant effect of IL-1β neutralizing antibody on protein secretion, compared with isotype control). Data represent mean±SEM values from n=3 primary ASM cell cultures.
3.3.5 Pam3CSK4 does not affect TNFα-induced NLRP3 and caspase-1 mRNA expression in ASM cells

NLRP3 is an important component of the inflammasome and has been shown to be upregulated under inflammatory conditions. Caspase-1 also plays an important role in inflammasome function to cleave precursor pro-IL-1β into mature IL-1β. Collectively, our data thus far demonstrates that Pam3CSK4-mediated augmentation of TNFα-induced IL-6 and IL-8 mRNA and protein level is not mediated by IL-1β secretion, thus, these results indicate that components of the NLRP3 inflammasome may not be upregulated in ASM cells upon TLR2 activation. To address this we measured NLRP3 and caspase-1 mRNA expression in response to Pam3CSK4 and TNFα, alone and in combination, over time (0-24 h). As shown in Figure 3.5A, neither Pam3CSK4, nor TNFα had a significant effect on NLRP3 mRNA expression over 24 h. Moreover, the temporal kinetics of NLRP3 mRNA expression is not affected in response to Pam3CSK4 + TNFα in combination. We also measured the temporal kinetics of caspase-1 mRNA expression in response to Pam3CSK4 + TNFα, alone and in combination (Figure 3.5B) and found TNFα induced a small (~3-fold) but significant effect on caspase-1 mRNA expression at 8 h (P<0.05) but Pam3CSK4 did not upregulate TNFα-induced caspase-1 mRNA expression. Taken together, these results suggest that because NLRP3 and caspase-1 are not present in ASM cells, the NLRP3 inflammasome is not activated upon TLR2 ligation; thus IL-1β is not secreted from ASM cells.
Figure 3.5 Pam3CSK4 does not affect TNFα-induced NLRP3 and caspase-1 mRNA expression in ASM cells. (A-B) Growth-arrested ASM cells were pretreated with vehicle or Pam3CSK4 (1 μg/ml) for 1 h, followed by treatment with vehicle or TNFα (10 ng/mL) for 0, 1, 2, 4, 8, and 24 h. (A) NLRP3 and (B) caspase-1 mRNA expression was quantified by real-time RT-PCR and results expressed as fold increase compared to vehicle-treated cells at 0 h (mean±SEM values from (A) n=6 or (B) n=4 primary ASM cell cultures). Statistical analysis was performed using two-way ANOVA then Bonferroni's post-test (where * denotes a significant effect of treatment on caspase-1 mRNA expression, compared to vehicle-treated cells (P<0.05)).
3.4 Discussion

The inflammasomes are a group of protein complexes thought to be responsible for inflammation associated respiratory diseases including asthma. Inflammasome-mediated activation of caspase-1 is crucial for maturation and secretion of IL-1β, which plays the pivotal role in inflammation associated asthma. However, the underlying molecular mechanism is yet to be revealed. In our present study, we address this issue. In this study, we uncover the molecular pathway/s underlying TLR2-induced activation of the inflammasome and investigate if, and how, the inflammasome induces cytokine secretion in airway smooth muscle cells in vitro. Our data demonstrate that the Pam3CSK4 mediated augmentation of TNFα-induced IL-6 and IL-8 mRNA and protein concentrations are not mediated by IL-1β secretion via NLRP3 inflammasome activation in ASM cells. NLRP3 and caspase-1 are not expressed in ASM cells in vitro. Thus, the NLRP3 inflammasome is not activated in ASM upon TLR2 ligation.

TLRs are receptors that are known to bind PAMPs found in bacteria, viruses, fungi and other microbes and play an important role in the innate immune response. Respiratory infections are key factors underlying asthma exacerbations, moreover it has been shown that ASM cells express TLR2, 3 and 4, activated by bacterial cell wall components and viruses (Sukkar et al., 2006). By propagating and amplifying inflammation in response to bacterial and viral pathogens, airway structural cells play an important immunomodulatory role in asthma. Although Pam3CSK4 is primarily known as a TLR2 agonist, its ability to activate the NLRP3 inflammasome to release IL-1β has been demonstrated in macrophages (Kanneganti et al., 2006). This led us to examine the effects of IL-1β neutralization on Pam3CSK4 and TNFα-induced cytokine upregulation in ASM cells.
Recent *in vivo* and *in vitro* studies have implicated the NLRP3 inflammasome as a potential therapeutic target in asthma (Ather et al., 2011; Besnard et al., 2011; Birrell and Eltom, 2011; dos Santos et al., 2012), although debate continues (Kool et al., 2011; Allen et al., 2012). To date, several reports demonstrated the presence of the functional NLRP3 inflammasome in asthmatic airway epithelia (Allen et al., 2009; Hirota et al., 2012; Tran et al., 2012). The NLRP3 inflammasome mediated production of IL-1β in airway epithelial cells leads to airway neutrophilic inflammation (Hirota et al., 2012), which is the predominant phenotype of airway inflammation in acute exacerbations (ten Brinke et al., 2004; Cowan et al., 2010). Allen *et al.* demonstrated that the NLRP3 inflammasome expressed in airway epithelial cells and macrophages is required for inflammation after influenza infection (Allen et al., 2009). Thus, a growing body of research suggests that the NLRP3 inflammasome may be activated in the airways to promote increased cytokine expression and a proinflammatory milieu during infections.

Increasing evidence demonstrates that ASM cells play a role in the amplification of inflammatory responses during infectious exacerbations in asthma via augmented cytokine production (Elias et al., 1997; Morris et al., 2006; Oliver et al., 2006; Sukkar et al., 2006; Manetsch et al., 2012b). Our study is the first to examine the involvement of the NLRP3 inflammasome and possible IL-1β secretion in Pam3CSK4 and TNFα-induced cytokine upregulation in ASM cells. We first demonstrated that IL-1β induces a significant increase in IL-6 and IL-8 protein secretion by ASM cells in a concentration dependent manner. We also demonstrated that the IL-1β-induced upregulation of IL-6 and IL-8 secretion is completely inhibited by IL-1β neutralizing antibody. These results show that the IL-1β neutralizing antibody can successfully neutralize the effects of IL-1β on cytokine upregulation in ASM cells. However,
the Pam3CSK4-induced upregulation of IL-6 and IL-8 protein secretion was not inhibited by the neutralization of IL-1β. This finding suggests that IL-1β secretion is not involved in TLR2 ligand-induced cytokine upregulation in ASM cells.

In support of this, we also demonstrated that ASM cells treated with Pam3CSK4 and TNFα do not secrete detectable concentrations of IL-1β protein over 24 h, although gene expression is significantly augmented. For the NLRP3 inflammasome in ASM cells to be active in a classic manner, the production and secretion of mature IL-1β should be observed in response to Pam3CSK4 (Martinon et al., 2009), because IL-1β secretion is widely accepted as a surrogate marker for NLRP3 inflammasome activation. However, TLR2 ligation by Pam3CSK4 appears unable to induce assembly of the NLRP3 inflammasome and the processing of IL-1β secretion. Together, our results provide evidence that the NLRP3 inflammasome is not activated to promote IL-1β maturation and secretion in response to Pam3CSK4 and TNFα in ASM cells. This rules out the possibility that IL-1β secreted as a result of Pam3CSK4 enhanced, TNFα-induced IL-1β mRNA expression observed at an early time point (4 h) acts in an autocrine manner to boost the further augmentation of IL-6, IL-8, and IL-1β gene expression at later time points (24 h) via a positive feedback loop.

The results presented here indicate important cell-type specificity in airway inflammation, because we demonstrate that the robust up-regulation of proinflammatory cytokines by invading pathogens may not be mediated by activation of the NLRP3 inflammasome and IL-1β secretion in ASM. Using primary cultures of human ASM cells, we demonstrated that Pam3CSK4 robustly up-regulates TNFα-induced IL-1β mRNA expression, but does not significantly change the expression of NLRP3 and caspase-1 mRNA. Importantly, IL-1β is not secreted from ASM cells. Thus IL-1β is unable to act in an autocrine manner to induce an inflammatory milieu that
mimics exacerbation in ASM cells by promoting IL-6 secretion and upregulation of the neutrophil chemoattractant chemokine, IL-8.

We explored the role of the NLRP3 inflammasome and the secretion of IL-1β from ASM cells in response to Pam3CSK4 activation, and although TLR2 ligation robustly enhanced cytokine expression, this upregulation was IL-1β independent. Exacerbated asthmatic symptoms are not effectively attenuated by current pharmacological management. By addressing molecular mechanisms of exacerbation, our experiments have revealed a potentially important cell type difference, that is, whereas the NLRP3 inflammasome is activated in human airway epithelium (Hirota et al., 2012), the TLR2-mediated enhancement of proinflammatory cytokine secretion in the underlying ASM layer is NLRP3 inflammasome independent.

In our published paper, there are some more supporting data (not shown here as those are done by our collaborator in other lab) generated by using in situ model of human tracheas, which are align with our in vitro data and further strengthen our hypothesis (Hirota et al., 2013). Therefore, we believe that our findings in both in vitro and in situ models have clinical significance and reinforce future investigations using contemporary experimental models. Although the functional relevance of the restricted localization of the NLRP3 inflammasome in the airways is unclear at present, crosstalk between airway structural cells (and inflammatory cells) remains a possibility. Moreover, in our study we have used only one stimulus that may be not sufficient to stimulate active IL-1β, as such in many cells a secondary stimulus is needed to stimulate the activation step. And also, we cannot rule out that other inflammasomes may be playing a role given that caspase-1 mRNA was increased in our setting. Thus, further studies are warranted to develop new therapeutic strategies to combat asthma exacerbations in the future.
Chapter 4

TLR2 activation causes desensitization of β2-adrenoreceptor via COX-2 production

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Chapter 4
TLR2 activation causes desensitization of $\beta_2$-adrenoreceptor via COX-2 production

4.1 Introduction

Both viruses and bacteria are considered as the most important factors contributing asthma exacerbation. Viruses are identified in 80% of asthma exacerbations in children and 45–80% in adults (Hayden, 2004). Rhinovirus, which causes the majority of common colds, is responsible for at least half of all asthma exacerbations (Corne et al., 2002). Bacterial infections are increasingly recognized as playing an important role in asthma exacerbations [reviewed in (Edwards et al., 2012)], although they have been less well investigated to date. Moreover the expression of TLR2 receptor in ASM cells that is activated by bacterial cell wall components such as LPS and lipopeptides strongly suggests their involvement in the amplification of airway inflammatory responses during infectious exacerbation.

When a person with asthma suffers from a viral or bacterial infection, the molecular pathways by which asthma medicines act are changed making them less sensitive. As bronchodilators, $\beta_2$-agonists are considered the first line therapy of asthma that relax the muscles surrounding the airways and opens the airways that become tighten during asthma exacerbation. There are two types of $\beta_2$-agonists available, short-acting $\beta_2$-agonists are often referred to as rescue inhalers and are used to quickly relieve of asthma symptoms and long acting $\beta_2$-agonists taken regularly to control chronic symptoms and prevent asthma attacks. In well-controlled asthma, $\beta_2$-agonists
relax the airways by interacting with β2-AR. β2-AR are one of the most well characterized seven transmembrane receptor belongs to GPCRs family, consist of three extracellular loops, with one amino-terminus, and three intracellular loops, with a carboxy-terminus [reviewed in (Shore and Moore, 2003)]. Binding of a β2-agonist to the β2-AR ultimately relax the airways by rapidly increasing cAMP levels in ASM cells.

However, these molecular pathways are changed by infection and mounting evidences suggest that β2-adrenergic relaxant mechanisms may be dysfunctional in airways (Pang et al., 1998b; Shore and Moore, 2003). Recent studies have revealed that viral infection desensitizes the β2-AR and reduces cAMP generation in response to β2-agonists (Trian et al., 2010). These changes are due to interaction of viral products with specific TLRs, resulting in COX-2-mediated prostaglandin production (including PGE2) and repression of β2-AR activity (Van Ly et al., 2013). To date, however, whether bacterial infection directly affects β2-AR function in a similar, TLR/COX-2-dependent manner has not been investigated. This is the aim of the current study.

Previous reports support that in human ASM cells β2-AR receptor desensitization can be either homologous or heterologous [reviewed in (Shore and Moore, 2003)]. In our present study we attempt to determine whether TLR2 agonist Pam3CSK4, mimic bacterial infection, affects β2-AR function in a similar manner as viral infection heterologously via COX-2 production. In parallel study we also showed the homologous desensitization of β2-AR by short acting and long acting β2-agonists, salbutamol and formoterol respectively.

In addition to bronchodilatory actions in the airways, β2-agonists also have anti-inflammatory effects via cAMP-dependent upregulation of immunomodulatory molecules. One such molecule is the MAPK-deactivating protein MKP-1 (Manetsch et al., 2012a; Manetsch et al., 2013). MKP-1 expression is mediated via β2-AR/cAMP-dependent mechanisms (Manetsch et al., 2012a);
thus, we propose that upregulation of this critical anti-inflammatory molecule by β2-agonists would be severely curtailed in bacterial infection. Therefore in this study we demonstrated the impact of TLR2 ligand associated β2-AR desensitization by impaired cAMP production as well as reduced gene expression of the critical anti-inflammatory molecule MKP-1 in response to β2-agonists.

4.2 Materials and methods

4.2.1 Cell culture

For detailed description, see Chapter 2.2.1.

4.2.2 Chemicals

Pam3CSK4 was purchased from InVivoGen (San Diego, CA, USA), tumour necrosis factor α (TNFα) from R&D Systems (Minneapolis, MN, USA) and celecoxib and PGE2 from Cayman Chemical Company (Ann Arbor, MI, USA). Unless otherwise specified, all chemicals used in this study were purchased from Sigma-Aldrich (St.Louis, MO).

4.2.3 Real-time RT-PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) and reverse transcribed using the RevertAid First strand cDNA Synthesis Kit (Fermentas Life Sciences). Real-time RT-PCR was performed on an ABI Prism 7500 with COX-2 (Hs0015133_m1) and MKP-1 (DUSP1: Hs00610256_g1) TaqMan® Gene Expression Assays and the eukaryotic 18S rRNA endogenous control probe (Applied Biosystems) subjected to the following cycle parameters: 50°C for 2 min, 1 cycle; 95°C for 10 min, 1 cycle; 95°C for 15 s, 60°C for 1 min, 40 cycles and mRNA
expression (fold increase) quantified by delta delta Ct calculations. For detailed description, see Chapter 2.2.3.

4.2.4 cAMP and PGE$_2$ assay

Measured by enzyme immunoassay according to the manufacturers’ instructions (Prostaglandin E$_2$ EIA 514010 and cAMP EIA 58100: Cayman). For detailed description, see Chapter 2.2.5 and 2.2.6.

4.2.5 Statistical analysis

Statistical analysis was performed using either the Student’s unpaired t test or one or two-way ANOVA, followed by the Bonferroni post hoc test. P<0.05 was considered sufficient to reject the null hypothesis for all analyses. Data represent means ± SEMs.

4.3 Results

4.3.1 TLR2 ligand engagement upregulates TNFα-induced COX-2 mRNA expression and increases PGE$_2$ secretion

Our previous studies have modelled rhinovirus-induced asthma exacerbation in vitro in ASM cells to reveal the molecular events responsible for β$_2$-AR desensitization (Trian et al., 2010; Trian et al., 2011; Van Ly et al., 2013). To date however, whether bacterial infection directly affects β$_2$-AR function had not been explored. We addressed this by modelling bacterial exacerbation in vitro. ASM cells were treated with the synthetic bacterial lipoprotein Pam3CSK4 to mimic bacterial infection via TLR2 activation and with TNFα to simulate inflammation. We then measured the temporal kinetics of COX-2 mRNA expression and PGE$_2$ secretion.
Upregulation of COX-2 mRNA expression by TNFα was sustained in the presence of Pam3CSK4, with TLR2 activation significantly increasing TNFα-induced COX-2 expression at 24 h (Figure 4.1A: P<0.05). Secretion of the COX-2 product PGE₂ increased in a similar manner (Figure 4.1B); TLR2 ligand engagement by Pam3CSK4 significantly upregulated TNFα-induced PGE₂ secretion at 24 h (P<0.05).
Figure 4.1 TLR2 ligand engagement upregulates TNFα-induced COX-2 mRNA expression and increases PGE2 secretion. Growth-arrested ASM cells were pretreated with vehicle or Pam3CSK4 (1 μg/mL) for 1 h, followed by treatment with vehicle or TNFα (10 ng/mL) for 0, 1, 2, 4, 8, and 24 h. (A) COX-2 mRNA expression was quantified by real-time RT-PCR (results expressed as fold increase compared to vehicle-treated cells at 0 h) and (B) PGE2 secretion was measured by EIA. Statistical analysis was performed using two-way ANOVA then Bonferroni's post-test (where * denotes a significant effect of Pam3CSK4 on TNFα-induced COX-2 mRNA expression and PGE2 secretion (P<0.05)). Data are mean±SEM values from (A) n=7 and (B) n=4 primary ASM cell cultures.
4.3.2 PGE₂ induces heterologous β₂-AR desensitization as measured by inhibition of β₂-agonist-induced cAMP production

Because COX-2 products, such as PGE₂, are responsible for rhinovirus-induced β₂-AR desensitization (Van Ly et al., 2013), our results suggest that bacteria and viruses may induce a common molecular mechanism to downregulate β₂-AR on ASM cells in a heterologous manner. To confirm this, we pretreated ASM cells for 24 h with a range of concentrations of PGE₂ (0.1–100 nM) and measured cAMP production in response to two β₂-agonists: short-acting β₂-agonist salbutamol (Figure 4.2A) and long-acting β₂-agonist formoterol (Figure 4.2B). These experiments were performed in parallel with control experiments to demonstrate homologous β₂-AR desensitization by β₂-agonists. In confirmation of earlier reports (Shore and Moore, 2003), salbutamol induced a 5-fold increase in cAMP production that was sensitive to homologous β₂-AR desensitization (Figure 4.2A: P<0.05). Notably, pretreatment with PGE₂ induced heterologous β₂-AR desensitization in a concentration-dependent manner (P<0.05) (5, 12). Similarly, formoterol or PGE₂ also significantly repressed cAMP generation in response to formoterol (Figure 4.2B: P<0.05).
Figure 4.2 PGE\(_{2}\) induces heterologous β\(_2\)-AR desensitization as measured by inhibition of β\(_2\)-agonist-induced cAMP production. Growth-arrested ASM cells were pretreated for 24 h with vehicle, salbutamol (10 µM), formoterol (0.01 µM), or a range of PGE\(_{2}\) concentrations (0.1-100 nM). Desensitization of the β\(_2\)-AR was assessed by measuring production of cAMP in response to stimulation with (A) 10 µM salbutamol or (B) 0.01 µM formoterol for 15 min, compared to vehicle, in the presence of the pan-phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX: 30 min pretreatment at 10 µM). Results are expressed as a percentage of β\(_2\)-agonist-induced cAMP. Statistical analysis was performed using one-way ANOVA then Bonferroni’s post-test (where § or * denotes a significant effect of β\(_2\)-agonists or PGE\(_{2}\) on cAMP production, respectively (P<0.05)). Data are mean±SEM values from n=5 primary ASM cell cultures.
4.3.3 Celecoxib inhibits PGE$_2$ secretion but not COX-2 mRNA expression

We are first to show that TLR2 activation also leads to β$_2$-AR desensitization in ASM cells. As this occurs in a COX-2-dependent, PGE$_2$-mediated manner we now wished to test the effectiveness of the specific COX-2 inhibitor celecoxib. Celecoxib inhibits COX-2 enzymatic activity, not expression; therefore we confirmed that celecoxib had no effect on COX-2 mRNA expression (Figure 4.3A). In contrast, celecoxib did have the expected inhibitory effect on PGE$_2$ secretion. This is shown in Figure 4.3B, where celecoxib pretreatment completely represses PGE$_2$ secretion induced by Pam3CSK4 + TNFα (Figure 4.3B: P<0.05).
Celecoxib inhibits PGE$_2$ secretion but not COX-2 mRNA expression. Growth-arrested ASM cells were pretreated with vehicle or 10 µM celecoxib for 1 h. Cells were then treated with vehicle or Pam3CSK4 (1 µg/mL) for 1 h, followed by vehicle or TNFα (10 ng/mL) for 24 h. (A) COX-2 mRNA expression was quantified by real-time RT-PCR (results expressed as fold increase compared to vehicle-treated cells) and (B) PGE$_2$ secretion was measured by EIA. Statistical analysis was performed using two-way ANOVA then Bonferroni's post-test (where * denotes a significant effect of celecoxib on PGE$_2$ secretion ($P<0.05$)). Data are mean±SEM values from n=4 primary ASM cell cultures.
4.3.4 Pam3CSK4 + TNFα cause tachyphylaxis of β2-AR agonists mediating MKP-1 gene expression and this can be reversed by celecoxib

We next examined the effects of TLR2 activation on MKP-1 responses. Notably, pretreating cells with Pam3CSK4 + TNFα resulted in a significant reduction in β2-AR agonist-induced MKP-1 gene expression (Figure 4.4A: P<0.05). Figure 4.4B represents control experiments confirming PGE2-mediated β2-AR desensitization. These data demonstrate that Pam3CSK4 + TNFα causes tachyphylaxis of β2-AR agonist-induced MKP-1 gene expression in a PGE2-dependent manner. This could be reversed by celecoxib, selective COX-2 inhibitor, to allow β2-agonist-induced MKP-1 gene expression to be reinstated (Figure 4.4C).
Figure 4.4 Pam3CSK4 + TNFα cause tachyphylaxis of β2-AR agonists mediating MKP-1 gene expression and this can be reversed by celecoxib. Growth-arrested ASM cells were pretreated for 24 h with vehicle, Pam3CSK4 (1 µg/mL) + TNFα (10 ng/mL), or PGE2 (100 nM). (A) The impact of TLR2-mediated β2-AR desensitization on anti-inflammatory actions of β2-agonists was assessed by measuring MKP-1 mRNA expression by RT-PCR in response to stimulation with 10 µM salbutamol or formoterol (0.01 µM) for 1 h, in comparison to vehicle (results expressed as fold increase compared to vehicle- or Pam3CSK4 + TNFα-treated cells). (B) Control experiments confirm PGE2-mediated β2-AR desensitization results in reduced β2-agonist-mediated MKP-1 expression, in comparison to vehicle-treated cells. (C) In parallel experiments, cells were pretreated with vehicle or 10 µM celecoxib for 1 h, and treated under identical conditions as above (A) to demonstrate that tachyphylaxis of β2-AR agonists was reversed by celecoxib allowing β2-agonist-induced MKP-1 gene expression to be reinstated. Statistical analysis was performed using one-way ANOVA then Bonferroni’s post-test (where * denotes a significant repression of β2-agonist-induced MKP-1 mRNA expression and § denotes a significant effect of celecoxib (P<0.05)). Data are mean±SEM values from n=3 primary ASM cell cultures.
4.4 Discussion

β2-AR agonist hyporesponsiveness is a hallmark feature in human asthma. The affinity of β2-agonists to bind with β2-AR is found to be much less sensitive in virus-induced asthma exacerbations (Reddel et al., 1999; Reddel et al., 2011; Rueter et al., 2012) and can be compromised by bacterial infection (Edwards et al., 2012). To date, the underlying mechanisms are not completely understood, and to ultimately improve asthma therapy, we require a greater understanding of the molecular events that occur at the cellular level that manifest clinically with reduced β2-agonist response during infection. Utilizing an in vitro model of bacterial exacerbation, we show that TLR2 activation in the presence of an inflammatory stimulus, TNFα, leads to β2-AR desensitization in ASM cells. TLR2 activation induced a significant time-dependent increase in expression of COX-2 mRNA and secretion of COX-2 product, PGE2. PGE2 induces heterologous desensitization of the β2-AR on ASM cells thereby limiting the bronchodilatory actions of β2-agonists by repressing the amount of cAMP and also curtailing the anti-inflammatory effects of β2-agonists by reducing the amount of anti-inflammatory protein, MKP-1. Consistent with our previous findings that COX-2 product, such as PGE2, is responsible for rhinovirus-induced β2-AR desensitization (Trian et al., 2010; Van Ly et al., 2013), these results show that bacteria also induce same prostanoid-dependent molecular mechanism to downregulate the β2-AR on ASM cells.

To demonstrate the impact of β2-AR desensitization, we measured the effect on two important ways in which β2-agonists induce their beneficial effects in asthma: (i) bronchorelaxation and (ii) anti-inflammatory actions. Firstly, as β2-agonists exert their bronchodilatory action by increasing the level of cAMP, we show that β2-agonist-induced cAMP level is significantly repressed when the β2-AR is heterologously desensitized by PGE2. Although Pam3CSK4 +
TNFα induced the expected increased secretion of PGE\textsubscript{2} in this setting, the measured levels released from a very small amount of lung tissue and diluted within the surrounding media were markedly lower than the concentration of exogenous PGE\textsubscript{2} shown to induce desensitization of salbutamol-mediated relaxation. Nevertheless, we propose that the local concentrations of PGE\textsubscript{2} generated within the whole lung \textit{in vivo} following infection would be sufficient to contribute to the loss of dilator responsiveness. This is supported by a previous \textit{in vivo} study in an infection model where \textit{influenza A} infection-induced sustained increases in BAL levels of PGE\textsubscript{2}, and COX2 \textit{/_/_} mice had reduced BAL inflammatory cells and cytokines compared with wild-type mice (Carey et al., 2005). Thus, we have begun to achieve a better understanding of the molecular mechanisms of infection-induced asthma exacerbations that will ultimately allow the development of new therapeutics to specifically treat infection-induced asthma exacerbations.

Because COX-2 products, including PGE\textsubscript{2}, are also responsible for \textit{rhinovirus}-induced β\textsubscript{2}-AR desensitization (Van Ly et al., 2013), our results suggest that bacteria and virus induce a common molecular mechanism responsible for β\textsubscript{2}-agonist tachyphylaxis.

Secondly, our recent studies have demonstrated that one of the key ways in which β\textsubscript{2}-agonists have an anti-inflammatory effect in airways is via upregulation of anti-inflammatory protein, MKP-1 (Manetsch et al., 2012a; Manetsch et al., 2013). As this upregulation is mediated via β\textsubscript{2}-AR/cAMP-dependent mechanisms (Manetsch et al., 2012a), we now show that β\textsubscript{2}-agonist-induced MKP-1 mRNA expression is significantly repressed when the β\textsubscript{2}-AR is heterologously desensitized by PGE\textsubscript{2}. The extent of repression of the response to salbutamol and formoterol by PGE\textsubscript{2} was also consistent with their relative capacity induce cAMP-dependent MKP-1 expression. Being a full agonist, formoterol appeared to be relatively less sensitive to β\textsubscript{2}-AR desensitization than the partial agonist salbutamol. As salbutamol lacks receptor reserve, PGE\textsubscript{2}-
mediated desensitization had a greater impact on its capacity to increase expression of MKP-1 and potentially to limit airway inflammation. Importantly, we also demonstrated that TLR2 activation has a direct effect on β2-agonist-induced MKP-1 response. Pam3CSK4 + TNFα caused tachyphylaxis of β2-AR agonist-induced MKP-1 gene expression, and this could be reversed by celecoxib. These results suggest that celecoxib could be therapeutic in infectious exacerbation as treatment reverses the tachyphylaxis of salbutamol and formoterol on MKP-1 gene expression.

Utilizing a model of rhinovirus-induced asthma exacerbation in vitro in ASM cells (Trian et al., 2010; Trian et al., 2011; Van Ly et al., 2013), we previously learned how key molecular steps responsible for the action of β2-agonists are severely curtailed by viral infectious stimuli. Whether bacterial infection directly affects β2-AR function had not been explored. To address this, we modelled bacterial exacerbation in vitro with the synthetic bacterial lipoprotein Pam3CSK4 (a TLR2 agonist) to mimic bacterial infection and TNFα to simulate inflammation. TLR2 is a pattern-recognition receptor important for mediating the response to bacterial products, and Pam3CSK4 has been widely used to mimic bacterial infections (gram + bacteria) (Beckett et al., 2012; Manetsch et al., 2012b). Using this model, we now show that TLR2 activation results in a time-dependent increase in TNFα-induced COX-2 mRNA expression. COX-2 converts arachidonic acid to prostanoids (including prostaglandins), and in confirmation of earlier reports (Pang et al., 1998b), COX-2 upregulation in ASM cells consequently results in secretion of PGE2. Secreted PGE2 then acts back on the ASM in an autocrine manner to cause heterologous β2-AR desensitization (Pang et al., 1998b; Shore and Moore, 2003; Van Ly et al., 2013). Homologous desensitization was confirmed in parallel experiments (Hall et al., 1993); ASM cells were pretreated with β2-agonists, and the resultant cAMP formed in response to
salbutamol and formoterol was significantly attenuated. Moreover, it was interesting to note that
the sensitivity to heterologous $\beta_2$-AR desensitization induced by PGE$_2$ differed between the two
$\beta_2$-agonists tested. Inhibition of salbutamol-induced cAMP generation was evident after
pretreatment with a 10-fold lower concentration of PGE$_2$ than was required to inhibit the
formoterol response, perhaps reflecting the lack of receptor reserve for salbutamol, which is a
partial agonist at $\beta_2$-ARs.

These findings suggest that in ASM cells the increased effect of TLR2 agonist, Pam3CSK4 on
COX-2 mediated PGE$_2$ secretion further contribute in heterologous desensitization of $\beta_2$-AR.

In our published paper, there are some more supporting data (not shown here as those are done
by our collaborator in other lab) showing similar events in mouse lung slices *ex vivo* model, which
further strengthen our hypothesis (Alkhouri et al., 2014). Collectively, our findings in both
*in vitro* and *ex vivo* models successfully demonstrate the role of TLR2 agonist in $\beta_2$-AR
desensitization in asthma. Therefore, we believe the data presented in this chapter will provide
greater understanding in the context of $\beta_2$-AR desensitisation and reinforce further investigation
using contemporary experimental models to ascertain its clinical significance. Understanding
these events may prove to be an important step in improving the efficacy of $\beta_2$-agonists for the
treatment of asthma and will help to develop efficacious pharmacotherapeutic strategies to treat
respiratory disease without unwanted side effects in the near future.
Chapter 5

Sphingosine 1-phosphate increases COX-2 expression and PGE$_2$ secretion: effects on $\beta_2$-adrenergic receptor desensitization

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Chapter 5

Sphingosine 1-phosphate increases COX-2 expression and PGE$_2$ secretion: effects on $\beta_2$-adrenergic receptor desensitization

5.1 Introduction

In Chapter 4, we successfully demonstrate that TLR2 agonist, Pam3CSK4 induces COX-2 expression in ASM cells and contributes in heterologous desensitization of $\beta_2$-AR via PGE$_2$ secretion. Here, in this chapter, we aim to explore the effect of another inflammatory mediator, S1P, on COX-2 upregulation and its impact on $\beta_2$-AR desensitization. Increased COX-2 protein level has potential in regulating several aspects of inflammatory diseases including airway inflammation such as asthma and COPD. As “inflammation-associated” enzyme, induction of COX-2 is triggered by a number of cytokines and inflammatory mediators present in various inflammatory cells. In acute and chronic inflammatory states the induced COX-2 is primarily responsible for the synthesis of the prostanoids and along with COX-2 that prostanoids are associated in pathological process of several inflammatory conditions.

S1P is a bioactive sphingolipid found elevated in BAL from individuals with allergic asthma and was associated with inflammatory cell influx into the airways (Ammit et al., 2001). S1P exerts potent effects in in vitro and in vivo models of asthmatic inflammation and airway remodelling (Ammit et al., 2001; Roviezzo et al., 2007; Nishiuma et al., 2008; Che et al., 2014; Rahman et al., 2014). Moreover, Fuerst et al. reported that S1P potently regulated calcium mobilization and gene expression (including COX-2) in ASM grown from bronchial biopsies from healthy and asthma individuals (Fuerst et al., 2014). Notably, an S1P analog (FTY720; fingolimod) was
recently shown to be linked to exacerbation of asthma symptoms (van Rossum et al., 2014). Thus, although COX-2/PGE₂ expression has been linked to S1P, its role in hyporesponsiveness of the β₂-AR in ASM cells has not been examined. S1P has been shown to induce β₂-AR desensitization in a guinea pig model of bronchoconstriction (Makino et al., 2012) and was recently shown to increase COX-2/PGE₂ in tracheal ASM cells in vitro (Hsu et al., 2015). Herein we investigate the effect of S1P on COX-2 upregulation alone and in combination with TNFα in bronchial ASM cells and provide the first demonstration of β₂-AR desensitization by S1P in vitro.

Bronchodilatory β₂-agonists are the most commonly used drugs to relieve acute bronchoconstriction in people with asthma or COPD. β₂-agonists exert their effects by binding with the β₂-AR, a seven transmembrane GPCR and ultimately relax the airways by rapidly increasing cAMP levels in ASM cells. However, heterologous and homologous β₂-AR desensitization can occur in diverse range of clinically-relevant contexts, including β₂-agonist overuse, inflammation, bacterial and viral infectious exacerbation (Corne et al., 2002; Shore and Moore, 2003; Edwards et al., 2012). As this can result in hyporesponsiveness to β₂-agonists, β₂-AR desensitization is an important issue limiting effective treatment of chronic respiratory disease; thus further investigation into the causes and underlying molecular mechanisms are warranted.

Accumulating evidence has demonstrated the key role played by the enzyme COX-2 and its prostanoid products (PGE₂ in particular) in mediating β₂-AR desensitization. A number of pro-inflammatory cytokines elevated in BAL from people with asthma have been shown to induce COX-2 expression and result in PGE₂ secretion from ASM cells in vitro. Cytokines investigated include TNFα, interleukin 1β and interferon γ, added alone or in combination (cytomix) (Belvisi
et al., 1997; Pang and Knox, 1997; Pang et al., 1998b; Singer et al., 2003). PGE$_2$ is known to exert homologous desensitization of β$_2$-AR (Hall et al., 1993) and we have recently confirmed COX-2-dependent/PGE$_2$-mediated hyporesponsiveness to β$_2$-agonists in models of viral- and bacterial-induced exacerbation (Trian et al., 2010; Van Ly et al., 2013; Alkhouri et al., 2014).

As a part of our ongoing research on unveiling the molecular events of COX-2 upregulation as well as its impact in β$_2$-AR desensitization, in this study we investigate the effect of S1P alone and in combination with TNFα on COX-2 upregulation in bronchial ASM cells and provide the first demonstration of β$_2$-AR desensitization by S1P in vitro. We show the bioactive sphingolipid S1P induces hyporesponsiveness to the β$_2$-agonists salbutamol and formoterol, and demonstrate that S1P augments COX-2 expression and PGE$_2$ secretion induced by TNFα in an additive manner. Notably, S1P-induced effects are repressed by the COX-2 selective inhibitor, celecoxib and dexamethasone, thus, representing feasible pharmacotherapeutic strategies for restoring β$_2$-AR sensitivity.

5.2 Materials and methods

5.2.1 ASM cell culture

For detailed description, see Chapter 2.2.1.

5.2.2 Chemicals

TNFα was purchased from R&D Systems (Minneapolis, MN) and celecoxib from Cayman Chemical Company (Ann Arbor, MI). S1P (Biomol) was purchased from Enzo Life Sciences (Farmingdale, NY). Unless otherwise specified, all chemicals were from Sigma-Aldrich (St.
Louis, MO). Unless otherwise specified, all chemicals used in this study were purchased from Sigma-Aldrich (St.Louis, MO).

5.2.3 Real-time RT-PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen Australia, Doncaster, VIC, Australia) and reverse transcribed using the RevertAid First strand cDNA Synthesis Kit (Fermentas Life Sciences, Hanover, MD). Real-time RT-PCR was performed on an ABI Prism 7500 with COX-2 (Hs0015133_m1) TaqMan® Gene Expression Assays and the eukaryotic 18S rRNA endogenous control probe (Applied Biosystems, Foster City, CA) subjected to the following cycle parameters: 50°C for 2 min, 1 cycle; 95°C for 10 min, 1 cycle; 95°C for 15 s, 60°C for 1 min, 40 cycles and mRNA expression (fold increase) quantified by delta delta Ct calculations.

5.2.4 PGE₂ assay

PGE₂ was measured by enzyme immunoassay (Prostaglandin E₂ EIA 514010: Cayman Chemical Company) according to the manufacturer’s instructions. For detailed description, see Chapter 2.2.6.

5.2.5 Western blotting

Western blotting was performed using mouse monoclonal antibodies against COX-2 (29: Santa Cruz Biotechnology, Santa Cruz, CA), compared to α-tubulin as the loading control (clone DM 1A, Santa Cruz Biotechnology). Primary antibodies were detected with goat anti-mouse horse radish peroxidase–conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA)
and visualized by enhanced chemiluminescence (Perkin Elmer, Wellesley, MA). For detailed description, see Chapter 2.2.2.

5.2.6 COX-2 siRNA

ASM cells were transiently transfected using nucleofection with 1 μg COX-2-specific ON-Target SMART pool siRNA, consisting of a pool of four individual siRNA (Dharmacon: Thermo Fisher Scientific, Waltham, MA) or a scrambled siRNA control (ON-Target plus Control Non-targeting siRNA: Dharmacon), using methods established in our earlier publication (Quante et al., 2008). ASM cells were plated in 6-well plates for 16 h after transfection, before being growth-arrested with 2 mL DMEM media with 0.1% BSA for a further 24 h. Cells were then stimulated with S1P (1 μM) before COX-2 mRNA was measured by RT-PCR at 1 h, COX-2 protein detected by Western blotting at 24 h and secreted PGE$_2$ at 24 h measured by enzyme immunoassay.

5.2.7 cAMP assay

Growth-arrested ASM cells were pretreated for 24 h with vehicle, 100 nM PGE$_2$, vehicle, S1P (1 μM), S1P (1 μM) + TNFα (10 ng/ml). Desensitization of the β$_2$-AR was assessed by measuring production of cAMP in response to stimulation with 10 μM salbutamol or 10 nM formoterol for 15 min, compared to vehicle, in the presence of IBMX (30 min pretreatment at 10 μM) (Alkhouri et al., 2014). Cell lysates were prepared and intracellular cAMP was measured by enzyme immunoassay (cAMP EIA 581001: Cayman Chemical Company) according to the manufacturer’s instructions. For detailed description, see Chapter 2.2.5.
5.2.8 Statistical analysis

Statistical analysis was performed using Student's unpaired $t$ test, one-way ANOVA then Fisher’s post-hoc multiple comparison test, or two-way ANOVA then Bonferroni's post-test. P values $< 0.05$ were sufficient to reject the null hypothesis for all analyses.

5.3 Results

5.3.1 S1P upregulates COX-2 mRNA expression and protein upregulation to increase PGE$_2$ secretion from ASM cells

To explore the role of S1P in $\beta_2$-AR desensitization, we first treated ASM cells with S1P (1 µM) over 24 h and measured the temporal kinetics of COX-2 mRNA expression, protein upregulation and PGE$_2$ secretion. Stimulation of ASM cells with S1P significantly upregulated COX-2 mRNA expression at 1 and 2 h (Figure 5.1A). COX-2 protein upregulation followed with a peak at 8 h (Figures 5.1B and 5.1C) and resulted in increased levels of PGE$_2$ by 24 h (Figure 5.1D) (P $< 0.05$).
Figure 5.1 S1P upregulates COX-2 mRNA expression and protein upregulation to increase PGE$_2$ secretion from ASM cells. Growth-arrested ASM cells were treated with vehicle or S1P (1 µM) for 0, 1, 2, 4, 8, and 24 h. (A) COX-2 mRNA expression was quantified by real-time RT-PCR (results expressed as fold increase compared to vehicle-treated cells at 0 h). (B, C) COX-2 protein was detected by Western blotting (with α-tubulin as the loading control), where results are representative Western blots (72 kDa) (B), while (C) demonstrates densitometric analysis (results expressed as fold increase compared to vehicle-treated cells at 0 h). (D) PGE$_2$ secretion was measured by EIA. Statistical analysis was performed using one-way or two-way ANOVA then Bonferroni’s post-test (where * denotes a significant effect of S1P (P<0.05)). Data are mean±SEM values from n=3 primary ASM cell cultures.
5.3.2 Celecoxib has no effect on S1P-induced COX-2 mRNA, but significantly inhibited S1P-induced PGE\textsubscript{2} secretion

We then treated cells with the selective COX-2 inhibitor, celecoxib, and examined its effect on COX-2 mRNA expression and PGE\textsubscript{2} secretion. As expected, celecoxib had no effect on S1P-induced COX-2 mRNA expression (Figure 5.2A). In contrast, celecoxib pretreatment significantly repressed S1P-induced PGE\textsubscript{2} secretion (Figure 5.2B: P<0.05). These results confirm that celecoxib inhibits COX-2 enzymatic activity, but not its mRNA expression, and demonstrate that S1P-induced PGE\textsubscript{2} secretion occurs in a COX-2-dependent manner.

**Figure 5.2** Celecoxib has no effect on S1P-induced COX-2 mRNA, but significantly inhibited S1P-induced PGE\textsubscript{2} secretion. Growth-arrested ASM cells were pretreated with vehicle or 10 µM celecoxib for 1 h before treatment with S1P (1 µM) for 0, 1, 2, 4, 8, and 24 h. (A) COX-2 mRNA expression was quantified by real-time RT-PCR (results expressed as fold increase compared to vehicle-treated cells at 0 h). (B) Repression of S1P-induced PGE\textsubscript{2} secretion (results expressed as a percentage of S1P-induced PGE\textsubscript{2} secretion at 24 h). Statistical analysis was performed using Student's unpaired t test (where § denotes significant repression by celecoxib). Data are mean±SEM values from n=3 primary ASM cell cultures.
5.3.3 Dexamethasone represses S1P-induced COX-2 mRNA expression and PGE$_2$ secretion

Corticosteroids are first line anti-inflammatory therapies in asthma and we have already shown that they have repressive effects on S1P-induced cytokine secretion (Che et al., 2014; Rahman et al., 2014). Earlier publications have shown that cytokine-induced COX-2 expression and PGE$_2$ secretion can be repressed by the corticosteroid dexamethasone (Belvisi et al., 1997; Pang and Knox, 1997). To assess whether corticosteroids repress S1P-induced effects, ASM cells were pretreated with 100 nM dexamethasone for 30 min before stimulation. As shown in Figure 5.3A, dexamethasone abolished S1P-induced COX-2 mRNA expression, with significant repression observed at the 1, 2 and 4 h time point (P<0.05). Moreover, we observed that pretreatment with dexamethasone significantly inhibited S1P-induced PGE$_2$ secretion at 24 h (Figure 5.3B: P<0.05).
Figure 5.3 Dexamethasone represses S1P-induced COX-2 mRNA expression and PGE$_2$ secretion. Growth-arrested ASM cells were pretreated with vehicle or 100 nM dexamethasone for 30 min before treatment with S1P (1 µM) for 0, 1, 2, 4, 8, and 24 h. (A) COX-2 mRNA expression was quantified by real-time RT-PCR (results expressed as fold increase compared to vehicle-treated cells at 0 h). (B) Repression of S1P-induced PGE$_2$ secretion (results expressed as a percentage of S1P-induced PGE$_2$ secretion at 24 h). Statistical analysis was performed using one-way or two-way ANOVA then Bonferroni's post-test (where § denotes significant repression by dexamethasone). Data are mean±SEM values from n≥3 primary ASM cell cultures.
5.3.4 COX-2 knockdown by siRNA represses S1P-induced COX-2 mRNA expression, protein upregulation and PGE$_2$ secretion

To further support the role of S1P-induced COX-2 expression on PGE$_2$ secretion, we examined the impact of specifically knocking down COX-2 with siRNA on S1P-induced COX-2 mRNA expression, protein upregulation and PGE$_2$ secretion (Figure 5.4). ASM cells were transiently transfected using nucleofection with scrambled control or COX-2 siRNA, growth-arrested, then stimulated with S1P (1 µM). As shown in Figure 5.4A, the peak of COX-2 mRNA expression at 1 h was significantly reduced by COX-2 siRNA (P<0.05). S1P-induced COX-2 protein expression was significantly reduced by siRNA knockdown (Figure 5.4B), with densitometric analysis revealing a significant 66.9±9.8% reduction of COX-2 protein expression at 24 h in cells transfected with siRNA against COX-2, compared to scrambled control (Figure 5.4C: P<0.05). This resulted in corresponding decrease in the amount of PGE$_2$ secreted (Figure 5.4D: P<0.05). Collectively, these results confirm that S1P significantly enhances COX-2 expression and PGE$_2$ secretion, as this was repressed by the selective COX-2 inhibitor, celecoxib, the corticosteroid dexamethasone, or siRNA knockdown of COX-2 expression.
Figure 5.4 COX-2 knockdown by siRNA represses S1P-induced COX-2 mRNA expression, protein upregulation and PGE$_2$ secretion. ASM cells were transiently transfected using nucleofection with scrambled control or COX-2 siRNA, growth-arrested, then stimulated with S1P (1 µM). (A) COX-2 mRNA was measured by RT-PCR at 1 h (results expressed as % of S1P-induced COX-2 mRNA expression, scrambled control). (B, C) COX-2 protein was detected by Western blotting (with α-tubulin as the loading control) at 24 h (results are representative Western blots (72 kDa) (B), while (C) demonstrates densitometric analysis). (D) Secreted PGE$_2$ at 24 h was measured by enzyme immunoassay (results expressed as % of S1P-induced PGE$_2$ secretion, scrambled control). Statistical analysis was performed using Student's unpaired $t$ test (where § denotes a significant effect of siRNA against COX-2, compared to scrambled control). Data are mean±SEM values from n=3 primary ASM cell cultures.
5.3.5 TNFα enhances S1P-induced COX-2 mRNA expression and protein upregulation and increases PGE₂ secretion

We (Alkhouri et al., 2014) and others (Belvisi et al., 1997; Pang and Knox, 1997) have shown that the pro-inflammatory cytokine TNFα found elevated in BAL increases COX-2 mRNA expression and induces PGE₂ secretion from ASM cells. Comparatively, TNFα alone is a relatively modest inducer of COX-2 mRNA expression and PGE₂ secretion (Pang and Knox, 1997; Comer et al., 2014), but robust upregulation can be observed in combination with other cytokines, IL-1β in particular (Belvisi et al., 1997), or as cytomix (Belvisi et al., 1997; Singer et al., 2003). We propose that the effects of S1P would be enhanced in the presence of TNFα and in order to test this we treated growth-arrested ASM cells with S1P (1 µM) or TNFα (10 ng/mL), alone or in combination. As shown in Figure 5.5A, the temporal kinetics of COX-2 mRNA expression induced by TNFα is similar to that observed when ASM cells are stimulated with S1P alone. However, when cells are stimulated with both mediators together there is an additive effect, with TNFα significantly enhancing S1P-induced COX-2 mRNA expression at 4 and 8 h (Figure 5.5A: P<0.05). COX-2 protein upregulation at 24 h (Figure 5.5B), aligned with the mRNA data, and densitometric analysis confirmed that TNFα significantly enhanced S1P-induced COX-2 protein upregulation (Figure 5.5C: P<0.05). These data concur with the temporal kinetics of PGE₂ secretion. As shown in Figure 5.5D, when added in combination, we observed that TNFα significantly increased S1P-induced PGE₂ secretion at 4, 8 and 24 h (P<0.05).
Figure 5.5 TNFα enhances S1P-induced COX-2 mRNA expression and protein upregulation and increases PGE₂ secretion. Growth-arrested ASM cells were treated with vehicle, S1P (1 µM), TNFα (10 ng/ml), or S1P + TNFα, for 0, 1, 2, 4, 8, and 24 h. (A) COX-2 mRNA expression was quantified by real-time RT-PCR (results expressed as fold increase compared to vehicle-treated cells at 0 h); (B, C) COX-2 protein was detected by Western blotting at 24 h (compared to α-tubulin as a loading control), where (B) is a representative blot (72 kDa) and (C) is densitometric analysis of COX-2 protein upregulation (results normalised with α-tubulin expressed as fold increase compared to vehicle-treated cells); (D) PGE₂ secretion was measured by EIA. Statistical analysis was performed with two-way ANOVA then Bonferroni's post-test or Student's unpaired t test (where * denotes a significant effect of TNFα on S1P-induced effects (P<0.05)). Data are mean±SEM values from n=3 primary ASM cell cultures.
5.3.6 S1P induces heterologous β₂-adrenergic desensitization as measured by inhibition of β₂-agonist-induced cAMP production; in a manner independent of adenylate cyclase

Taken together, our data thus far shows that S1P increases COX-2 upregulation and significantly enhances PGE₂ secretion from ASM cells. Moreover, when added in combination with TNFα, COX-2 expression and PGE₂ secretion is substantially increased in an additive manner. PGE₂ is a known inducer of β₂-AR desensitization and so we now wished to demonstrate that S1P, alone and in combination with TNFα, caused β₂-AR desensitization and resulted in β₂-AR hyporesponsiveness to two widely-used β₂-agonists in asthma and COPD, short-acting salbutamol and long-acting formoterol. ASM cells were exposed to a concentration of PGE₂ known to induce heterologous desensitization, i.e. 100 nM (Alkhouri et al., 2014), in parallel with S1P, or S1P + TNFα, compared to vehicle. We then stimulated cells for 15 min with either salbutamol (Figure 5.6A) or formoterol (Figure 5.6B) and measured the cAMP produced in response to β₂-agonists as a measure of β₂-AR desensitization. As shown in Figure 5.6A, salbutamol significantly increased cAMP production in ASM cells and this was significantly repressed by pretreatment with PGE₂. These data concur with our previous study (Alkhouri et al., 2014). Notably, pretreating cells with S1P, or S1P + TNFα, also resulted in a significantly less cAMP, indicative of β₂-AR desensitization (P<0.05). There was also evidence of hyporesponsiveness to the β₂-agonist formoterol, as shown in Figure 5.6B; where formoterol induced 121.0±9.1 pmol cAMP/mL that was significantly repressed by PGE₂, S1P, or S1P + TNFα (P<0.05). This is independent of adenylate cyclase involvement, as shown in Figure 5.6C, where the adenylate cyclase activator, forskolin, significantly increases cAMP secretion from ASM cells and this was unaffected by PGE₂ alone, S1P, or S1P in combination with TNFα. These data support the assertion that S1P induces heterologous β₂-AR desensitization via PGE₂,
in a manner that may be upstream of adenylate cyclase. Collectively, these studies provide the first evidence that, like other inflammatory mediators found elevated in asthma, the bioactive sphingolipid S1P induces β₂-adrenergic desensitization in ASM.
Figure 5.6 S1P induces heterologous β₂-adrenergic desensitization as measured by inhibition of β₂-agonist-induced cAMP production; in a manner independent of adenylate cyclase. Growth-arrested ASM cells were pretreated for 24 h with vehicle, 100 nM PGE₂, vehicle, S1P (1 μM), S1P (1 μM) + TNFα (10 ng/mL). (A, B) Desensitization of the β₂-adrenergic receptor was assessed by measuring production of cAMP (pmol/mL) in response to stimulation with (A) 10 μM salbutamol or (B) 10 nM formoterol for 15 min, compared to vehicle, in the presence of the pan-phosphodiesterase inhibitor IBMX. (C) To exclude the involvement of adenylate cyclase, we measured production of cAMP (pmol/ml) in response to stimulation with the adenylate cyclase activator forskolin (10 μM) for 15 min, compared to vehicle, in the presence of IBMX. Statistical analysis was performed using one-way ANOVA then Bonferroni's post-test (where * denotes a significant effect on β₂-agonist-induced cAMP production (P<0.05)). Data are mean±SEM values from n=3 (A, B) and n=4 (C) primary ASM cell cultures.
5.4 Discussion

S1P is a bioactive sphingolipid found elevated in the BAL from people with allergic asthma. It has potent effects on airway inflammation and herein we show that S1P robustly upregulates COX-2 expression to result in PGE₂ production in bronchial ASM cells. We also provide the first evidence to demonstrate that S1P induces β₂-AR desensitization to result in reduced cAMP production to clinically-used β₂-agonists, salbutamol and formoterol. Moreover, S1P acts in an additive manner to enhance the COX-2/PGE₂-mediated upregulation in response to the pro-inflammatory cytokine TNFα. Thus, with this study we are the first to implicate this mediator in β₂-AR desensitization.

β₂-AR desensitization is an important issue in respiratory medicine. Mechanisms have been elucidated in part, and are clearly linked to COX-2 upregulation in ASM and the subsequent autocrine effects of PGE₂. A range of pro-inflammatory cytokines have been shown to increase COX-2/PGE₂ [reviewed in (Shore and Moore, 2003)]. Collectively, these studies reinforce the notion that a key characteristic of a pro-inflammatory cytokine in airway inflammation, in addition to their established ability to initiate potentiate and amplify the pro-asthmatic cascade, is their capacity to dampen the ability of β₂-agonists to exert their bronchodilatory effects. Moreover, cytokine responses can be robustly potentiated by activation of pattern-recognition receptors on ASM cells, with our recent studies showing that activation of TLRs for viral and bacterial products augments COX-2 expression/PGE₂ secretion and amplifies β₂-AR desensitization (Trian et al., 2010; Van Ly et al., 2013; Alkhouri et al., 2014). These studies may provide a mechanistic basis for clinically significant hyporesponsiveness to β₂-agonists observed in infectious exacerbation (Reddel et al., 1999).
Our study is the first to examine the impact of S1P on β2-AR desensitization in ASM cells. S1P is a bioactive sphingolipid found in increased levels in the BAL of people with allergic asthma, with effects on asthmatic inflammation and bronchoconstriction in vitro (Ammit et al., 2001; Che et al., 2014; Rahman et al., 2014) and in vivo (Roviezzo et al., 2007; Nishiuma et al., 2008; Fuerst et al., 2014). It is a legitimate target in asthma and our current study reveals that in addition to its known pro-inflammatory and bronchoconstrictive functions, S1P can also induce β2-AR desensitization. In this way, S1P can accelerate the pro-asthmatic phenotype, while disabling the brake.

COX-2 is an inducible gene and is known to be rapidly expressed in ASM cells in response to a diverse range of mediators; viz, cytokines (including TNFα, interleukin 1β and interferon γ, added alone or in combination as cytomix) (Belvisi et al., 1997; Pang and Knox, 1997; Pang et al., 1998b), TLR activators (Trian et al., 2010; Van Ly et al., 2013; Alkhouri et al., 2014) and now S1P (Hsu et al., 2015) and this study. Thus, the COX-2/PGE₂ pathway has emerged as a legitimate therapeutic target pathway in order to restore β2-AR responsiveness. Understanding the molecular mechanisms responsible will reveal future pharmacotherapeutic strategies. COX-2 mRNA expression can repressed by corticosteroids, as we have shown for S1P, and in confirmation of studies with COX-2 induced by other stimuli (Belvisi et al., 1997; Pang and Knox, 1997; Singer et al., 2003). Targeting COX-2 more specifically offers great promise and a notable recent study, Comer et al. demonstrate that cytokine stimulation of ASM cells from people with asthma have enhanced COX-2 mRNA and protein and increased secretion of PGE₂, compared to non-asthmatic controls, and this is due to epigenetic regulation involving miR-155, a microRNA found elevated in asthma (Comer et al., 2014). Prostanoid products of COX-2 enzymatic action can also be blocked with the use of non-selective (Belvisi et al., 1997; Pang and
Knox, 1997) and selective COX-2 inhibitors, such as celecoxib (Van Ly et al., 2013; Alkhouri et al., 2014; Comer et al., 2014). We could also target the specific receptors for PGE$_2$, and this may also relevant given that increased E-prostanoid receptor surface expression has been reported in ASM cells from people with asthma (Burgess et al., 2004). Further studies are warranted.

With this study we have highlighted the potential impact of S1P on $\beta_2$-AR receptor function. We have revealed that S1P can increase COX-2 mRNA and protein expression, which in turn leads to enhanced secretion of PGE$_2$ from ASM cells *in vitro* and suggested that S1P may contribute to $\beta_2$-AR desensitization in patients with asthma. This new knowledge may be exploited to discover novel targets for restoring the efficacy of bronchodilatory $\beta_2$-agonists in the future.
Chapter 6

IL-17A increases TNFα-induced COX-2 protein stability and augments PGE$_2$ secretion from airway smooth muscle cells

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Chapter 6

IL-17A increases TNFα-induced COX-2 protein stability and augments PGE₂ secretion from airway smooth muscle cells

6.1 Introduction

In Chapter 4 and 5, we demonstrate that both TLR2 agonist and S1P induce COX-2 expression in ASM cells and contribute in heterologous desensitization of β₂-AR via PGE₂ secretion. We further aim to extend our study herein by exploring the effects of another important inflammatory mediator, IL-17A on COX-2 expression and PGE₂ secretion and eventually on β₂-AR desensitization. COX-2 is an inducible enzyme that can be rapidly, but transiently, upregulated by a diverse range of stimuli, including pro-inflammatory mediators and infectious stimuli acting via TLRs (Trian et al., 2010; Van Ly et al., 2013; Alkhouri et al., 2014; Rumzhum et al., 2015b). Although IL-17A has been implicated in infectious exacerbation in respiratory disease, its impact on COX-2/PGE₂ has not been explored to date.

IL-17A orchestrates airway inflammation in asthma and COPD. This pivotal cytokine is found in elevated levels in respiratory diseases, including severe asthma (Chesne et al., 2014) and COPD (Caramori et al., 2014), and directs pulmonary immunity and inflammation (McAleer and Kolls, 2014). Recent evidence has implicated IL-17A as a key driver of disease exacerbation in severe asthma (Brandt et al., 2013) and in vivo models of infectious exacerbation (Essilfie et al., 2011; Lunding et al., 2015; Roos et al., 2015).

Herein we examine the impact of IL-17A on COX-2 mRNA expression, protein upregulation and subsequent PGE₂ secretion from ASM cells. Modelling inflammation with TNFα we show
that although IL-17A has no effect when added alone, it substantially amplifies TNFα-mediated responses in ASM cells. Interestingly, the molecular mechanism responsible for COX-2 upregulation differs from those previously reported in ASM cells (Alkhouri et al., 2014; Rumzhum et al., 2015b). That is, IL-7A had no effect on COX-2 mRNA expression; rather it enhances TNFα-induced COX-2 protein stability. We reveal that steady-state COX-2 protein production in response to TNFα stimulation is relatively transient (reaching a peak at 8 h, then subsiding; indicative of proteasomal degradation and confirmed by pretreatment with proteasome inhibitors). However, in the presence of IL-17A, TNFα-induced COX-2 protein levels are enhanced and sustained for to 24 h. This was confirmed by cycloheximide chase experiments where we show that TNFα-induced COX-2 protein stability is greatly enhanced in the presence of IL-17A. This is the first report to highlight the role that enhanced COX-2 protein stability may play in the context of respiratory disease driven by inflammation; because by increasing TNFα-induced COX-2 protein stability, IL-17A serves to substantially enhance secretion of PGE₂ from ASM cells. PGE₂ is a multifunctional prostanoid with both beneficial and adverse effects in airway pathogenesis (Sastre and del Pozo, 2012) and in this study we examined the impact of IL-17A on β₂-AR desensitization on ASM cells.

6.2 Material and methods

6.2.1 ASM cell culture

For detailed description, see Chapter 2.2.1.
6.2.2 Chemicals

TNFα and IL-17A were purchased from R&D Systems (Minneapolis, MN) and celecoxib from Cayman Chemical Company (Ann Arbor, MI). Unless otherwise specified, all chemicals were from Sigma-Aldrich (St. Louis, MO).

6.2.3 PGE₂ assay

PGE₂ was measured by enzyme immunoassay (Prostaglandin E₂ EIA 514010: Cayman Chemical Company) according to the manufacturer’s instructions. For detailed description, see Chapter 2.2.6.

6.2.4 Real-time RT-PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen Australia, Doncaster, VIC, Australia) and reverse transcribed using the RevertAid First strand cDNA Synthesis Kit (Fermentas Life Sciences, Hanover, MD). Real-time RT-PCR was performed on an ABI Prism 7500 with COX-2 (Hs0015133_m1) TaqMan® Gene Expression Assays and the eukaryotic 18S rRNA endogenous control probe (Applied Biosystems, Foster City, CA) subjected to the following cycle parameters: 50°C for 2 min, 1 cycle; 95°C for 10 min, 1 cycle; 95°C for 15 s, 60°C for 1 min, 40 cycles and mRNA expression (fold increase) quantified by delta delta Ct calculations.

6.2.5 Western blotting

SDS-PAGE was performed with 8% (COX-2) or 12% (IκB-α) separating gels and proteins were electrophoretically transferred to nitrocellulose membranes (Pall Corporation, Port Washington,
Western blotting was performed using mouse monoclonal antibodies against COX-2 (sc-19999) or rabbit polyclonal antibodies against IκB-α (sc-371), compared to α-tubulin as the loading control (clone DM 1A: sc-32293). All primary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA) and were detected with goat anti-mouse or goat anti-rabbit HRP-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA) and visualized by enhanced chemiluminescence (Perkin Elmer, Wellesley, MA). For detailed description, see Chapter 2.2.2.

**6.2.6 cAMP assay**

Desensitization of the β2-AR was assessed by measuring production of cAMP in response to stimulation with the β2-agonists salbutamol (10 µM) and formoterol (10 nM) for 15 min, in the presence of IBMX (30 min pretreatment at 10 µM), in accordance with previously published methods (Alkhouri et al., 2014; Rumzhum et al., 2015b). cAMP was measured by enzyme immunoassay (cAMP EIA 581001: Cayman Chemical Company) according to the manufacturer’s instructions. For detailed description, see Chapter 2.2.5.

**6.2.7 Statistical analysis**

Statistical analysis was performed using Student's unpaired t test, one-way ANOVA then Fisher’s post-hoc multiple comparison test, or two-way ANOVA then Bonferroni’s post-test. P values<0.05 were sufficient to reject the null hypothesis for all analyses.
6.3 Results

6.3.1 IL-17A augments TNFα-induced PGE₂ secretion, but does not increase TNFα-induced COX-2 mRNA expression

IL-17A is known to modulate airway inflammation in respiratory disease. Our recent studies in ASM cells have revealed the important role played by the prostanoid PGE₂ in mediating β₂-AR desensitization (Alkhouri et al., 2014; Rumzhum et al., 2015b). This occurs in a COX-2-dependent manner and to date, whether IL-17A exerts an influence on COX-2 upregulation/PGE₂ secretion in ASM cells was unknown. To address this, we first treated cells with IL-17A (10 ng/mL) alone, or in combination with TNFα (10 ng/mL), and measured the temporal kinetics of PGE₂ secretion, compared to vehicle-treated cells. As shown in Figure 6.1A, IL-17A alone had no effect on PGE₂ secretion, while stimulation with TNFα, a known inducer of PGE₂ (Pang and Knox, 1997; Alkhouri et al., 2014; Rumzhum et al., 2015b), resulted in significantly elevated levels of PGE₂ by 24 h (P<0.05). Importantly, when cells were stimulated with TNFα in the presence of IL-17A, PGE₂ secretion was robustly enhanced with significant and sustained amounts of PGE₂ secretion observed as early as 4 h (Figure 6.1A: P<0.05).

We were intrigued to understand the underlying molecular mechanism. PGE₂ is produced by the action of the enzyme COX-2. COX-2 can be upregulated by increased gene expression (via transcriptional or post-transcriptional means), and/or post-translationally via increased protein stability. To date, the effect of IL-17A on COX-2 gene expression in ASM cells was unknown. To understand the mechanism responsible for the upregulation of TNFα-induced PGE₂ secretion in ASM cells by IL-17A, we examined the impact of IL-17A on COX-2 mRNA expression and whether it played a role in potentiating the effects of TNFα. In Figure 6.1B we demonstrate the temporal regulation of COX-2 mRNA expression; and, in corrobororation of our earlier studies
(Alkhouri et al., 2014; Rumzhum et al., 2015b), TNFα increases COX-2 mRNA expression with significant expression (P<0.05) observed as early as 1 h, sustained until 4 h, and then shown to decline by 8 h. As predicted by the lack of PGE$_2$ secretion, IL-17A alone did not induce COX-2 mRNA expression. More unexpectedly however, IL-17A added in combination with TNFα did not enhance COX-2 mRNA expression.

Figure 6.1 IL-17A augments TNFα-induced PGE$_2$ secretion, but does not increase TNFα-induced COX-2 mRNA expression. Growth-arrested ASM cells were treated with vehicle, IL-17A (10 ng/mL), TNFα (10 ng/mL), or IL-17A + TNFα (both at 10 ng/mL), for 0, 1, 2, 4, 8, and 24 h. (A) PGE$_2$ secretion was measured by EIA. (B) COX-2 mRNA expression was quantified by real-time RT-PCR (results expressed as fold increase compared to vehicle-treated cells at 0 h). Statistical analysis was performed using two-way ANOVA then Bonferroni’s post-test (where * denotes a significant effect of TNFα on PGE$_2$ secretion or COX-2 mRNA expression, and § denotes a significant effect of IL-17A on TNFα-induced PGE$_2$ secretion (P<0.05)). Data are mean±SEM values from n=5 primary ASM cell cultures.
6.3.2 IL-17A increases TNFα-induced COX-2 protein upregulation

Because these data indicate that the augmentation of TNFα-induced PGE₂ by IL-17A is not via potentiation of TNFα-induced COX-2 mRNA expression, we then examined COX-2 protein upregulation after growth-arrested ASM cells were treated with vehicle, IL-17A, TNFα, or IL-17A + TNFα (Figure 6.2A). In accordance with the lack of effect of IL-17A on COX-2 mRNA expression and PGE₂ secretion, IL-17A alone did not induce COX-2 protein (data not shown). As shown by Western blotting (Figure 6.2A), there was a peak of TNFα-induced protein upregulation observed at 8 h, that later declined at 24 h. This was confirmed by densitometric analysis (Figure 6.2B: P<0.05), where TNFα induced a significant 10.6±5.5-fold increase in COX-2 protein upregulation at 8 h, that then declined by 24 h. Thus, the temporal kinetics of COX-2 protein upregulation after TNFα stimulation (Figure 6.2) aligns well with COX-2 mRNA expression (Figure 6.1B): that is, COX-2 mRNA induced by TNFα stimulation was consequently translated into COX-2 protein; albeit in a transient manner. In contrast, while IL-17A had no effect on TNFα-induced COX-2 mRNA (Figure 6.1B), IL-17A robustly upregulated TNFα-induced COX-2 protein in a sustained manner (Figure 6.2). Western blotting revealed that IL-17A enhanced TNFα-induced COX-2 protein upregulation at 4 h, 8 h and 24 h (Figure 6.2A). This was confirmed by densitometric analysis (Figure 6.2B: P<0.05). Notably, at 24 h, IL-17A added in combination with TNFα induced a 33.8±1.7-fold increase in COX-2 protein, in comparison to 3.9±1.7-fold with TNFα alone at the same time point (Figure 6.2B: P<0.05). Taken together, our data shows that IL-17A increases TNFα-induced COX-2 protein stability, but not TNFα-induced COX-2 mRNA gene expression in ASM cells.
Figure 6.2 IL-17A increases TNFα-induced COX-2 protein upregulation. Growth-arrested ASM cells were treated with vehicle, IL-17A (10 ng/mL), TNFα (10 ng/mL), or IL-17A + TNFα (both at 10 ng/mL), for 0, 1, 2, 4, 8, and 24 h. COX-2 protein was detected by Western blotting (compared to α-tubulin as a loading control), where (A) is a representative blot (72 kDa) (vehicle and IL-17A did not induce COX-2 protein – data not shown) and (B) is densitometric analysis of COX-2 protein upregulation (results normalised with α-tubulin then expressed as fold increase compared to vehicle-treated cells at 0 h). Statistical analysis was performed by two-way ANOVA then Bonferroni's post-test (where * denotes a significant effect of TNFα on COX-2 protein upregulation, and § denotes a significant effect of IL-17A on TNFα-induced COX-2 protein upregulation (P<0.05)). Data are mean±SEM values from n=4 primary ASM cell cultures.
6.3.3 Proteasome inhibitors increase TNFα-induced COX-2 protein and PGE₂ secretion

COX-2 is an inducible gene known to be expressed by a wide variety of inflammatory stimuli, but COX-2 protein upregulation in cells is typically transient [reviewed in (Kang et al., 2007)]. Post-translational regulatory mechanisms have begun to emerge (Haddad et al., 2012), and in cell types apart from ASM, degradation by the 26S proteasome has been shown to play a role (Rockwell et al., 2000; Haddad et al., 2012; Brender and Barki-Harrington, 2014). Our data suggests that IL-17A increases the stability of COX-2 protein induced by TNFα. But to date, whether protein stability contributes to steady-state levels of COX-2 protein in ASM cells was unknown. Thus, in order to show that TNFα-induced COX-2 protein is subject to proteasomal degradation in ASM, we pretreated cells with two inhibitors of the proteasome - bortezomib and MG-132 – and examined whether TNFα-induced COX-2 protein levels are increased. Growth-arrested ASM cells were pretreated for 30 min with bortezomib (10 nM) or MG-132 (10 µM), before stimulation with TNFα for up to 24 h. In the representative Western blots shown in Figure 6.3A, blocking the proteasome with bortezomib or MG-132 enhances COX-2 protein upregulation induced by TNFα. This is confirmed by densitometric analysis in Figure 6.3B, where the level of TNFα-induced COX-2 protein at 24 h (3.3±0.9-fold) was significantly increased by bortezomib to 8.9±1.8-fold and MG-132 to 19.5±2.5-fold (P<0.05). These data support our assertion that TNFα-induced COX-2 is degraded by the proteasome. Thus, it follows that if COX-2 protein stability is enhanced, production of COX-2-mediated prostanoid products, including PGE₂, will be increased. In support, Figure 6.3C shows that the TNFα-induced PGE₂ secretion at 24 h is significantly enhanced after bortezomib or MG-132 pretreatment (P<0.05).
Figure 6.3 Proteasome inhibitors increase TNFα-induced COX-2 protein and PGE₂ secretion. Growth-arrested ASM cells were pretreated for 30 min with vehicle, bortezomib (10 nM), or MG-132 (10 µM) before stimulation with TNFα (10 ng/mL) for 0, 1, 2, 4, 8, and 24 h. (A, B) COX-2 protein was detected by Western blotting at 24 h (compared to α-tubulin as a loading control), where (A) is a representative blot (72 kDa) and (B) is densitometric analysis of COX-2 protein upregulation (results normalised with α-tubulin then expressed as fold increase compared to TNFα-treated cells at 0 h). Statistical analysis was performed with two-way ANOVA then Bonferroni’s post-test (where § denotes a significant effect of proteasome inhibition onTNFα-induced COX-2 protein upregulation (P<0.05)). (C) PGE₂ secretion at 24 h was measured by EIA (results expressed as fold increase compared to vehicle-treated cells at 0 h). Statistical analysis was performed one-way ANOVA then Fisher’s post-hoc multiple comparison test (where § denotes a significant effect of proteasome inhibition onTNFα-induced PGE₂ secretion (P<0.05)). Data are mean±SEM values from n=6 primary ASM cell cultures.
6.3.4 IL-17A enhances TNFα-induced COX-2 protein stability

To confirm that IL-17A enhances TNFα-induced COX-2 protein stability, we performed cycloheximide chase experiments. We treated growth-arrested ASM cells for 8 h with TNFα ± IL-17A (to achieve peak levels of COX-2 steady-state protein production; as demonstrated earlier in Figure 6.2A). Cells were then washed and treated with cycloheximide (20 µg/mL) to block further protein translation; in accordance with previous published methods (Bradshaw et al., 2011). Cell lysates were prepared at 1, 2, 4, 6, and 16 h after the addition of cycloheximide (designated as 0 h). COX-2 was then quantified by Western blotting (normalized to α-tubulin), analysed by densitometry and expressed as a percentage of COX-2 at 0 h. As shown in Figure 6.4, the percentage of COX-2 remaining 2, 4, 6 and 16 h after protein translation had been halted by cycloheximide treatment was significantly greater in cells treated with IL-17A + TNFα, compared to TNFα alone (P<0.05). Thus, IL-17A enhances TNFα-induced protein stability. Future studies examining the molecular mechanisms responsible for enhanced COX-2 protein stability exerted by IL-17A are warranted, but herein we were intrigued to examine whether IL-17A stabilizes other proteins relevant to airway inflammatory diseases. To address this, we focussed on IκB-α, the inhibitory protein regulated by the 26S proteasome that complexes with pro-inflammatory NF-κB to hold it in check. When ASM cells are stimulated by TNFα, IκB-α protein was degraded (as shown in Figure 6.5, and in confirmation of our previous reports (Moutzouris et al., 2010; Manetsch et al., 2012b)). NF-κB is then released from the inhibitory complex and is now competent to activate transcriptional pathways and promote ASM synthetic function (Moutzouris et al., 2010; Manetsch et al., 2012b). Interestingly, we show that TNFα-induced degradation of inhibitory IκB-α was unaffected by IL-17A (Figure 6.5). These results address, in part, the specificity of the IL-17A-mediated COX-2 response by showing that under
identical conditions, other proteins shown to be regulated proteasomally (utilizing IκB-α as an example) are unaffected by IL-17A.

Figure 6.4 IL-17A increases TNFα-induced COX-2 protein stability. Growth-arrested ASM cells were treated with TNFα (10 ng/mL) or IL-17A + TNFα (both at 10 ng/mL) for 8 h. Cells were then washed and treated with 20 µg/ml cycloheximide to inhibit further protein translation. Cell lysates were then prepared at 0, 1, 2, 4, 6 and 16 h and COX-2 protein quantified by Western blotting (compared to α-tubulin as a loading control). COX-2 protein levels at each time point was measured by densitometry and expressed as a percentage of COX-2 at 0 h (i.e. time of treatment with cycloheximide). Statistical analysis was performed by two-way ANOVA then Bonferroni’s post-test (where § denotes a significant effect of IL-17A on TNFα-induced COX-2 protein stability (P<0.05)). Data are mean±SEM values with non-linear regression curve fit from n=6 primary ASM cell cultures.
Figure 6.5 TNFα-induced IκB-α degradation was unaffected by IL-17A. Growth-arrested ASM cells were treated with vehicle, IL-17A (10 ng/mL), TNFα (10 ng/mL), or IL-17A + TNFα (both at 10 ng/mL), for 0, 15, 30, 45 and 60 min. IκB-α was analysed by Western blotting compared to α-tubulin as a loading control (35-41 kDa). Results are representative of n=3 primary ASM cell cultures.

6.3.5 Heterologous β2-adrenergic desensitization as measured by inhibition of β2-agonist-induced cAMP production in ASM cells: effects of IL-17A ± TNFα

PGE2 is known to induce heterologous β2-AR desensitization on ASM cells. Because we have shown that IL-17A robustly increased COX-2 protein/PGE2 induced by TNFα, we sought to examine the impact of IL-17A ± TNFα on β2-AR desensitization, as measured by inhibition of β2-agonist-induced cAMP production. As shown in Figures 6.6A and 6.6B, ASM treated with the β2-agonists, salbutamol or formoterol, respectively, significantly increased cAMP release from ASM cells. This could be significantly repressed by pretreatment with 100 nM PGE2, in
confirmation of our previous publications (Alkhouri et al., 2014; Rumzhum et al., 2015b). These results demonstrate the known ability of PGE$_2$ to heterologously desensitize the β$_2$-AR. As IL-17A did not increase PGE$_2$, it was unsurprising that there was no significant repression of cAMP produced induced by salbutamol (Figure 6.6A) or formoterol (Figure 6.6B). What was surprising however was that IL-17A in combination with TNFα, treatment conditions known to robustly increase PGE$_2$, had no effect on β$_2$-agonist-induced cAMP (Figures 6.6A and 6.6B). If anything, cAMP production appeared to increase. In parallel studies we confirmed that indeed this is the case, and as shown in Figure 6.6C, pretreatment of ASM cells with IL-17A + TNFα resulted in significantly increased cAMP production (P<0.05). PGE$_2$ is a multifunctional prostanoid and can increase cAMP in ASM cells via receptor-mediated pathways. Our study shows that treating ASM cells with IL-17A + TNFα results in a robust and sustained production of PGE$_2$. Thus we propose that the resultant PGE$_2$ acts to increase cAMP, confounding interpretation of hyporesponsiveness to β$_2$-agonists. This was confirmed by utilization of a COX-2 selective inhibitor where pretreatment with celecoxib significantly reduced cAMP produced by IL-17A in combination with TNFα (Figure 6.6D: P<0.05).
Figure 6.6 Heterologous β2-adrenergic desensitization as measured by inhibition of β2-agonist-induced cAMP production in ASM cells: effects of IL-17A ± TNFα. (A, B) Growth-arrested ASM cells were pretreated for 24 h with vehicle, 100 nM PGE2, IL-17A (10 ng/mL), or IL-17A + TNFα (both at 10 ng/mL). Desensitization of the β2-AR was assessed by measuring production of cAMP (pmol/mL) in response to stimulation with (A) 10 µM salbutamol or (B) 10 nM formoterol for 15 min, compared to vehicle, in the presence of the pan-phosphodiesterase inhibitor IBMX. In parallel studies using conditions outlined above: (C) cells were pretreated for 24 h with vehicle, IL-17A, or IL-17A + TNFα, and cAMP production (pmol/mL) measured; (D) cells were pretreated for 1 h with vehicle or celecoxib (10 µM) and the effect on % IL-17A + TNFα-induced cAMP assessed. Statistical analysis was performed using Student's unpaired t test (where * denotes a significant effect on cAMP production and § denotes significant repression (P<0.05)). Data are mean±SEM values from n=6 primary ASM cell cultures.
6.4 Discussion

In this study we show that TNFα upregulates COX-2 by inducing mRNA expression in ASM cells. We reveal that the transient upregulation of COX-2 protein induced by TNFα in ASM cells is due to degradation by the proteasome. We show that two inhibitors of the 26S proteasome - bortezomib (Velcade®) and MG-132 - can block degradation of TNFα-induced COX-2 and enhance PGE₂ production. We have shown that IL-17A acts to increase TNFα-induced PGE₂ by enhancing COX-2 protein stability, but not COX-2 gene expression. This is a novel finding in ASM cells and this new knowledge improves our understanding of how IL-17A impacts on the COX-2/PGE₂ regulatory network.

Given the impact of the COX-2 enzyme in health and disease, it unsurprisingly that there are multiple molecular mechanisms that allow COX-2, and its resultant prostanoids, to be controlled in precise spatiotemporal regulatory networks. COX-2 gene expression is known to be regulated transcriptionally (Appleby et al., 1994) and/or post-transcriptionally via increased mRNA stability (Lasa et al., 2000). Somewhat more recently, mechanisms responsible for the post-translational regulation of COX-2 protein stability have begun to emerge (Mbonye et al., 2008; Haddad et al., 2012; Brender and Barki-Harrington, 2014). Although IL-17A is known to increase gene expression by enhancing mRNA stability (Hartupee et al., 2007), and we have previously shown that IL-17A significantly enhanced TNFα-induced IL-6 and IL-8 mRNA expression via post-transcriptional means in ASM cells (Henness et al., 2004; Henness et al., 2006), in this study we show that IL-17A has no effect on COX-2 mRNA gene expression, instead IL-17A acts to enhance the stability of COX-2 protein induced by TNFα.

Diverse stimuli are known to induce COX-2 (Kang et al., 2007) and the half-life (t₁/₂) of the protein is known to be relatively short-lived. This allows the protein to be rapidly upregulated.
and labile prostanoids produced, and then rapidly switched off to limit the amount of prostanoids produced. In cell types apart from ASM, the $t_{1/2}$ for COX-2 varies from 2-7 h [reviewed in (Kang et al., 2007)] where Kang et al. comments that “COX-2 protein degradation is specifically programmed to limit the COX-2”. Herein we perform cycloheximide chase experiments and demonstrate, for the first time in ASM cells, that the $t_{1/2}$ of TNFα-induced COX-2 (defined here as time (h) taken to reach 50% of COX-2 protein levels observed before cycloheximide treatment) is between 1-2 h (as shown in Figure 6.4). In contrast, COX-2 protein produced after stimulation with IL-17A + TNFα is comparatively stable and does not degrade to levels below 50% over the evaluation period examined (up to 16 h post-cycloheximide treatment). In the presence of IL-17A, TNFα-induced COX-2 protein is sustained and remains at plateau of ~ 70%, compared to ~ 30% in the absence of IL-17A. The mechanisms responsible for enhanced protein stability may involve post-translational modifications and endoplasmic reticulum-associated degradation systems (Mbonye et al., 2006; Mbonye et al., 2008), and in cell types apart from ASM, degradation by the 26S proteasome has been shown to play a role (Rockwell et al., 2000; Haddad et al., 2012; Brender and Barki-Harrington, 2014). Further studies are warranted as these molecular mechanisms may be amenable to pharmacological intervention as members of the proteasomal machinery may prove to be important drug targets to combat inflammatory disease in the future (Skaar et al., 2014).

We show that IL-17A has a substantial impact on PGE₂ secretion from ASM cells by increasing TNFα-induced COX-2 protein upregulation in a sustained manner. PGE₂ is a multifunctional prostanoid with both beneficial and adverse effects in respiratory disease. Early studies revealed that PGE₂ has bronchodilatory and anti-inflammatory effects in human airways (Kawakami et al., 1973; Pavord and Tattersfield, 1995). This is consistent with the ability of PGE₂ to act via
receptor-mediated pathways to enhance cAMP (Billington et al., 2013). However, PGE_2 can also cause desensitization of the β_2-AR on ASM cells [reviewed in (Shore and Moore, 2003; Billington et al., 2013)], and we have shown that pro-inflammatory mediators and infectious stimuli (acting via TLRs) (Trian et al., 2010; Van Ly et al., 2013; Alkhouri et al., 2014; Rumzhum et al., 2015b) cause PGE_2-dependent heterologous desensitization of the β_2-AR in vitro. In this way, PGE_2 may curtail the beneficial bronchodilatory actions of β_2-agonists. Given that hyporesponsiveness to bronchodilators is a hallmark feature of exacerbation in respiratory disease (Reddel et al., 1999), and IL-17A has been shown to drive infectious exacerbation (Essilfie et al., 2011; Brandt et al., 2013; Lunding et al., 2015; Roos et al., 2015), we sought to examine the impact of IL-17A on β_2-AR desensitization. Utilizing our established method (Alkhouri et al., 2014; Rumzhum et al., 2015b) for assessing β_2-AR desensitization in ASM cells by measuring β_2-agonist-induced cAMP production, we confirmed that PGE_2 caused desensitization, and as expected due to its inability to induce PGE_2, IL-17A did not. Consistent with the aforementioned ability of PGE_2 to induce cAMP, it was not possible to demonstrate an effect of IL-17A + TNFα on β_2-AR desensitization, as IL-17A + TNFα significantly induced cAMP in a COX-2/PGE_2-dependent manner.

In summary, our study extends the role and function of IL-17A as a multifaceted fine-tuning cytokine in respiratory disease. We show that IL-17A potentiates COX-2 upregulation in inflammation modelled in vitro and can enhance PGE_2 secretion. PGE_2 is experiencing a somewhat of a renaissance in the respiratory field, driven by the development of selective receptor antagonists and transgenic mouse-models (Birrell et al., 2015; Maher et al., 2015). These research tools may allow precise delineation of the “good” and “bad” effects of PGE_2 in
in this context of infectious exacerbation and may pave the way for novel pharmacotherapeutic strategies to treat severe asthma and COPD in the future.
Chapter 7

Role of specific PGE$_2$ receptors
Chapter 7
Role of specific PGE\textsubscript{2} receptors

7.1 Introduction

In Chapter 4 - Chapter 6, we successfully demonstrate the role of COX-2-induced PGE\textsubscript{2} on \(\beta\textsubscript{2}\)-AR desensitization in \textit{in vitro} ASM cell model. In this chapter, we aim to identify the role of specific PGE\textsubscript{2} receptors in the context of \(\beta\textsubscript{2}\)-AR desensitization. COX-2 has multiple prostanoid products, among which studies have shown that PGE\textsubscript{2} predominates in some inflammatory contexts (Belvisi et al., 1997; Pang et al., 1998a; Van Ly et al., 2013). Rather than stored, PGE\textsubscript{2} is de novo synthesized. Immediately after synthesis it is inactivated by the actions of the enzyme, 15-hydroxyprostaglandin dehydrogenase (PGDH) (Tai et al., 2006). PGE\textsubscript{2} shows pleiotropic autocrine, paracrine and endocrine functions (Reid et al., 2003). The actions of PGE\textsubscript{2} are mediated via four distinct E prostanoid (EP) receptor subtypes, EP\textsubscript{1}, EP\textsubscript{2}, EP\textsubscript{3} and EP\textsubscript{4}, belonging to the homologous GPCR superfamily (Narumiya et al., 1999; Vancheri et al., 2004). PGE\textsubscript{2} is a multifunctional protein and the diverse effects of PGE\textsubscript{2} are presumably associated with the presence of several EP receptors that mediate different and sometimes opposing downstream effects. PGE\textsubscript{2} signalling through EP\textsubscript{2} and EP\textsubscript{4} receptors promotes the accumulation of cAMP. This leads to smooth muscle relaxation. PGE\textsubscript{2} signalling through EP\textsubscript{1} and EP\textsubscript{3} receptors activation increase intracellular calcium or decrease cAMP generation which could be associated with smooth muscle contraction (An et al., 1993; Coleman et al., 1994; Qian et al., 1994; Breyer et al., 2001; Vancheri et al., 2004; Kay et al., 2006; Foudi et al., 2008; Machado-Carvalho et al., 2014; Claar et al., 2015). Hence, controlling the balance between the contractile and relaxant response to PGE\textsubscript{2} in favour of protective relaxation is very crucial.
Previous report suggests that further research on EP receptors, specifically EP$_2$ and EP$_4$, may lead to novel therapeutic strategies for the treatment of asthma and COPD (Billington et al., 2013). Previous reports have revealed the expression of EP$_2$ and EP$_4$ receptors in ASM cells (Clarke et al., 2005b; Billington et al., 2013). Considering the potential role of EP$_2$ and EP$_4$ receptors in inflammatory lung diseases, in this study, we target EP$_2$ and EP$_4$ receptors and aim to provide a stronger molecular framework behind the observed functional responses in ASM cells generated by PGE$_2$. EP$_2$ receptor was first cloned in human by Regan et al. in 1994 (Regan et al., 1994). Its cDNA encodes a 358-amino-acid polypeptide and signals through increased cAMP. EP$_2$ receptor is widely distributed in lung tissue and possesses a relaxant role in bronchioles, suggesting that EP$_2$ agonists could be used to treat pulmonary diseases (Pavord et al., 1991; Coleman et al., 1994; Narumiya et al., 1999). The human EP$_4$ receptor cDNA encodes a 488-amino-acid polypeptide and is widely distributed in thymus, ileum, lung, spleen, adrenal, and kidney tissues (Breyer et al., 2001). Similar to EP$_2$ receptor, activation of the EP$_4$ receptor leads to an increase in cAMP levels. Thereby, research also suggests that EP$_4$ receptors are most similar to EP$_2$ prostanoid receptors not only in pharmacological terms, but also in many physiological settings. EP$_2$ and EP$_4$ receptors are co-located, making identification of individual roles difficult (Wilson et al., 2006). Several reports examine EP receptor subtype functions in ASM and highlight the potential therapeutically beneficial effects of EP$_2$ and EP$_4$ agonists on airway remodelling (Guo et al., 2005; Kong et al., 2008; Buckley et al., 2011; Yan et al., 2011; Benyahia et al., 2012). In elucidating their specific roles, we used PF-04418948 and GW627368X as selective EP$_2$ and EP$_4$ receptor antagonists respectively (Wilson et al., 2006; af Forselles et al., 2011).
7.2 Material and methods

7.2.1 ASM cell culture

For detailed description, see Chapter 2.2.1.

7.2.2 Chemicals

PGE$_2$, PF-04418948 and GW-627368X were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Unless otherwise specified, all chemicals used in this study were purchased from Sigma-Aldrich (St.Louis, MO).

7.2.3 Real-time RT-PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) and reverse transcribed using the RevertAid First strand cDNA Synthesis Kit (Fermentas Life Sciences). Real-time RT-PCR was performed on an ABI Prism 7500 with MKP-1 (DUSP1: Hs00610256_g1) TaqMan® Gene Expression Assays and the eukaryotic 18S rRNA endogenous control probe (Applied Biosystems) subjected to the following cycle parameters: 50°C for 2 min, 1 cycle; 95°C for 10 min, 1 cycle; 95°C for 15 s, 60°C for 1 min, 40 cycles and mRNA expression (fold increase) quantified by delta delta Ct calculations. For detailed description, see Chapter 2.2.3.

7.2.4 cAMP assay

Desensitization of the $\beta_2$-AR was assessed by measuring production of cAMP in response with a range of concentrations of PGE$_2$ (0.1–100 nM) for 24 h, in the presence of IBMX (30 min pretreatment at 10 $\mu$M), in accordance with previously published methods (Alkhouri et al., 2014; Rumzhum et al., 2015b). cAMP was measured by enzyme immunoassay (cAMP EIA 581001:...
Cayman Chemical Company) according to the manufacturer’s instructions. For detailed description, see Chapter 2.2.5.

7.2.5 Statistical analysis
Statistical analysis was performed using Student's unpaired t test, one-way ANOVA then Fisher’s post-hoc multiple comparison test, or two-way ANOVA then Bonferroni's post-test. P values < 0.05 were sufficient to reject the null hypothesis for all analyses.

7.3 Results
7.3.1 PGE$_2$ induces MKP-1 expression in ASM cells
Previous study described that PGE$_2$ induces cAMP production (Ammit et al., 2002), and as cAMP upregulation induces MKP-1 expression in ASM cells (Patel et al., 2015a) it makes sense to show PGE$_2$-induced MKP-1 mRNA expression in ASM and, more importantly, to show whether it is mediated via EP$_2$ and/or EP$_4$ receptors. As mentioned before, we used selective antagonists, PF-04418948 to block the EP$_2$ receptor, and GW 627368X to block the EP$_4$ receptor. We found that PGE$_2$ (0.1-1000 nM) dose-dependently increases MKP-1 mRNA expression (Figure 7.1). Notably, pretreatment of cells with PF-04418948 or GW 627368X (both at 1 μM) for 30 min, prior to 1 h treatment with a range of concentrations of PGE$_2$ (0.1-1000 nM), didn’t show any differences in MKP-1 mRNA expression (Figure 7.1). This suggests that neither EP$_2$ nor EP$_4$ receptors are solely responsible for PGE$_2$-induced MKP-1 expression, or perhaps other receptors or mechanisms are involved.
Figure 7.1 PGE\textsubscript{2} induces MKP-1 expression and the role of EP\textsubscript{2} and EP\textsubscript{4} receptors. Growth-arrested ASM cells were pretreated for 30 min with vehicle or PF-04418948, EP\textsubscript{2} antagonist, (1μM) or GW 627368X, EP\textsubscript{4} antagonist (1μM) followed by a range of PGE\textsubscript{2} concentrations (0.1-100 nM). After 1 h MKP-1 mRNA expression was measured by RT-PCR. Statistical analysis was performed using one-way ANOVA and Fisher’s PLSD (where * denotes a significant upregulation of MKP-1 mRNA compared to vehicle control (P<0.05)). Data are mean±SEM values from n=4 primary ASM cell cultures.
7.3.2 Role of EP receptor antagonists on PGE₂-induced β₂-AR desensitization (short protocol)

In chapter 4, we measured PGE₂-induced cAMP levels to describe β₂-AR receptor desensitization where cells were allowed to grow for 48 h before serum starvation (short protocol). Following the same condition herein, we aim to explore the pathways for PGE₂-induced β₂-AR desensitization, in particular, the role of EP₂ or EP₄ receptor is identified. To confirm this, ASM cells were pretreated for 24 h with a range of concentrations of PGE₂ (0.1–100 nM) and cAMP production was measured in response to two β₂-agonists: long-acting β₂-agonist formoterol (Figure 7.2A) and short-acting β₂-agonist salbutamol (Figure 7.2B). In accordance with previous findings (Chapter 4-6), this result also showed that pretreatment with PGE₂ induced heterologous β₂-AR desensitization in a concentration-dependent manner (P<0.05) in response to both the β₂-agonists. Interestingly, we found that antagonism of the EP₂ receptor, but not the EP₄ receptor, significantly blocks PGE₂-induced heterologous β₂-AR desensitization and restores cAMP production. This finding suggests that EP₂ receptor may be involved in β₂-AR receptor desensitization.
Figure 7.2 Role of EP receptor antagonists on PGE\(_2\)-induced β\(_2\)-AR desensitization (short protocol). Growth-arrested ASM cells were pretreated for 24 h with vehicle, or a range of PGE\(_2\) concentrations (0.1-100 nM). Desensitization of the β\(_2\)-AR was assessed by measuring production of cAMP in response to stimulation with (A) 0.01 μM formoterol or (B) 10 μM salbutamol for 15 min compared to vehicle, in the presence of the pan-phosphodiesterase inhibitor IBMX (30 min pretreatment at 10 μM). Cells were pretreated for 30 min with PF-04418948, EP\(_2\) antagonist, (1μM) or GW 627368X, EP\(_4\) antagonist (1μM) before PGE\(_2\) treatment. Results are expressed as a percentage of β\(_2\)-agonist-induced cAMP. Statistical analysis was performed using two-way ANOVA then Bonferroni’s post-test (where * denotes a significant effect of β\(_2\)-agonists or PGE\(_2\) on cAMP production, respectively (P<0.05)). Data are mean±SEM values from n=4 primary ASM cell cultures.
7.3.3 Role of EP receptor antagonists on PGE₂-induced β₂-AR desensitization (standard protocol)

We mostly followed the standard protocol (ASM cells are allowed one week to grow before serum starvation) in this thesis. Data in Figure 7.2 demonstrated that EP₂ antagonist reverse PGE₂-induced β₂-AR desensitization by restoring cAMP production. As such the data in Figure 7.2 generated using short protocol; hence we aim here to explore the effect of both antagonists (EP₂ and EP₄) in PGE₂-induced β₂-AR desensitization following the standard protocol. ASM cells were pretreated with PGE₂ (0.1–100 nM) for 24 h and cAMP production was measured in response to two β₂-agonists: long-acting β₂-agonist formoterol (Figure 7.3A) and short-acting β₂-agonist salbutamol (Figure 7.3B). In parallel, the cells were treated with specific EP₂ or EP₄ receptor antagonists, 30 min before PGE₂ treatments. Although, both protocols (short and standard) produced similar results in terms of PGE₂-induced β₂-AR desensitization, variable results obtained in terms of antagonism. Unlike short protocol, in standard protocol none of the EP₂ or EP₄ receptor sems to be involved in β₂-AR desensitization.
Figure 7.3 Role of EP receptor antagonists on PGE₂-induced β₂-AR desensitization (standard protocol). Growth-arrested ASM cells were pretreated for 24 h with vehicle, or a range of PGE₂ concentrations (0.1-100 nM). Desensitization of the β₂-AR was assessed by measuring production of cAMP in response to stimulation with (A) 0.01 µM formoterol or (B) 10 µM salbutamol for 15 min, compared to vehicle, in the presence of the pan-phosphodiesterase inhibitor IBMX (30 min pretreatment at 10 µM). Cells were pretreated for 30 min with PF-04418948, EP₂ antagonist, (1µM) or GW 627368X, EP₄ antagonist (1µM) before PGE₂ treatment. Results are expressed as a percentage of β₂-agonist-induced cAMP. Statistical analysis was performed using two-way ANOVA then Bonferroni's post-test (P<0.05)). Data are mean±SEM values from n=4 primary ASM cell cultures.
7.4 Discussion

The human lung is a rich source of prostaglandins, particularly PGE$_2$ (Karim et al., 1967). Despite intense research for several decades focusing on the functional effects of PGE$_2$ in the lung and other tissues, this interesting molecule still offers a vast scope of study to uncover the complex biology associated with it. PGE$_2$ is a multifunctional protein and its actions are mediated via complex crosstalk between EP$_{1-4}$ receptors (Figure 7.4) (Chell et al., 2006). Previous reports have revealed the expression of EP$_2$ and EP$_4$ receptors in ASM cells that upon ligand binding lead to the secretion of cAMP (Clarke et al., 2004; Clarke et al., 2005a; Clarke et al., 2005b). Anti-inflammatory protein, MKP-1 is a promising target to attenuate the inflammatory responses mediated by MAPK activation (Franklin and Kraft, 1997; Abraham and Clark, 2006). MKP-1 serves a crucial negative feedback role in regulating pro-remodelling signal transduction. The anti-inflammatory action of MKP-1 is shown to be mediated in a cAMP dependent manner in ASM cells (Manetsch et al., 2012a; Manetsch et al., 2013; Patel et al., 2015a). Moreover, MKP-1 expression is found to be CREB responsive (Cho et al., 2009). This study is the first to explore the direct effect of PGE$_2$ on MKP-1 expression. As a cAMP elevating agent PGE$_2$ dose dependently upregulates MKP-1 mRNA expression. Moreover, this effect found unaffected using EP$_2$ or EP$_4$ antagonists, indicating that these receptors are not involved in PGE$_2$-induced MKP-1 secretion. Further experiments to reveal the molecular pathways associated with PGE$_2$-induced MKP-1 protein level and identify the cAMP mediated downstream signalling pathways are warranted.
Figure 7.4 PGE$_2$ signalling pathways through EP$_{1-4}$ receptors (Chell et al., 2006).

We have also attempted to explore the underlying molecular pathways of PGE$_2$-induced β$_2$-AR using two different established protocols for cAMP experiments; short (cells are allowed to grow for 48 h) and standard (cells are allowed to grow for one week). Although using the short protocol it appears EP$_2$ may be involved in β$_2$-AR receptor desensitization as EP$_2$ antagonist restores the cAMP level which was repressed by PGE$_2$, but, the standard protocol did not reproduce the same result. However, in both protocols it was found that stimulation with PGE$_2$ induces heterologous desensitization of β$_2$-AR in ASM cells. It is not clear what causes the apparent disparity between these studies in terms of receptor antagonism, but due to the difference in cell plating time among these two protocols, a number of possibilities can be considered including, differential production of cAMP and difference in cell cycle progression.
Previous reports also suggest that identifying the particular receptor(s) involved in prostanoid-mediated events is complicated due to the complex association of the ligands with the receptor subtypes and similarity in second messenger pathways (Tsuboi et al., 2002). In this study, we only focused on EP$_2$ and EP$_4$ receptors, which didn’t appear to play a major role in mediating the PGE$_2$ responses in the context of β$_2$-AR desensitization in our in vitro ASM cell model. However, our findings don’t rule out the role of other two EP receptors, EP$_1$ & EP$_3$, in the same experimental settings. Further, great advances in last few years have identified selective, well characterized compounds to study independent receptor functions and knock-out mice generation for all prostanoid receptors and subtypes. This will help to elucidate the physiological and potential pathological roles of this receptor family (Kobayashi and Narumiya, 2002; Naganawa et al., 2006; Murase et al., 2008; Maubach et al., 2009; Singh et al., 2009; af Forselles et al., 2011). Future experiments to understand the further levels of EP receptor regulation are warranted.
Chapter 8

General Discussion and Conclusion
Chapter 8
General Discussion and Conclusion

8.1 Overview

Millions of people worldwide are affected by asthma and its prevalence is increasing day by day. It is the most common chronic disease both in children and adults and a major cause of disability and poor quality of life: physically, emotionally, socially and professionally, for those who are affected. Asthma prevalence is high (> 10%) in developed countries and rates are increasing in developing regions as they become more westernized. It is, therefore, regarded as a global burden (Braman, 2006). Along with loss of health and work productivity, its global prevalence also causes economic hardship due to increasing health resource utilization and consumption of considerable health care costs. Despite considerable efforts having been taken over the past decades into understanding the many aspects of asthma, the basic causes of the disease mechanism and the reasons for its increased prevalence remain largely unknown.

Asthma is a chronic pulmonary inflammatory diseases characterized by airway inflammation, airway hyperresponsiveness and airway remodelling. It is a long term disease that has no cure although good therapeutic management can control its severity by maintaining good lung function. Currently, corticosteroids and β₂-agonists are the most commonly used therapy against the inflammatory condition caused by asthma. However, glucocorticoid insensitivity is a considerable problem in respiratory disease, as is β₂-AR desensitization. Acute exacerbations of asthma are of particular importance in these cases, where the drugs become much less effective or the receptors become irresponsible. Viruses and bacteria are considered the most common cause of asthma exacerbation. It is therefore very important to understand the molecular
mechanisms underlying receptor hyporesponsiveness in the infectious asthmatic condition to improve the efficacy of drug treatment. A better insight into the pathways and mechanisms associated with infectious asthmatic conditions would allow us to use this knowledge for the future development of anti-inflammatory therapy with the most targeted but least side effects. In this project, we therefore aimed to explore the underlying molecular mechanisms involving TLR2 receptor activation, COX-2 protein upregulation and β2-AR desensitization in ASM cells during infectious asthma exacerbation.

8.2 Our approaches

In this thesis we have focussed on the role of the ASM in asthma and asthma exacerbation. The primary ASM cell lines were established from human bronchi obtained from patients undergoing surgical resection for carcinoma or lung transplant donors in accordance with procedures approved by the Sydney South West Area Health Service and the Human Research Ethics Committee of the University of Sydney.

Apart from mucus plugging and swelling of airway tubes, ASM cells are believed to be the main effector cells that amplify airway narrowing and airway remodelling by changing their (ASM cells’) proliferative, contractile and synthetic functions via a number of inflammatory signalling pathways. Moreover, ASM cells play a role in the amplification of inflammatory responses during infectious exacerbations in asthma via augmented cytokine production. Further, recent detection of functional TLRs expression in ASM cells has great potential in utilizing ASM cells as a model of infectious asthma exacerbation. The main aim of this thesis, therefore, was to investigate the bacterial infections (TLR2 engagement by Pam3CSK4) generated outcomes in
ASM cells. The role of ASM cells in inflammasome activation and COX-2 secretion upon TLR2 ligation was identified.

Further studies investigated the molecular mechanisms underlying COX-2 upregulation and its regulation and impact on airway inflammation. COX-2 has long been regarded as playing a pivotal role in the pathogenesis of airway inflammation in respiratory diseases such as asthma and COPD. COX-2 can be rapidly and robustly expressed in response to a diverse range of pro-inflammatory cytokines and mediators. Thus, increased levels of COX-2 protein and prostanoid metabolites serve as key contributors to pathobiology in respiratory diseases typified by dysregulated inflammation. Bronchodilators, β2-agonists are the front line therapy in bronchial asthma. However, it is well-recognized that the β2-AR on human ASM cells is subject to heterologous desensitization induced by a COX-2 product, PGE2. These studies aimed to investigate the clinical consequences of COX-2-mediated upregulation of PGE2 in ASM cells in the context of β2-AR desensitization.

This was addressed by five projects:

1. **Identifying the possible role of TLR2-induced activation of inflammasome in ASM cell cytokine secretion**
   - TNFα-induced IL-1β mRNA expression and augmentation by Pam3CSK4
   - Pam3CSK4 potentiates TNFα-induced IL-6 and IL-8 protein secretion, but IL-1β is not secreted from ASM cells
   - Neutralization of IL-1β in conditioned media has no effect on cytokine secretion
   - Pam3CSK4 does not affect TNFα-induced NLRP3 and caspase-1 mRNA expression in ASM cells
This part of the study provides a better understanding of how infectious inflammatory stimuli affect pro- and anti-inflammatory mechanisms in ASM cells. Although the TLR2 ligand, Pam3CSK4, robustly up-regulated ASM cytokine expression in response to TNFα and significantly enhanced IL-1β mRNA expression, we were unable to detect IL-1β in the cell supernatants. Thus, we suggested that IL-1β was not secreted and therefore was unable to act in an autocrine manner to promote the amplification of ASM inflammatory responses. In conclusion, these data demonstrate that the enhanced synthetic function of ASM cells, induced by infectious exacerbations of airway inflammation, is NLRP3 inflammasome and IL-1β independent. Although NLRP3 is found to be present in human airway epithelium, these results prove that this protein is not detected in the nearby ASM bundles, suggesting that activation of the NLRP3 inflammasome by invading pathogens may prove cell type specific in exacerbations of airway inflammation in asthma.

2. Investigation of TLR2-induced β2-AR desensitization via COX-2 production

- TLR2 ligand engagement upregulates TNFα-induced COX-2 mRNA expression and increases PGE₂ secretion
- PGE₂ induces heterologous β₂-AR desensitization as measured by inhibition of β₂-agonist-induced cAMP production
- Celecoxib inhibits PGE₂ secretion but not COX-2 mRNA expression
- Pam3CSK4 + TNFα cause tachyphylaxis of β₂-AR agonists mediating MKP-1 gene expression and this can be reversed by celecoxib

This study gives an insight into the functional outcomes associated with TLR2-induced COX-2 in the context of β₂-AR desensitization. Utilizing an in vitro model of bacterial exacerbation in ASM cells we showed that activation of TLR2 in the presence of an inflammatory stimulus leads
to $\beta_2$-AR desensitization. This occurs via TLR2 dependent upregulation of COX-2 mRNA expression and increased secretion of PGE$_2$. Importantly, PGE$_2$ causes heterologous $\beta_2$-AR desensitization and reduces cAMP and MKP-1 production in response to short-acting (salbutamol) and long-acting (formoterol) $\beta_2$-agonists. Thus, infectious bacterial stimuli act in a PGE$_2$-dependent manner to severely curtail the beneficial actions of $\beta_2$-agonists.

3. Examination of COX-2 expression and PGE$_2$ secretion by S1P and its effects on $\beta_2$-AR desensitization

- S1P upregulates COX-2 mRNA expression and protein upregulation to increase PGE$_2$ secretion from ASM cells
- COX-2 knockdown by siRNA represses S1P-induced COX-2 mRNA expression, protein upregulation and PGE$_2$ secretion
- TNF$\alpha$ enhances S1P-induced COX-2 mRNA expression and protein upregulation and increases PGE$_2$ secretion
- S1P induces heterologous $\beta_2$-AR as measured by inhibition of $\beta_2$-agonist-induced cAMP production; in a manner independent of adenylate cyclase

It was shown that S1P plays an important role in the pathophysiology of asthma and also induces $\beta_2$-AR desensitization in bronchial ASM cells and exerts hyporesponsiveness to $\beta_2$-agonists. ASM cells were treated with S1P (1 $\mu$M) for up to 24 h and then the temporal kinetics of COX-2 mRNA expression, protein upregulation and PGE$_2$ secretion were examined. S1P significantly enhanced COX-2 expression and PGE$_2$ secretion and this was repressed by the selective COX-2 inhibitor, celecoxib, the corticosteroid dexamethasone, or siRNA knockdown of COX-2 expression. In combination with another pro-inflammatory mediator found elevated in asthmatic airways, the cytokine TNF$\alpha$, it was observed that S1P-induced COX-2 mRNA expression and
Chapter 8

protein upregulation and also PGE$_2$ secretion from ASM cells was significantly enhanced. Notably, S1P-induced heterologous $\beta_2$-AR as measured by inhibition of cAMP production in response to the short-acting $\beta_2$-agonist, salbutamol, and the long-acting $\beta_2$-agonist, formoterol. Taken together, these data indicate that S1P represses $\beta_2$-adrenergic activity in ASM cells by increasing COX-2 mediated PGE$_2$ production, and suggest that this bioactive sphingolipid found elevated in asthma may contribute to $\beta_2$-AR desensitization.

4. IL-17A increases TNF$\alpha$-induced COX-2 protein stability and augments PGE$_2$ secretion from ASM cells

- IL-17A augments TNF$\alpha$-induced PGE$_2$ secretion, but does not increase TNF$\alpha$-induced COX-2 mRNA expression
- IL-17A increases TNF$\alpha$-induced COX-2 protein upregulation
- Proteasome inhibitors increase TNF$\alpha$-induced COX-2 protein and PGE$_2$ secretion
- IL-17A enhances TNF$\alpha$-induced COX-2 protein stability
- Heterologous $\beta_2$-AR desensitization as measured by inhibition of $\beta_2$-agonist-induced cAMP production in ASM cells: effects of IL-17A ± TNF$\alpha$

IL-17A plays an important role in respiratory diseases and is a known regulator of pulmonary inflammation and immunity. Recent studies have linked IL-17A with exacerbation in asthma and COPD. In this study, we showed that IL-17A induces a robust and sustained upregulation of COX-2 protein and PGE$_2$ secretion from ASM cells. COX-2 can be regulated at transcriptional, post-transcriptional and/or post-translational levels. We have elucidated the underlying molecular mechanisms responsible for the sustained upregulation of TNF$\alpha$-induced COX-2 by IL-17A in ASM cells and showed that it is not via increased COX-2 gene expression. Instead, TNF$\alpha$-induced COX-2 upregulation is subject to be regulated by proteasome, and IL-17A acts to
increase TNFα-induced COX-2 protein stability by preventing its proteosomal degradation. In this way, IL-17A acts to amplify the COX-2-mediated effects of TNFα and greatly enhances PGE₂ secretion from ASM cells. As PGE₂ is a multifunctional prostanoid with diverse roles in respiratory disease, the experimental results demonstrate a novel function for IL-17A in airway inflammation.

**Identifying the role of specific EP receptors**

- Role of EP₂ and EP₄ receptors in PGE₂-induced upregulation of MKP-1
- Role of EP₂ and EP₄ receptors in PGE₂-induced β₂-AR desensitization

This study aimed to explore the different functional outcomes related molecular pathways generated by the diverse functional molecule PGE₂. Considering that PGE₂ synthesis is initialized by COX-2 and then binds with four distinct receptors, EP₁-₄, each with exclusive expression patterns throughout the body; it is predictable that this prostanoid is implicated in many and sometimes seemingly opposing functions in humans. PGE₂ is a bronchorelaxant via releasing cAMP by binding with EP₂ and EP₄ receptors. MKP-1 is a critical anti-inflammatory protein that is produced by cAMP mediated pathways. As a cAMP elevating agent, this study attempted to show the effect of PGE₂ on MKP-1 mRNA expression directly and for the first time herein, it was shown that in ASM cells PGE₂ dose dependently induces MKP-1 mRNA expression. However, we were unable to antagonize this effect by using specific EP₂ and EP₄ receptor antagonists. This indicates that these receptors might not be involved in this process. In previous studies we demonstrated that COX-2 mediated PGE₂ is responsible for β₂-AR desensitization. Further studies were therefore attempted to elucidate the role of the specific receptors responsible for this effect. Two different established protocols were followed. According to short protocol, it appears that EP₂ but not EP₄ is associated with β₂-AR desensitization. Using the
standard protocol, however, these results were not in agreement. The precise mechanism by which PGE₂ contributes to the inflammatory response by causing β₂-AR desensitization has not yet been identified, although some preliminary evidences that warrant further work to clarify which class of receptor is involved have been provided. Collectively, the results gained from these studies have provided answers to the initial research aims. It has been established that TLR2 activation by Pam3CSK4 in an in vitro ASM cell model does not activate NLRP3 inflammasomes but does induce COX-2 and PGE₂ production. It was also shown that inflammatory stimuli, S1P and IL-17A, also upregulate COX-2-induced PGE₂ in this in vitro model. Reduced responsiveness of β₂-AR is a characteristic feature in asthma exacerbation. Our studies demonstrate that the TLR2 agonist, Pam3CSK4, and S1P cause β₂-AR desensitization in the presence of TNFα. The study using IL-17A has provided intriguing evidence to suggest that TNFα-induced COX-2 upregulation is subject to be regulated by the proteasome, and IL-17A acts to increase TNFα-induced COX-2 protein stability. Further, some preliminary evidences have been provided to identify the specific role of EP₂ and EP₄ receptors in the context of airway inflammation. The main findings from this thesis are outlined in a schematic diagram (Figure 8.1).
8.3 Conclusions and future directions

A growing body of evidence related to TLR2 signalling has demonstrated important relationships between these pathways and infectious asthma exacerbation. Activation of TLRs by microbial components triggers expression of several genes that are involved in immune responses. The aim of this thesis was to identify the signalling cascades that lead to bacteria-induced asthma exacerbation. In particular, the involvement of TLR2 receptor activation and COX-2 protein upregulation in disease exacerbation has been examined. The experimental results suggest that manipulation of TLR pathways, thereby controlling the expression of further inflammatory mediators, will provide great therapeutic potential in the treatment of asthma and COPD. Evidence has been provided that TLR2 ligand engagement is unable to induce inflammasome activation. This proves that inflammasome activation appears to be cell-type specific in airway
inflammation. It would be of interest to investigate further crosstalk between airway structural cells and inflammatory cells in the context of the functional relevance of the restricted localization of the NLRP3 inflammasome in the airways.

Further, it has been shown that TLR2 engagement increased COX-2 expression and therefore, PGE\textsubscript{2} release by ASM cells. A previous report suggests that COX-2 products, including PGE\textsubscript{2}, are responsible for \textit{rhinovirus}-induced β\textsubscript{2}-AR desensitization (Van Ly et al., 2013). In accordance with that, this study supports the premise that bacterial infection directly affects β\textsubscript{2}-AR function in a similar manner to viral infection. To date, β\textsubscript{2}-agonists are considered the first line therapy for asthma, and β\textsubscript{2}-AR present in ASM cells are the most important target of β\textsubscript{2}-agonist therapy. However, an increasing body of evidence suggests that several cytokines present in the airway microenvironment are some of the most important factors that influence β\textsubscript{2}-AR responsiveness in ASM (Shore and Moore, 2002). This study has shown that, as with TLR2 agonist, S1P has the potential to repress β\textsubscript{2}-adrenergic activity via increasing COX-2 mediated PGE\textsubscript{2} production in ASM cells. However, the study with 1L-17A suggested that due to its inability to induce PGE\textsubscript{2}, IL-17A did not cause β\textsubscript{2}-AR desensitization.

The data presented has increased the knowledge of the mechanistic basis for the β\textsubscript{2}-AR dysfunction in asthma and may prove to be an important step in improving the efficacy of these agents, as well as provide new ways to discover the roles of bacterial infection in asthma pathogenesis and exacerbation. We suggest that more targeted therapies are needed to prevent β\textsubscript{2}-AR desensitization in response to viral and bacterial infections.

According to accumulating research it is obvious that not only COX-2 but also its products, prostanoids, play an important role in the pathophysiology of several pulmonary dysfunctions. Moreover, prostanoids show their diverse functions, either pro-inflammatory or anti-
inflammatory, by binding with several receptor subtypes. There are at least eight types and subtypes of membrane prostanoid receptor forms characterized in mouse and human, including two subtypes of the PGD receptor (DP₁ and DP₂), four subtypes of the PGE receptor (EP₁, EP₂, EP₃, and EP₄), the PGF receptor (FP), the PGI receptor (IP) and the TxA receptor (TP) (Narumiya and FitzGerald, 2001). Binding of receptor specific ligands with their respective receptors can result in varying responses in different cells and tissue types that may be either beneficial or deleterious. In brief, PGD₂ is a potent bronchoconstrictor and vasodilator acting via DP₁, DP₂ and the TP receptor (Johnston et al., 1995; Larsson et al., 2011). PGI₂ is capable of restraining allergic airway inflammation in mice by inhibiting dendritic cell activation of CD4+ Th2 cells, Th2 cell differentiation and eosinophil migration through the IP receptor. Among other factors, prostanoid thromboxane A₂ (TXA₂) is considered to be an inflammatory and bronchoconstrictive mediator in asthma and PGF₂α plays an important role in pulmonary fibrosis (Claar et al., 2015). The activity of PGE₂ via signalling through EP₂ and EP₄ receptors promotes the accumulation of cAMP. This leads to smooth muscle relaxation, whereas EP₁ and EP₃ receptor activation increases intracellular calcium or decreases cAMP generation that could be associated with smooth muscle contraction (An et al., 1993; Coleman et al., 1994; Qian et al., 1994; Breyer et al., 2001; Vancheri et al., 2004; Kay et al., 2006; Foudi et al., 2008; Machado-Carvalho et al., 2014; Claar et al., 2015). Our results demonstrate both the inflammatory and anti-inflammatory effects of PGE₂ by causing β₂-AR receptor desensitization and by releasing the anti-inflammatory protein, MKP-1, respectively. This could be attributed to the association of different EP receptors with various physiological functions.

The complexity of COX-2 and prostaglandin in the pathogenesis of inflammatory lung diseases is well reviewed by Park et al. (Park and Christman, 2006). Up until now the most common way
to target COX-2 has been to inhibit its enzymatic activity via non-selective (e.g. NSAIDs) or selective inhibitors (such as coxibs). The complete inhibition of COX enzyme with non-selective drugs inhibits both COX-1 and COX-2, producing severe side effects by blocking the good prostanoids production via COX-1. Taking this into account, a number of selective COX-2 inhibitors have been discovered but as some COX-2 products also exhibit protective role in various circumstances, it is crucial to gain further knowledge about the mechanism of COX-2 inhibitors in airway cells.

As the challenge now lies in discovering the potential target to overcome β2-AR desensitization, our project aimed to identify a specific receptor mediating the COX-2/ PGE2-induced β2-AR desensitization in ASM cells. We propose that selectivity profiling of each receptor subtype is as equally important as exploring specific receptor agonists or antagonists with the most targeted but least side effects in the context of inflammatory respiratory diseases. Further studies using individual prostanoid receptors in knocked-out mice will offer a new avenue for greater understanding the impact of COX-2 products in clinically relevant respiratory disease.

We hypothesised that TLR2 receptor activation may upregulate inflammasomes and COX-2 in ASM cells. We found that NLRP3 inflammasomes were not activated in ASM upon TLR2 ligation. However, our experimental settings cannot rule out the presence of other inflammasomes. This warrants future investigation. Moreover, although earlier report suggests that in some in vitro settings a secondary signal is necessary to stimulate active inflammasomes secretion (Netea et al., 2009), we didn’t attempt that in our study. That may explain our negative results. Therefore, we believe that there is a wide scope for future experiments in this area. Intriguingly, in a parallel study, we found that COX-2 protein is upregulated in ASM cells upon TLR2 ligation. Considering the potential role of COX-2 in airway inflammation, we further
hypothesized that COX-2 may cause β2-AR receptor desensitization via PGE2 secretion. Our data demonstrated that, not only TLR2 ligand, but also S1P, caused β2-AR desensitization via the COX-2-dependant PGE2-mediated pathway. Another important inflammatory mediator, IL-17A, although having no effect on β2-AR desensitization, induced stabilization of COX-2 protein, which is a novel finding. In our studies, we investigated the direct effects of S1P and IL-17A on COX-2 upregulation. Previous reports, however, suggest that these inflammatory cytokines can be released or have a potential link with infectious asthmatic conditions (Essilfie et al., 2011; van Rossum et al., 2014; Lunding et al., 2015; Roos et al., 2015). Thus, further investigations linking infection and S1P & IL-17A mediated COX-2 upregulation is necessary. We believe these research findings will advance our knowledge in the context of β2-AR desensitisisation and merits further investigation using contemporary experimental models to translate into clinical outcomes. However, the studies performed in this thesis are not out of limitation. In this project, we used mimic bacterial infection PAM3CSK4, although it is an established TLR2 ligand, we feel that the use of the actual pathogen may strengthen our findings. Moreover, due to rare availability of asthmatic cells, we used non-asthmatic ASM cells in our studies. Research findings support that there are some intrinsic differences between asthmatic and non-asthmatic cells in terms of cell proliferation and expression of various matrix proteins, as well as altered calcium handling (Johnson et al., 2001; Johnson et al., 2004; Mahn et al., 2010). This should be considered in future studies.

In conclusion, this thesis provides further understanding of the TLR2 receptor activation and COX-2 upregulation in ASM cells. The research carried out explored the role of COX-2, induced by TLR2 agonist and some other inflammatory mediators, in β2-AR desensitization. Further, this is the first evidence showing IL-17A-induced COX-2 regulation in ASM cells. Overall, we
strongly believe that, according to the results of this project, we are now well-positioned to unravel some important complexities associated with asthma exacerbation and hopefully develop efficacious pharmacotherapeutic strategies to treat respiratory disease without unwanted side effects. Hopefully, this will happen in the near future.
Chapter 9
References
Chapter 9

References


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