The impact of preciser MKP-1 regulation and modulation on cytokine expression in asthma and airway remodelling
Prabhala, Pavan

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The impact of precise MKP-1 regulation and modulation on cytokine expression in asthma and airway remodelling

by

Pavan Prabhala

A thesis submitted for the degree of

Doctor of Philosophy

Faculty of Pharmacy

University of Sydney, Sydney

January 2016
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Declaration

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 associate-supervision of Dr Nicole M. Verrills, School of Biomedical Sciences and Pharmacy (medical biochemistry), 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.

There are no conflicts of interests to report

Pavan Prabhala 06.05.2016
Publications

Journal Articles

Nowshin N. Rumzhum, Brijeshkumar S. Patel, **Prabhala P**, Ingrid C. Gelissen, Brian G. Oliver, and Alaina J. Ammit. IL-17A increases TNFα-induced COX-2 protein stability and augments PGE2 secretion from airway smooth muscle cells. Allergy, November 2015 doi: 10.1111/all.12810


Reviews

Presentations

Poster Presentations

National


Patel BS, Prabhala P, Oliver BG, Ammit AJ. Inhibitors of PDE4, but not PDE3, increase expression of anti-inflammatory MKP-1 in airway smooth muscle cells. American Journal of Respiratory Cell and Molecular Biology, submitted for poster presentation to the Australian health and medical research congress (Melbourne, November 2014)

International

Pres

Oral Presentation

Prabhala P, Bunge K, Rahman MM, Ge Q, Clark AR, Ammit AJ. Temporal regulation of cytokine mRNA expression by tristetraprolin: dynamic control by p38 MAPK and MKP-1. *American Journal of Physiology, Lung Cellular and Molecular Physiology*, Faculty of Pharmacy postgraduate conference (Sydney, November 2014)
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<tbody>
<tr>
<td>AHR</td>
<td>Airway hyper-responsiveness</td>
</tr>
<tr>
<td>AIHW</td>
<td>The Australian institute of health and welfare</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
</tr>
<tr>
<td>AP-2</td>
<td>Activator protein-2</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td>ARE</td>
<td>Adenosine + uridine-rich elements</td>
</tr>
<tr>
<td>ASM</td>
<td>Airway smooth muscle</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees celsius</td>
</tr>
<tr>
<td>CCL</td>
<td>Chemokine ligand</td>
</tr>
<tr>
<td>CAF-1</td>
<td>CCR4 associated factor</td>
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<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>Cdc</td>
<td>Cell division cycle phosphatases</td>
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<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
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<tr>
<td>C/EBP</td>
<td>CCAAT/enhancer binding protein</td>
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<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclo-oxygenase</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP response elements</td>
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<td>CREB</td>
<td>cAMP response-element binding protein</td>
</tr>
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<td>Cys</td>
<td>Cysteine</td>
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<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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</tr>
<tr>
<td>DUSP</td>
<td>Dual-specificity phosphatase</td>
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<tr>
<td>DSPc</td>
<td>Dual specificity phosphatase catalytic domain</td>
</tr>
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<td>ECM</td>
<td>Extra cellular matrix</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>GINA</td>
<td>Global initiative for asthma</td>
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<td>GM-CSF</td>
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<td>Human Antigen-R</td>
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<tr>
<td>ICAM</td>
<td>Intracellular adhesion molecule</td>
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<td>ICS</td>
<td>Inhaled corticosteroids</td>
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<td>KIM</td>
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<td>MAPK</td>
<td>Mitogen-activated protein kinases</td>
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<td>Abbreviation</td>
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<td>μg</td>
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<td>MK</td>
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<td>mRNA</td>
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<td>Ribonucleic acid</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
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<td>rpm</td>
<td>Rounds per minute</td>
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<tr>
<td>RT</td>
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<td>SDS</td>
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<td>TEMED</td>
<td>N,N,N',N' tetramethylethylenediamine</td>
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<tr>
<td>Th</td>
<td>T helper cells</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<td>TNFα</td>
<td>Tumour necrosis factor α</td>
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<td>TRIS</td>
<td>Tris(hydroxymethyl)aminomethane</td>
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<td>TTP</td>
<td>Tristetraprolin</td>
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<td>3’-untranslated region</td>
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<td>WB</td>
<td>Western blotting</td>
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<tr>
<td>WT</td>
<td>Wild type</td>
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Abstract

Asthma is a chronic inflammatory disease of the lungs and airways, which is typically characterised by reversible airway obstruction, airway hyper responsiveness and structural remodelling. β2 adrenoceptor agonists and inhaled corticosteroids are the main therapeutic treatments to asthma however these treatments are not always effective especially in patients with severe asthma. Hence alternative strategies need to be uncovered and investigated in order to manage these patients adequately. As an alternative strategy we aimed to look at the molecular level by exploring and harnessing the underlying temporal mechanics of endogenous anti-inflammatory molecules present during the inflammatory process. In this study we investigate the p38 MAPK-mediated regulatory pathway and the interaction of two anti-inflammatory proteins; mitogen-activated protein kinase phosphatase 1 (MKP-1) and tristetraprolin (TTP), along this pathway in the context of asthmatic inflammation.

Antibody validation is an important step for the generation of reliable and reproducible results. In the context of asthmatic inflammation primary cultures of ASM and MDA-MB-231 cells were utilized in vitro. Seven antibodies were tested under different experimental conditions and their sensitivity was measured using western blot analysis. The best results were achieved using antibody 5 and 7 5% skim milk as a blocking agent in congruence with overnight antibody incubation.

Once we had obtained reliable results from the antibodies we investigated the p38 MAPK-mediated interaction between MKP-1 and TTP. Utilizing primary cultures of ASM cells in vitro we explored the temporal regulation of IL-6 cytokine mRNA expression upon stimulation with tumor necrosis factor α (TNFα) and dexamethasone in a preventative protocol (added 1h after TNFα addition). Intriguingly, the temporal profile of mRNA expression was biphasic, however this expression was altered in the presence of dexamethasone, which acts to induce MKP-1 in a
temporally distinct manner. We showed that the biphasic nature of TNFα-induced IL-6 mRNA expression was regulated temporally by TTP. Importantly, TTP function is controlled by p38 MAPK and our study reveals that its expression in ASM cells is p38 MAPK-dependent. Moreover, its anti-inflammatory activity is controlled by p38 MAPK-mediated phosphorylation. Since MKP-1 is a MAPK deactivator; thus, by controlling p38 MAPK phosphorylation status in a temporally-distinct manner, endogenous and corticosteroid induced MKP-1 ensures that TTP is modulated and made functional at precisely the correct time to repress cytokine expression. Together, p38 MAPK, MKP-1 and TTP form a regulatory network that exerts significant control on cytokine secretion in pro-asthmatic inflammation through precise temporal signalling.

Since MKP-1 is an integral part of the anti-inflammatory negative feedback loop that is able to curtail the pro-inflammatory actions of p38 MAPK, and it is possible for its activity to be modulated, it represents a potential therapeutic target. This is evidenced through the use of dexamethasone in the previous study. However, this reduction in pro-inflammatory functions is temporary as MKP-1 is rapidly degraded via the proteasome. Targeting this molecule, and preventing its degradation represents another way of keeping it active for longer, and hence a potential long acting anti-inflammatory strategy. Compounds that can bind to MKP-1 at the catalytic region and prevent it from degradation were identified using a homology model based on the crystal structure of MKP-2. Utilizing primary ASM cell cultures, 2 of these compounds were tested using western blot and ELISA analysis. MKP-1 is known to reduce the levels of p38 MAPK keeping MKP-1 active for longer. The results showed that both compounds were able to increase MKP-1 levels, however they did this at different concentrations. This increase in MKP-1, coincided with a subsequent drop in the levels of phosphorylated p38 MAPK. Together this data suggests that harnessing the power of the endogenous anti-inflammatory molecule MKP-1 can be a potential anti-inflammatory therapeutic.
Chapter 1:

General Introduction
1 Chapter 1: General Introduction

1.1 Asthma

Asthma is chronic inflammatory condition of the airways that is associated with serious health and socioeconomic concerns. Reports from the World Health Organisation (WHO) and the Global Initiative for Asthma (GINA) indicate that asthma affects about 300 million individuals of all ethnic backgrounds worldwide (according to the last global study undertaken by the WHO (Bousquet et al., 2007)). This is unfortunately the latest global data available. The prevalence of asthma is expected to increase with projections predicting approximately 100 million new cases of asthma by the year 2025 (Masoli et al., 2004). Asthma is an under diagnosed and undertreated disease leading to poor long term care and a subsequent increase in financial burden, especially on those who have the most severe form of the disease. This increase in financial burden is not limited to the individual, but also to the associated health care system (Gold et al., 2014). Globally, developed nations have a higher prevalence of asthma than many developing nations. In Australia and North America for example, approximately 10% of the population have been diagnosed with asthma (AIHW, 2011) (which are some of the highest prevalence rates in the world) and hence it has been characterised as a national health priority area by the Australian government. In the presence of these high asthma rates a need arises to find a solution to this increasing problem.

Despite significant research into many aspects of asthma the underlying cause still remains unclear. Asthma is a chronic airway disease that presents with clinical manifestations such as wheezing, shortness of breath, coughing and chest tightness. However the duration and intensity if these symptoms varies substantially amongst individuals (Levy et al., 2006). The temporal course of these symptoms is also varied and may be present or absent at any point during the patient’s life. Medication such as inhaled corticosteroids in combination with β2-agonists have been used for many
years and have proven to be effective in reducing asthmatic episodes in mild and moderate asthmatics, however this therapy is not very effective in treating patients with severe asthma, 10% of whom require maximal doses of inhaled corticosteroids and some of these patients even require oral corticosteroids. Even after receiving maximum oral doses of corticosteroids if they are unable to improve their forced expiratory volume (FEV$_1$) they are defined as patients who are steroid resistant (Chung et al., 1999).

It is these patients who are in the 10% of severe asthmatics that account for a majority of the socio-economic burden. Hence alternative strategies need to be uncovered and investigated in order to manage these patients adequately. The strategy that we have employed is to tackle this problem on a molecular level, where it is possible to identify pharmacotherapeutic targets with greater specificity. Hence we attempted to gain an increased understanding into the complex molecular pathways that lead to many of the clinical manifestations associated with chronic asthma. Using this strategy another avenue to enhance the prognosis of patients with severe asthma can be potentially elucidated. In order for us to explore this avenue we need to understand the pathology of asthma by identifying the mechanisms and molecules that drive its pathogenesis.

As noted above the underlying cause of asthma remains unclear, however what is understood are the risk factors that contribute to the pathophysiology of asthma. The strongest risk factors are commonly associated with genetic, environmental and lifestyle factors. Combined these factors may trigger asthmatic symptoms (Table 1.1).
### Table 1.1 Major risk factors for asthma

<table>
<thead>
<tr>
<th>Asthma trigger</th>
<th>Specific triggers</th>
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<td>Environmental irritants</td>
<td>Cold/ dry air</td>
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<td>Dust</td>
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<td>Tobacco smoke</td>
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<td>Car exhaust fumes</td>
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<td>Indoor allergens</td>
<td>House dust mites</td>
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<td>Pet and animal dander</td>
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<td>Incense</td>
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<td>Outdoor allergens</td>
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<td>Mould spores</td>
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<td>Chemical irritants</td>
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<td>Physical exercise</td>
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<td>Hormonal changes</td>
<td>Oestrogen</td>
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<td></td>
<td>Progesterone</td>
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<td>Follicle stimulating hormone</td>
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<td></td>
<td>Luteinising hormone</td>
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<td>Obesity</td>
<td>Obesity driven inflammation</td>
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<td>Viral and respiratory infections</td>
<td>Rhinovirus</td>
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<td>Influenza A</td>
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<td>Respiratory syncytial virus</td>
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<td>Medicines</td>
<td>Aspirin</td>
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<td>NSAIDs</td>
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### 1.1.1 The pathology of asthma

The pathophysiology of asthma is centred on airway inflammation and relates to airway dysfunction partly through the release of inflammatory mediators and partly through remodelling of the airways. However both act in concert with one another in a positively reinforced cycle to develop the chronic inflammatory phenotype. Usually, most asthma starts in relation to the sensitization to common household allergens described above in Table 1.1. When exposed to these allergens, dendritic cells are able to stimulate naïve T cells and induce differentiation into T helper 1 cells (Th1), T helper 2 cells (Th2), T helper 9 cells (Th9) and T helper 17 cells (Th17), which are then able to proliferate and cause the secretion/migration of cytokines (Bowen et al., 2008), eosinophils, basophils, mast cells, neutrophils and macrophages (Swain et al., 1990; Kroegel et al., 1994; Virchow et al., 1994; Metcalfe et al., 1997) (Figure 1.1). This release of inflammatory mediators acts in triggering more inflammatory mediators leading to persistent inflammation. This persistent inflammation can lead to mild, moderate or severe asthma depending on intensity and duration of the inflammatory assault. As stated above severe asthma represents the smallest proportion of asthmatics (10%) but they account for most of the burden associated with asthma. (Chung 2016). Hence, before we proceed it is
important to characterise the various phenotypes of asthma and keep them in mind although we will not be delving much deeper into the mechanistic differences associated with the different phenotypes. This release of cytokines is also responsible in determining the phenotype of asthma. The two main phenotypes are the eosinophilic and the non-eosinophilic asthma. Eosinophilic asthma is defined as asthma with elevated levels of sputum and bronchial eosinophils. This phenotype accounts for 50% of asthmatics. The other 50% fall into the non-eosinophilic asthma. This phenotype can be further subdivided into neutrophilic and paucigranulocytic asthma. This phenotype is determined low levels of Th2 cytokine expression and sometimes by the absence of elevated eosinophils (eosinophil numbers are similar to basal levels). Neutrophilic asthma is thought to be present when sputum neutrophils are expressed above a threshold for normal neutrophils. The paucigranulocytic phenotype of asthma is one in which both neutrophils and eosinophils are increased above threshold levels. As there are differences in phenotypes of asthma there are also differences in the symptoms they trigger, however for the purpose of this study we are going to merge these symptoms and classify it as severe asthma as a whole. One such symptom is airway remodelling. Remodelling of the airways represents the structural changes that occur in the airways as a consequence of persistent inflammatory assault on the airways. Remodelling of the airways has been shown to trigger an increase in smooth muscle mass, smooth muscle proliferation, angiogenesis, nerve proliferation, mucus cell metaplasia, fibrosis thickening of the basement membrane and decreased cartilage integrity. (Naylor, 1962; Roche et al., 1989; Aikawa et al., 1992; Carroll et al., 1993; Elias et al., 1999; Haraguchi et al., 1999; Tanaka et al., 2003). The cells that propagate this inflammation are epithelial cells, as the first point of contact and smooth muscle cells that have recently been shown to be associated with inflammatory mediator release resulting in remodelling and structural changes (Figure1.2).

Since there is an increased expression of inflammatory mediators in the affected airways it is important to investigate the regulation of these proteins. Improper regulation of these proteins is what
leads to the remodelling phenotype which subsequently leads to the clinical manifestations of asthma. There are endogenous anti-inflammatory regulators of inflammation, however these regulators are only transiently active, leaving inflammatory signals and inflammation unchecked when they are inactive. Hence it is valuable to gain an understanding into how and when these regulators are switched on and off in order to develop ways to target these regulators to keep them switched on. Glucocorticoids that are currently prescribed for asthma, work in part by targeting these regulators and, affecting their transcription. It has been shown that in the airways of asthmatics, transcription factors including the glucocorticoid receptor (GR), NF-κB, Activator Protein-1 (AP-1), cAMP response-element binding protein (CREB) and CCAAT/Enhancer Binding Protein (C/EBP), are activated by inflammatory stimuli (Noguchi et al., 1993; Sommer et al., 2000; Wang et al., 2008; Shipp et al., 2010; Zhang et al., 2012). Some of these factors are able to switch on these regulators while others are able to switch them off. Genes that increase the synthesis of inflammatory proteins are also regulated by some of these factors. Hence it is important to examine the inflammatory and anti-inflammatory dichotomy that exists endogenously within the airways in order to increase the efficacy of current medications or develop new therapeutics with enhanced specificity.

1.1.2 Airway cells involved in asthma pathogenesis

Epithelial cells represent the first point of contact between airway irritants and the airways. Hence they play an important role in the remodelling process. This irritant insult results in the breaking of tight junctions and compromising the integrity of the epithelium. It is also involved in the recruitment of inflammatory cells to the interstitial tissue, vascular congestion and mucus secretion (Holgate et al., 2000). In addition to epithelial integrity loss, allergens are able to diffuse into the airways and come into contact with ASM cells, further enhancing inflammation (Figure 1.2).
Asthma represents the complex interaction between inflammatory cells and resident airway cells. Another important resident cell type is the ASM cells, which constitute the main effector cells in modulating airway bronchomotor tone. However, this is not the only role played by ASM cells. ASM cells actively contribute to the inflammatory and remodelling mechanisms in the airways through the recruitment, activation and trafficking of many pro-inflammatory cytokines, chemokines and extracellular matrix (ECM) proteins (Panettieri, 2002; Lazaar and Panettieri, 2005b). Autocrine and paracrine signals derived from ASM cells enable ASM cells, as well as other cells such as eosinophils to proliferate and propagate inflammation (Johnson and Knox, 1999; Teran et al., 1999; Fanat et al., 2009; Halwani et al., 2013) (Figure 1.2). When the cells have proliferated in severe asthma, they represent the largest target cell type as well as the largest potential source of inflammatory mediators, owing to their sheer representative volume (Tliba et al., 2008). This has been confirmed in an in vivo study that reported an increase in ASM thickness following prolonged exposure to allergens (Salmon et al., 1999). Evidence suggests that ASM cells are also able to propagate inflammation by migrating down chemotactic gradients to come in contact with inflammatory mediators. There are also evidence to suggest that in asthma the mechanical contraction of ASM cells is aberrant and through mechano-transduction pathways they can alter the mechanical properties of the airway wall (Noble et al., 2014). Hence we can see that the ASM cells are a pivotal cell type that are able to induce a host of inflammatory signals and their respective transduction pathways. Therefore this cell type was utilized to model and explore the mechanisms that drive asthmatic inflammation.

### 1.2 Mitogen activated protein kinases

As addressed above asthma, is driven by a number of inflammatory mediators, which are able to propagate asthma through a myriad of signalling pathways. These signalling pathways are crucial
and represent the backbone upon which asthma is built. Hence understanding these pathways will improve our knowledge of respiratory inflammation and this knowledge can then be used to find potentially sophisticated pharmacotherapeutic targets. The main signal transduction pathways that promote inflammation in ASM cells are the mitogen activated protein kinase (MAPK) pathways. These MAPKs are able to respond to various stressors as outlined above (Table 1.1) and membrane receptors to regulate downstream molecular targets culminating in the regulation of the transcriptional process.

Three well-defined phosphoproteins, ERK (extracellular signal related kinase), JNK (c-Jun N-terminal kinase) and p38 MAPK, are the MAPKs central to the signalling cascades in ASM cells. The pathway resulting in the activation of MAPKs is derived from a relatively linear framework (see Figure 1.3). Stimuli such as cellular stressors or mitogens interact in a predominately cell surface receptor-mediator manner to trigger the phosphorylation of MAPKKK which then causes the phosphorylation of MAPKK (Figure 1.3). Once MAPKK is phosphorylated it is subsequently able to phosphorylate its own substrate MAPK. Activated MAPKs represent a point of divergence leading to the phosphorylation of numerous downstream transcription factors, protein kinases and proto-oncogenes (Ashwell, 2006; Hu et al., 2007; Balakathiresan et al., 2009) (Figure 1.3).

1.2.1 p38 MAPK

Although all three MAPKs are important and play an integral part in cell growth, survival, apoptosis and inflammation, the focus of this study was on p38 MAPK and its concomitant downstream signalling effectors. The p38 MAPK-mediated pathway regulates a plethora of cellular processes that drive chronic airway inflammatory disease (Chang et al., 2012). Extensive investigation of the p38 MAPK-driven molecular mechanisms responsible for respiratory disease pathogenesis has led to drug discovery programmes that target this pathway as a potential anti-inflammatory therapy.
Moreover, p38 MAPK has also been implicated in lack of corticosteroid sensitivity (Chang et al., 2012; Ammit, 2013). P38 MAPK has also been implicated in differentiating dendritic cells into Th17 cells and hence the propagation of neutrophils and thereby non-eosinophilic severe asthma.

There are four isoforms of p38 MAPK - α, β, δ, γ - transcribed by four separate genes and characterised by different expression patterns. p38α is expressed ubiquitously and along with p38β, these isoforms are considered to be responsible for inflammatory responses. Inflammation is a central component of many respiratory diseases, especially asthma and the use of selective pharmacological inhibitors has highlighted p38 MAPK as playing a major role in disease severity and progression. Extensive investigations utilizing inhibitors have yielded valuable information regarding the role of p38 MAPK in chronic respiratory disease. p38 MAPK regulates the immunomodulatory function of various airway cells through the control of chemokine and cytokine release (Amrani et al., 2001; Hallsworth et al., 2001; Henness et al., 2006; Quante et al., 2008). p38 MAPK is also responsible for neutrophil and eosinophil migration (Rousseau et al., 1997), altering ASM contractility, causing obstruction of air flow (Lakser et al., 2002; Bhavsar et al., 2008), releasing degradative enzymes, like matrix metalloproteinases (Woo et al., 2004) and causing remodelling of airway architecture by increasing smooth muscle proliferation (Nath et al., 2006). This is achieved through the activation of numerous downstream targets and transcription factors. Much of p38 MAPK activity is directed toward inflammation and propagating inflammatory mediators, however some of its activities are directed toward its own repression. This auto regulatory mechanism presents an exciting opportunity to regulate p38 MAPK. The magnitude and duration of p38 MAPK plays such an important role in its signal transduction pathway, that its activation is as important as its inactivation. Therefore, it would logically follow that inhibiting this mediator totally or in an isoform-specific manner, may prove to have clinical efficacy as an anti-inflammatory treatment in chronic respiratory disease, especially in patients who respond very poorly to inhaled corticosteroids.
First-generation inhibitors of p38 MAPK include pharmacological inhibitors such as SB203580, BIRB 796 and SB239063. These pyridinyl imidazole compounds competitively antagonize the ATP binding site and block the activity of p38 MAPK (Lee et al., 1994). Recent studies on p38 MAPK inhibitors have used these compounds as a template to create a new set of slightly remodelled and more selective p38 MAPK inhibitors. So-called generation 2 and generation 3 inhibitors. These inhibitors offer a broad range of anti-inflammatory effects because of their enhanced selectivity for p38 MAPK. Inhibiting the p38 MAPK pathway in animal models showed a dramatic reduction in levels of pro-inflammatory cytokines and thereby reasonably attenuated chronic inflammatory responses (Young et al., 1993; Lee et al., 1994; Abdul-Careem et al., 2007; Ronkina et al., 2010). These compounds have not yet been used in the treatment of asthma but they have been used marginally in clinical trials for rheumatoid arthritis (Haddad, 2001) and Crohn’s disease (Schreiber et al., 2006).

More recently, three molecules have been recently trialled as potential anti-inflammatory molecules in chronic obstructive pulmonary disease (COPD). Losmapimod, an oral inhibitor of p38 MAPK was shown to be effective in improving plasma fibrinogen levels and levels of hyperinflation in patients with COPD (Lomas et al., 2012). However it was also revealed to be ineffective in reducing in IL-6, IL-8 and C-reactive protein. Similarly another p38 MAPK inhibitor, dilmapimod, was administered as a single oral dose in a 4 week clinical trial and demonstrated reduction in phosphorylated heat shock protein and TNFα production (Singh et al., 2010). Similar to losmapimod, dilmapimod was also able to reduce serum fibrinogen and the forced vital capacity (FVC) but unable to alter levels of IL-6 or IL-8. Another 6 week clinical study has suggested that a small molecule p38 MAPK inhibitor, PH-797804, was effective in increasing some measurable airway criteria such as forced expiratory volume (FEV1). This study also reported that PH-797804 reduced systemic fibrinogen and circulating cytokines in patients with moderate to severe COPD in comparison to the placebo patients. It was also observed that this p38 MAPK inhibitor was effective
in the presence of a bronchodilator, salbutamol (Abdul-Careem et al., 2007). However, the major limitation of these studies is in their attenuated size and their inability to impact on inflammatory disease on multiple levels.

Efficacy of these trials can be used as a yard stick to determine if p38 MAPK inhibitors can be used as monotherapy or in combination with corticosteroids. However, there are important lessons to be learned from trials of p38 MAPK inhibitors in rheumatoid arthritis. The trials have been largely unsuccessful in rheumatoid arthritis due to unexpected inefficacy and side effects. The side effects associated with the use of p38 MAPK inhibitors, such as hepato-toxicity, dizziness and skin rash, may occur as a result of potential non-specific inhibition (Heinrichsdorff et al., 2008). This non-specific inhibition is intuitive as the human genome codes over 500 functionally different kinases which share a high degree of sequence homology, hence when targeting one kinase it is more than likely to also have some off target effects, especially at higher doses. Out of the three generations of p38 MAPK inhibitors, generation 1 presented with the most toxicity especially hepato-toxicity, which was thought to be a by-product of the induction of cytochrome p450 enzymes (Godl et al., 2003). Generation 2 inhibitors were less toxic but still led to adverse events, with one study indicating that 58% of the patients receiving a p38 MAPK inhibitor experienced an adverse event (Cohen et al., 2009). They also showed that the severity of these adverse events increased with increasing inhibitor concentration. Another study on VX-702 reported that the most common adverse events were respiratory and gastrointestinal infections (Damjanov et al., 2009). Oral bioavailability data is not readily available however from the literature it can be stated that the presence of side effects due to oral administration of the drug suggests good oral bioavailability. This was attributed to the fact that p38 MAPK was a potent suppressor of the innate immune response to viral and bacterial pathogens. When these side effects were noted along with unexpected inefficacy, these molecules lost their sheen and most of them were discontinued at the level of phase two clinical trials (Genovese 2009). The lack of efficacy could also be attributed to the recruitment of alternate
pathways to induce inflammatory avenues like NF-κB (inflammation escaping via alternative pathways) (Hammaker and Firestein, 2010). Notably, Clark and Dean (Clark and Dean, 2012) have raised the important issue of p38 MAPK-driven anti-inflammatory proteins to explain the apparent failure of p38 MAPK inhibitors in the rheumatology clinic. They state “It is likely that the failure of p38 MAPK inhibitors is connected to the important role of the p38 MAPK pathway in the negative feedback control of inflammatory responses” (Clark and Dean, 2012). That is, inhibiting p38 MAPK also blocks the endogenous anti-inflammatory proteins present that naturally curtail the inflammatory response. Important anti-inflammatory molecules, including interleukin 10 (IL-10), mitogen-activated protein kinase phosphatase 1 (MKP-1) and Tristetraprolin (TTP), are upregulated in response to increased p38 MAPK activation. Hence, p38 MAPK inhibitors represent a double-edged sword. Going further upstream in the MAPK signalling pathway (see Figure 1) could be an option as the therapeutic efficacy could be higher, but there is a greater risk of incurring further non-specific side effects. Another possible option could be to target the anti-inflammatory molecules that regulate the MAPK pathway. What these p38 MAPK inhibitors have highlighted is a need for deeper understanding of the signalling pathways that control the production of pro-inflammatory cytokines, in order to determine the most optimal target along the p38 MAPK pathway. Preventing the suppression or prolonging the activation of the transiently activated anti-inflammatory molecules could be of therapeutic benefit. This is the promise of TTP.

There are two molecules along this pathway that have endogenous anti-inflammatory properties and appear to be linked through precise molecular regulation. One of these molecules is part of the mitogen activated protein kinase phosphatase (MKPs) family known as MKP-1, and the other is an mRNA destabilising protein downstream of p38 MAPK known as TTP. Hence, this study aimed to examine the link of these anti-inflammatory proteins to p38 MAPK and to each other.
1.3 Mitogen activated protein kinase phosphatase 1

There are many dual specificity phosphatases that make up a large family of proteins. Of this family, the MKPs are a subgroup of about 11 structurally similar members (Faroq and Zhou, 2004). This subgroup can be further subdivided into 2-3 distinct groups based on their substrate specificity and their subcellular localisation. Nuclear MKPs are transcribed by immediate early genes and are able to transiently accumulate and dissipate. Cytosolic MKPs usually have different kinetics and are upregulated slowly at a later time point (Keyse, 2000; Crowell et al., 2014). The first MKP was isolated based on the structure of a protein phosphatase identified in the vaccinia virus (Guan et al., 1991; Keyse and Emslie, 1992). It is the structure of this identified phosphatase that is conserved among all the MKP family members. All MKPs share a common structure for the C-terminal catalytic domain (containing key cysteine, arginine and aspartate residues). They also share the N-terminal domain containing a kinase interaction motif (KIM) sandwiched between two regions of sequence similarity to the cdc-25 phosphatase (Dickinson and Keyse, 2006) (Figure 1.4). Although all MKPs have sequence similarity, they still have differences in amino acid sequences that account for their different substrate specificities.

MKP-1 is the prototypical member of the MKP family of dual specificity phosphatases (also known as DUSPs). It was first identified as an immediate early gene (Sun et al., 1993), as it is rapidly induced after mitogenic stimulation. It serves as a crucial negative feedback regulator in both innate immunity and inflammation as evidenced by the increase in cytokine levels and pro-inflammatory mediators in an MKP-1 knock out mouse (Salojin et al., 2006; Wang et al., 2007). MAPKs are activated by dual phosphorylation at the threonine and tyrosine residues. MKP-1 functions to dephosphorylate the MAPKs, specifically; p38 MAPK and JNK (p38>>JNK>>ERK). This was confirmed using MKP-1 deficient mice, where it was revealed that an absence of MKP-1 caused an increase in the duration of phosphorylation of p38 MAPK and JNK. Studies in human ASM cells have confirmed the role of MKP-1 as a negative feedback regulator of the p38 MAPK pathway and
Since MKP-1 is able to regulate the p38 MAPK pathway it is indirectly responsible for the action of TTP, that is an mRNA destabilising protein. Notably, the Ammit group has shown that anti-asthma medications, such as corticosteroids and $\beta_2$-agonists, also act to repress inflammation via MKP-1 upregulation (Quante et al., 2008; Manetsch et al., 2012b; Che et al., 2014b; Rahman et al., 2014). However, MKP-1 is only transiently upregulated and is rapidly degraded by the proteasome (Moutzouris et al., 2010) in part because of its potency. As a negative feedback regulator protein of p38 MAPK, MKP-1 is upstream of many inflammatory mediators. Hence MKP-1 represents an exciting prospect to control the duration and intensity of inflammation, and highlights the importance of identifying ways to modify MKP-1.

1.3.1 Modification of MKP-1

Regulation of inflammatory mediators can occur at the transcriptional level, controlling gene expression, or it can occur at the post transcriptional level whereby mRNA stability determines whether a particular transcript is translated to protein or is rapidly degraded. Regulation can also occur at the post-translational level by modulating the stability of translated proteins. This typically involves either preventing the degradation of the protein via the proteasome or modifying it to increase its stability. Although all three levels can be targeted to regulate an inflammatory response, post-transcriptional modification presents a feasible opportunity to exert fine control over the whole process.

Transcriptional regulation of MKP-1 is stimulated in response to many diverse extracellular signals like growth factors, bacterial agents, cytokines and other cellular signals like heat shock, hypoxia and reactive oxygen species (Owens and Keyse, 2007; Caunt and Keyse, 2013). This study will be focused on MKP-1 transcription inducing agents like TNF$\alpha$, corticosteroids, and hydrogen peroxide ($H_2O_2$). These MKP-1 transcription inducing agents are able to trigger intracellular signalling
pathways, which bind to the MKP-1 gene promoter region in the nucleus. This MKP-1 gene promoter region contains binding sites for transcription factors; activator protein 1 (AP-1) activator protein 2 (AP-2), nuclear factor (NF) κB specificity protein (SP-1) and controlled amino acid treatment/ binding transcription factor and the cyclic AMP response element (CRE), the glucocorticoid receptor element (GRE), E-box, and vitamin D receptor element (VDRE) (Noguchi et al., 1993; Sommer et al., 2000; Wang et al., 2008; Shipp et al., 2010; Zhang et al., 2012) (Figure 1.5). While a number of sites have been identified above, only some of the sites have been reported to increase MKP-1 transcription. In our studies, and in accordance with existing literature it has been found that MKP-1 was only activated transiently and returns back to basal levels soon after stimulation. This study aimed to delineate the impact of this MKP-1 regulation back to basal levels.

1.3.1.1 Post-transcriptional regulation

Most of the research on post transcriptional control has been performed with respect to mRNA stability. This process represents an important regulatory mechanism to control the amount of protein that is translated. mRNA transcripts contain multiple regions within their 3’untranslated region which contain adenosine uridine rich areas (ARE). These cis-acting motifs are known to be involved in the regulation of mRNA stability. Along with cis-acting motifs there are also trans-acting RNA binding proteins that are able to stabilise or destabilise mRNA transcripts. TTP is one such protein, which will be discussed later in this chapter, is an mRNA destabilising protein. Whether or not TTP is able to destabilise the MKP-1 mRNA is not yet known. Other RNA binding proteins such as HuR and NF 90 (Figure 1.5) have been shown to control the stabilisation of mRNA transcripts (Kuwano et al., 2008). There is one study, which indicates that these trans-acting proteins are not directly responsible for stability regulation but act as cellular rafts transporting their target mRNAs to an area of the cell responsible for their maintenance or destruction (Shi et al., 2014).
There has recently been a surge in research focusing on microRNA (miR). Micro RNAs are endogenously expressed non-coding small RNAs. They function in a manner similar to \textit{trans}-acting proteins and are able to trigger gene silencing and translational repression by binding to the 3’ untranslated region of target mRNAs (Ambros, 2004; Bartel, 2004; Zhu et al., 2010). These miRs are numerous and can have conflicting effects. For example miR-101 was shown to have an inhibitory effect on MKP-1 mRNA in macrophages but miR-708 was shown to augment MKP-1 expression via CD 38 in ASM cells (Zhu et al., 2010; Dileepan et al., 2014). It is possible that their impact may be cell type dependent.

1.3.1.2 Post translational regulation

Classic literature states that MKPs bind to MAPK and dephosphorylate them at the threonine / tyrosine region. MAPKs are active when they are phosphorylated at these sites however they need to be phosphorylated at both sites in order to be active. There is evidence to suggest that the MAPKs need to dock to the MKPs first, causing the MKPs to change their conformation and thereby stimulate their phosphatase activity. The MAPKs are able to dock to the kinase interaction motif (KIM) found near the N-terminal end of the MKPs (Tanoue et al., 2000; Tanoue and Nishida, 2002). MKP-1 is perhaps the most well studied of the MKP family and a lot of the early work in regards to structure and functionality was tested on MKP-1. The interaction between MAPK and MKP is a complex one as they are able to regulate each other (they act as substrates for each other in the case of ERK). In this way both MAPK and MKP-1 are able to exercise some control over substrate specificity. A study published in 1999 was one of the first to observe this interaction, by showing an increase in molecular weight bands (via gel electrophoresis), that MKP-1 was phosphorylated in the presence of ERK (P44/42 MAPK). They were also able to show that this post-translational change had altered the decay kinetics of MKP-1 causing it to become more stable (Brondello et al., 1999).
Others found that MAPKs had multiple docking sites, suggesting another way for these MAPKs to ensure substrate specificity (Jacobs et al., 1999; Sharrocks et al., 2000). The area where ERK binds to MKP is known as the dual specificity phosphatase catalytic domain (DEF) motif. The most interesting finding was that these docking interactions only occurred at specific locations that contained serine or threonine residues followed by a proline residue. At these specific residues post-transcriptional modification was able to occur (phosphorylation) (Figure 1.5).

Following on from the previous work, one group investigated ERK MAPK and found that ERK was not only able to act as an MKP-1 stabiliser under some cellular environments but was able to promote the proteasomal decay of MKP-1 in other cellular environments. This distinction in cellular environments was made based on the stimulus that upregulated ERK, for example proteasomal decay of MKP-1 was enhanced when ERK was stimulated in the presence of a mitogen activated protein kinase kinase (MAPKK) compared with Pb (II) induced ERK. To identify the motif necessary for proteasomal degradation, truncated mutants of MKP-1 were created, which eliminated residues 1-59, effectively removing the KIM domain. It was found that the decay kinetics remained the same suggesting that the KIM motif is not essential to ERK directed MKP-1 degradation via the-ubiquitin proteasome pathway (Lin et al., 2003; Lin and Yang, 2006). Once the location of the interaction between ERK and MKP-1 had been narrowed down, a detailed MKP-1 protein sequence analysis was performed revealing that MKP-1 has four (S/T) P residue complexes, two of which exist at the very end of the C-terminal and two that lie near the DEF motif. Four serine residues were found; ser 296, ser 323, ser 359 and ser 364, next to proline residues and these locations were targeted for further analysis. They then preformed point mutations at these residues to change these serines to alanines and tested these mutants against wild type (WT) MKP-1 to test whether they were phosphorylated after ERK docking and found that all four sites were phosphorylated however only the ser 296 and ser 323 were involved in proteasomal regulation.
This was validated as it was shown that the stability of MKP-1 and MKP-2 was reduced in the presence of an ERK pathway inhibitor (Crowell et al., 2014). In addition, certain other post translational modifications like s-glutationylation (the mixed bonds that form between glutathione and cysteine residues in protein) results as a consequence of metabolic and oxidative stress. This glutationylation is able to deactivate MKP-1 and present it for proteasomal degradation (Kim et al., 2012). Another posttranslational modification that can occur is the oxidation of MKP-1. Oxidation is very interesting as oxidative stress is prevalent in patients who are smokers and those who have COPD. In this high oxidative environment there are a lot of reactive oxygen species that are present. Oxidation is possible at the cysteine residues of target proteins. MKP-1 has a cysteine located as part of its catalytic triad and as such if the cysteine would be modified through oxidation then MKP-1 would lose its activity. Some studies on the redox regulation of MKP-1 have backed this up and have shown that TNFα expression is able to be altered in the presence of oxidised MKP-1. This was in line with other data that showed monocyte migration and macrophage recruitment were increased in the presence of oxidised MKP-1 (Tephly and Carter 2007; Kim et al., 2012). Since MKP-1 is a p38 MAPK deactivator, its oxidation would increase p38 MAPK and the inflammatory cytokines that are triggered as a result. This falls in line with reports of increased p38 MAPK in COPD patients and patients who smoke (Renda et al., 2008). Much of the literature on MKP-1 modification deals with the structural relationship between MAPK and MKP-1 and not with the post translational regulation of MKP-1. Hence, this investigation aimed to study the some of these post-translational modifications on MKP-1 in the context of asthma in ASM cells.

1.4 Tristetraprolin

Another molecule responsible for the anti-inflammatory effects of p38 MAPK is tristetraprolin.
Tristetraprolin, also known as Nup475 (DuBois et al., 1990), Tis 11 (Varnum et al., 1991), G0S24 (Heximer and Forsdyke, 1993) or Zfp36(Taylor et al., 1991), is the prototypical member of the family of Cys-Cys-Cys-His class tandem zinc finger proteins. It is an immediate early response gene with low levels expressed within quiescent cells. It can be rapidly expressed and translated into protein to exert regulatory control upon cytokine mRNA expression. A role for TTP as a trans-acting anti-inflammatory protein was first elucidated when a TTP knockout mouse developed a pro-inflammatory phenotype due to the overexpression of the cytokine TNFα in macrophages resulting in cachexia, myeloid hyperplasia and a host of other inflammatory conditions (Taylor et al., 1996). Based on evidence from earlier work (Caput et al., 1986) it was concluded that TTP confers mRNA instability and degradation by binding the conserved adenosine/uridine-rich element (ARE) present within the 3’-untranslated region (UTR) of mRNA transcripts (Carballo et al., 1998; Lai et al., 1999b). The importance of this cis-element is highlighted by its presence in approximately 8% of the human transcriptome (Bakheet et al., 2006). Interestingly, TTP itself has this ARE present within its own 3’-UTR, suggesting that TTP could regulate itself in an effort to dampen its anti-inflammatory effects (Brooks et al., 2004; Tchen et al., 2004a).

TTP is a critical anti-inflammatory protein that functions to induce mRNA decay. Many clinically important proteins, including cytokines, are controlled by post-transcriptional mechanisms via regulation of mRNA stability (see recent review (Brooks and Blackshear, 2013)). Notably, many of these proteins have been implicated in asthma (Table 1.2, adapted from (Brooks and Blackshear, 2013)). Many of the characteristics associated with asthma, such as basement membrane thickening, increased interstitial matrix deposition, ASM mass increasing, increased airway fibrosis, and neovascularization in the sub epithelial mucosa can be attributed to elevated levels of the inflammatory mediators that give rise to these characteristics (Ammit, 2005). As outlined in Table 1.2, many of the proteins responsible for these disease characteristics are TTP targets (Tang and Fiscus, 2001; Mattos et al., 2002; Hirst, 2003; Batra et al., 2007; Kato and Schleimer, 2007; Barnes,
Chapter 1

2008; Ishmael, 2011; Kang et al., 2011; Rael and Lockey, 2011; Boonpiyathad et al., 2013; Banerjee et al., 2014; Masaki et al., 2014).

Table 1.2 Inflammatory mediators that are TTP targets

<table>
<thead>
<tr>
<th>TTP targets implicated in asthma</th>
<th>Function in asthma pathogenesis</th>
<th>Shown to be regulated by TTP in cell line</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-17</td>
<td>Inducing and mediating pro-inflammatory responses</td>
<td>Human T cell lines (Lee et al., 2012)</td>
</tr>
<tr>
<td>IL-6</td>
<td>Pro-inflammatory (neutrophil production)</td>
<td>Various cell lines (Neininger et al., 2002; Jalonen et al., 2006; Patil et al., 2008; Van Tubergen et al., 2011; Zhao et al., 2011; Brahma et al., 2012; Mercado et al., 2012; Chafin et al., 2013; Banerjee et al., 2014)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Many pro-inflammatory functions</td>
<td>Mouse RAW264.7 cells (Brook et al., 2006b)</td>
</tr>
<tr>
<td>TNFα</td>
<td>Many pro-inflammatory functions</td>
<td>Mouse macrophages (Taylor et al., 1996; Carballo et al., 1998)</td>
</tr>
<tr>
<td>CCL2</td>
<td>Recruitment of inflammatory mediators</td>
<td>Mouse macrophages, fibroblasts (Sauer et al., 2006)</td>
</tr>
<tr>
<td>CXCL 8</td>
<td>Recruitment of neutrophils</td>
<td>Various cell lines (Suswam et al., 2008; Balakathiresan et al., 2009; Bhattacharyya et al., 2011; Al Ghouleh and Magder, 2012; Galbiati et al., 2014)</td>
</tr>
<tr>
<td>Protein/Antigen</td>
<td>Function</td>
<td>Cells and Sources</td>
</tr>
<tr>
<td>----------------</td>
<td>----------</td>
<td>-------------------</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Increases levels of neutrophils and eosinophils</td>
<td>Mouse bone-marrow derived stromal cells (Carballo et al., 2000)</td>
</tr>
<tr>
<td>VEGF</td>
<td>Promotes angiogenesis</td>
<td>Various cell lines (Essafi-Benkhadir et al., 2007; Suswam et al., 2008; Essafi-Benkhadir et al., 2010; Lee et al., 2010; Bhattacharyya et al., 2011; Banerjee et al., 2014)</td>
</tr>
<tr>
<td>IL-13</td>
<td>Involved in airway remodelling</td>
<td>Mast Cells (Barnstein et al., 2006)</td>
</tr>
<tr>
<td>IL-23</td>
<td>Neutrophil infiltration</td>
<td>Mouse dendritic cells, macrophages (Qian et al., 2011)</td>
</tr>
<tr>
<td>CCL5</td>
<td>Chemotaxis of chemokines and cytokines</td>
<td>Mouse dendritic cells, peripheral blood mononuclear cells (Jin et al., 2012; Rosenberger et al., 2012)</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Recruitment of circulating pro-inflammatory mediators</td>
<td>Human pulmonary microvascular endothelial cells (Mercado et al., 2012; Colin et al., 2014)</td>
</tr>
<tr>
<td>COX-2</td>
<td>Regulates PGD2</td>
<td>Various cells (Boutaud et al., 2003; Phillips et al., 2004; Sully et al., 2004; Hammaker and Firestein, 2010; Bhattacharyya et al., 2011)</td>
</tr>
<tr>
<td>iNOS</td>
<td>Modulation airway and vascular smooth muscle</td>
<td>DLD-1 cells, rat cortical astrocytes (Linker et al., 2005; Lisi et al., 2011)</td>
</tr>
<tr>
<td>IL-2</td>
<td>Inducing and mediating pro-inflammatory responses</td>
<td>Mouse splenocytes (Ogilvie et al., 2005)</td>
</tr>
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<td>--------</td>
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<td>-----------------------------------------</td>
</tr>
<tr>
<td>CXCL1</td>
<td>Neutrophil chemoattractant</td>
<td>Mouse macrophages (Datta et al., 2008)</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Remodelling of the extra cellular matrix</td>
<td>3D cancer model, chick chorioallantoic membrane (Van Tubergen et al., 2013)</td>
</tr>
<tr>
<td>CCL3</td>
<td>Recruitment of chemokines and cytokines, inflammatory mediator release</td>
<td>Mouse macrophages, fibroblasts (Sauer et al., 2006; Kang et al., 2011)</td>
</tr>
</tbody>
</table>

### 1.4.1 TTP expression and activity is controlled by p38 MAPK

The function of TTP as an mRNA destabilising factor is controlled by p38 MAPK phosphorylation. TTP is an immediate-early gene (Carballo et al., 1998) that is rapidly and robustly expressed via p38 MAPK signalling (Mahtani et al., 2001b; Tchen et al., 2004a; Brook et al., 2006b; Hitti et al., 2006a). Critically, p38 MAPK also activates the downstream kinase MK2, which phosphorylates TTP protein on two key serine residues (Ser52 and Ser178) preventing the recruitment of the deadenylase complex (Marchese et al., 2010b) and stopping the initiation of mRNA decay. Hence, the anti-inflammatory function of the destabilising RNA binding protein TTP is dictated by the phosphorylation status of TTP at Ser52 and Ser178. This knowledge provides us with important points of regulatory control.
MK2 is a substrate of the p38 MAPK pathway and has been implicated in post-translational control of cytokines. This was demonstrated by using MK2 knockout mice which failed to produce TNFα protein when compared with WT equivalents. However, mRNA levels when compared with WT mice showed levels that were very similar to knockout mice, suggesting that the control exerted was at the translational level. This also suggested that control may be imparted via a downstream substrate of MK2. Production of cytokines was also impaired compared to wild types, with IL-6 mRNA half-life in particular, markedly attenuated (Kotlyarov et al., 1999; Neininger et al., 2002). TTP was shown to be a substrate for MK2 using MK2/TTP double knockout mice and by reconstituting the p38 MAPK transduction pathway using recombinant kinases to detect TTP phosphorylation (Mahtani et al., 2001b; Hitti et al., 2006a).

1.4.2 Phosphorylation of TTP

The main function of TTP protein is to decay target mRNAs however its activity is also profoundly affected by post-translational modification (Cao et al., 2007). Post-translational phosphorylation appears to be the main factor responsible for determining its stability, activity and its ability to form protein complexes. Phosphorylation of TTP has been extensively studied and mapped revealing that TTP is a hyper-phosphorylated protein in vivo with its mRNA destabilizing activity depending on its phosphorylation status. When TTP is phosphorylated it is unable to effect the stability of target mRNA. However, when dephosphorylated it is active and therefore able to confer its mRNA destabilizing ability. TTP is more stable when phosphorylated and unstable when dephosphorylated. This allows TTP to be rapidly degraded by the proteasome when dephosphorylated (Brook et al., 2006b; Hitti et al., 2006a). TTP is both a direct and indirect substrate for p38 MAPK leading to its phosphorylation directly through p38 MAPK and indirectly via MK2. It has also been shown that Ser220 on mouse TTP is phosphorylated via the ERK MAPK pathway (Taylor et al., 1995). This indicated that dual MAPK involvement may be required to confer some level of post-translational
modification to TTP (Brooks et al., 2004) specifically relating to TTP stability. The functions of all the various TTP phosphorylation sites are not completely understood, however, the sites for MK2 protein binding have been characterized as Ser52 and Ser178 (Chrestensen et al., 2004; Stoecklin et al., 2004). Phosphorylation at these sites enables TTP to form complexes with multifunction adaptor 14-3-3 proteins that ablate its function as an mRNA destabilizing protein (Chrestensen et al., 2004; Sun et al., 2007; Marchese et al., 2010b). This complex also serves to protect TTP from dephosphorylation in a protein phosphatase 2A (PP2A)-dependent manner (Sun et al., 2007) and prevent degradation of TTP via the proteasome (Deleault et al., 2008; Pfeiffer and Brooks, 2012). This is in line with data that showed TTP levels increased when the cells were in the presence of a proteasome inhibitor. This complex that forms alters the functionality of TTP without altering its ability to bind mRNA (Cao et al., 2003; Rigby et al., 2005; Marchese et al., 2010b; Clement et al., 2011). This suggests that phosphorylated TTP remains inactive whilst being bound to target mRNA until it is dephosphorylated. It is then able to recruit the deadenylase components Not1 and CAF1, which are then able to rapidly deadenylate the target mRNA resulting in their destabilization (Sandler et al., 2011; Colin et al., 2014). Phosphorylation of TTP also has an impact on its subcellular localization, with studies showing that upon stimulation with a mitogen, TTP is rapidly translocated from the nucleus to the cytoplasm (Taylor et al., 1996; Johnson et al., 2002; Brook et al., 2006b) (Figure 1.3). Taken together, these studies demonstrate the importance of TTP phosphorylation status in controlling TTP function and thus represents a novel area to target as part of future pharmacotherapeutic strategies. Hence, mRNA stability plays a crucial role in the pathogenesis of inflammatory diseases.

As a negative feedback regulator protein of p38 MAPK, MKP-1 is upstream of TTP. This suggests an exciting possibility that MKP-1 might have a dual role as an anti-inflammatory protein because it is able to dephosphorylate p38 MAPK itself and subsequently activate TTP in the process. Hence we focused our research on MKP-1 and its effect on p38 MAPK and its subsequent effect on TTP.
1.5 Corticosteroids

Inhaled corticosteroids are the mainstay of chronic asthma treatment and are used effectively at low doses for the majority of asthmatics. However, there is a subset of the asthmatic population with severe asthma, who need maximal doses of inhaled corticosteroids, and another small subset who are resistant to corticosteroids. Corticosteroid insensitivity and resistance are terms to describe patients who do not respond well to corticosteroids and usually require maximal doses of corticosteroids, either inhaled or administered orally as the worst case. The mechanism that governs corticosteroid insensitivity is complicated and even after many years of research, there is still a lot that can learned. A detailed account of the molecular mechanisms thought responsible for corticosteroid insensitivity and resistance have been outlined in recent reviews (Ammit, 2013; Barnes, 2013), but it will be briefly explored in the next section. One main way corticosteroids deliver their anti-inflammatory action is via MKP-1. Thus, targeting molecules along the p38 MAPK pathway might prove to have clinical efficacy. Hence we aimed to delineate the role of a corticosteroid in the MKP-1, p38 MAPK and TTP axis described at length above.

Inhaled corticosteroids (ICS) have been the mainstay of chronic asthma treatment and are used effectively at low doses for the majority of asthmatics, with dramatic reductions in both morbidity and mortalities associated with asthma. This reduction in morbidity is also associated with an improvement in quality of life for many mild and moderate asthmatics allowing sufferers to lead normal lives. This is attributed to increased lung function, lesser recurrence of acute asthmatic episodes, and a reduction in remodelling induced airway changes (O'Byrne et al., 2006). Due to the subset of asthmatics with severe asthma who need maximal doses of inhaled corticosteroids and the subset of severe asthmatics who are resistant to corticosteroids, there is renewed interest in gaining a
greater understanding into glucocorticoids and their mechanism of action so that we can bypass glucocorticoid insensitivity by targeting the pathway responsible for its anti-inflammatory action.

1.5.1 Mechanism of action of glucocorticoids

The glucocorticoid receptor (GR) is central to the action of glucocorticoids. This receptor / agonist binding interaction occurs in the cytoplasm after the glucocorticoids have diffused across the cell membrane. This interaction leads to the activation and subsequent detachment of the GR from chaperone proteins (hsp 90) (Pratt et al., 2004), allowing the GR to then translocate to the nucleus and exert its effects upon the transcriptional machinery. Once in the nucleus, the GR is able to homodimerise and bind the glucocorticoid response elements (GRE) on the DNA sequences associated with the glucocorticoid response (Rhen and Cidlowski, 2005). This interaction is able to effectively switch gene transcription on or off. Hence the problem of glucocorticoid insensitivity potentially lies along this mechanistic pathway, and understanding more about this pathway and the potential regions where aberrations could occur would give us a clue into combating this problem. There is a lot of literature exploring the basis of this problem with most of the research focusing on the modifications to the glucocorticoid receptor including phosphorylation, nitrosylation and ubiquitination, changes in the levels of glucocorticoid isoforms, GR mediated recruitment of transcriptional and co-activators and co-repressors, increase in transcription factors, changes in immune mechanisms and defective histone acetylation (Wenzel et al., 1997; Ito et al., 2001; Irusen et al., 2002; Bhavsar et al., 2010; Chang et al., 2012; Ammit 2013; Barnes 2013; Chang et al., 2015). Mechanisms are outlined further in Figure 1.6. Interestingly glucocorticoid resistance in not limited to only one phenotype of asthma and is in fact overrepresented by the non-eosinophilic population. As mentioned above this information is presented in this thesis to encourage the reader to keep in mind the existence of phenotypic diversity not to highlight every difference. Patients from the non-eosinophilic cohort have a poor short term response to inhaled corticosteroids (Pavord et al., 1997).
Since non-eosinophilic asthma can be due to neutrophilia. It could represent one aspect of corticosteroid resistance in non-eosinophilic asthma, with one study reporting an increase in neutrophil survival in the presence of high doses of inhaled corticosteroids (Cox 1995) and another showing that neutrophils are relatively resistant to steroid treatment (Wenzel et al., 1999).

This data suggests a role for neutrophilia in corticosteroid resistance. However there may be a caveat to with differences being noted in lung and blood neutrophils in COPD (Wenzel et al., 1997). The pro inflammatory mediator p38 MAPK was not present in the lung neutrophils and only present in the neutrophils in the blood. Since p38 MAPK is able to phosphorylate the glucocorticoid receptor it might be that the infiltrating neutrophils have a greater role in non-eosinophilic corticosteroid insensitivity.

If we target the abrogated mechanisms along this pathway, we could not only restore glucocorticoid sensitivity, we could also induce their intended anti-inflammatory actions. The direct anti-inflammatory action of glucocorticoids is in part attributed to the genes for anti-inflammatory molecules being encoded (Clark, 2003a), such as the gene for β2 adrenoceptor, the gene for MKP-1 and glucocorticoid-inducible leucine zipper (GILZ-1). The anti-inflammatory action is also attributed to negative interactions with pro-inflammatory transcription factors like activator protein 1 and NF-κB. The activation of the MKP-1 gene is important as it is able to block the p38 MAPK signalling pathway and hence, inhibit phosphorylation of nuclear regulatory proteins and the expression of multiple pro-inflammatory genes (Maneechotesuwan et al., 2009). This is highlighted; in an in vivo study that showed the glucocorticoid induced anti-inflammatory action was reduced in macrophages from MKP-1 gene knock-down mice (Abraham et al., 2006). Glucocorticoids are also able to exert their anti-inflammatory effects by indirectly inducing MKP-1 gene expression through histone acetylation and through the inactivation of macrophage migration inhibitory factor (MIF).

This was shown in ulcerative colitis and could also be present in asthma. Thus we highlight the importance of MKP-1 in the partial execution of the anti-inflammatory effects of glucocorticoids.
1.6 Aims of this study

The substantial in vitro evidence implicating the p38 MAPK signalling pathway, in the pathogenesis of respiratory diseases driven by inflammation has propelled studies targeting p38 MAPK pathway as a novel anti-inflammatory strategy in vivo. These studies revealed that p38 MAPK is an interesting target as a therapy, however there are side effects involved in targeting p38 MAPK directly. Hence there is promise in targeting two endogenous anti-inflammatory molecules along the p38 MAPK mediated inflammatory pathway. The p38 MAPK deactivating molecule MKP-1 and the RNA destabilising molecule TTP are both key anti-inflammatory proteins whose functions include significant repression of p38 MAPK mediated inflammation. Hence greater understanding of the post-translational modifications involved in MKP-1 and TTP are warranted, along with a closer look at the interaction between the major players on the p38 MAPK pathway. This pathway could represent an alternative anti-inflammatory strategy in chronic respiratory diseases. The mechanism of MKP-1 and TTP as anti-inflammatory molecules has been studied extensively however their precise temporal regulation, a crucial element of their regulation, has not been studied. Thus the overall aim of the study was to determine the interaction and the precise temporal control of endogenous anti-inflammatory proteins present during inflammation.

Objective 1 (Refer to chapter 3)

- Validation of MKP-1 antibody

Initially it was necessary to find a primary MKP-1 antibody suitable for gel electrophoresis as this would then be carried through the thesis. When the new antibodies were used with the existing protocol for MKP-1 western blotting analysis, there was no discernible band for MKP-1. Hence, the protocol needed to be updated to accommodate all MKP-1 antibodies.
Objective 2 (Refer to chapter 4)

- To identify the temporal profile of MKP-1, p38 MAPK and TTP in relation to one another
- To examine this potential p38 MAPK regulatory network with respect to cytokine mRNA via p38 MAPK and tristetraprolin phosphorylation status

This study investigated the mechanisms that regulate cytokine secretion, which is a conduit of inflammation, and the temporal profile of these mechanisms. It is known that p38 MAPK drives inflammatory signals and is transiently dephosphorylated by MKP-1 and that TTP is able to destabilise cytokine mRNA. However, the precise link between MKP-1 and TTP and what happens to each molecule with respect to time and in relation to each other and the effects this has on inflammation using ASM cells remains unknown.

Objective 3 (Refer to chapter 5)

- To examine the impact of corticosteroid-induced MKP-1 in a curative protocol
- To identify how corticosteroid-induced MKP-1 represses pro-inflammatory cytokine secretion: via TTP activity, not TTP mRNA and protein expression.

The previous objective aimed to investigate the precise temporal relationship that exists between MKP-1, p38 MAPK and TTP. In this chapter, what happens to this close temporal relationship in the presence of a corticosteroid, which has been shown to increase the level of MKP-1 in ASM cells, was investigated.

Objective 4 (Refer to chapter 6)
- To induce post translational modification of MKP-1 through *in silico* modelling.
- To gain insights into the regulation of p38 MAPK in the presence of elevated levels of MKP-1.

It is know that MKP-1 protein is able to be modified at the post-translational level, and that this modification has been shown to increase stability of the protein and the degradation of the protein depending on where the modification occurs. Currently the crystal structure of MKP-1 is not known therefore an *in silico* approach to build a model of MKP-1 and identify molecules that will interfere with this process of modification was used to examine how the kinetics of MKP-1 would be altered.
When exposed to an extracellular stimulus, dendritic cells are able to stimulate naïve T cells and differentiate them into T helper cells, which are then able to proliferate and cause the secretion/migration of cytokines and other inflammatory mediators. Structural remodelling occurs as a result of constant inflammatory assault. Remodelling of the airways is able to trigger an increase in smooth muscle mass, smooth muscle proliferation, angiogenesis, nerve proliferation, mucus cell metaplasia, fibrosis and thickening of the basement membrane and it has also been associated with decreased cartilage integrity. All of this together results in the chronic inflammatory phenotype.
Figure 1.2 Airway smooth cells and airway epithelial cells and their effect on airway remodelling

In the presence of cellular stressors and inflammatory assault, the epithelium is damaged and its integrity is lost causing these mitogens to infiltrate the first line of defense and trigger an inflammatory response from airway smooth muscle cells. The damaged epithelium layer also contributes to the slew of inflammatory mediators recruited. These inflammatory mediators act on the smooth muscle cells, which subsequently proliferate, leading to airway remodelling.
Chapter 1

Figure 1.3 p38 MAPK-dependent signalling in respiratory disease

The three members of the MAPK superfamily - ERK (extra-cellular signal related kinase), JNK (c-Jun N-terminal kinase) and p38 MAPK - are stimulated via a series of steps involving MAPKKK (mitogen-activated protein kinase kinase kinase) phosphorylating downstream MAPKK (mitogen-activated protein kinase kinase), then ultimately MAPKs. This figure focuses on downstream effectors of the p38 MAPK pathway. We propose that blocking p38 MAPK may have a negative impact on anti-inflammatory proteins such as TTP (tristetraprolin), which is regulated downstream of p38 MAPK. Once active TTP is able to elicit anti-inflammatory actions by destabilising cytokine mRNA after recruiting CAF 1 and CNOT 1. Abbreviations are: ATF (activating transcription factor); CAF 1 (CCR4 associated factor); CNOT 1 (CCR4-NOT complex); CREB (cAMP response element binding); EGFR (epidermal growth factor receptor); MK2/3 (mitogen activated protein kinase activated protein kinase); MSK (mitogen and stress activated kinase 1); NF-κB (nuclear factor-κB).
MKP-1 is able to be regulated at three levels, the transcriptional level, the post transcriptional level and the post translational level. In the presence of an extracellular stimuli certain transcription factors are activated such as activator protein 1 (AP-1) activator protein 2 (AP-2), nuclear factor (NF) κB specificity protein (SP-1) and controlled amino acid treatment/ binding transcription factor and the cyclic AMP response element (CRE), the glucocorticoid receptor element (GRE), E-box and vitamin D receptor element (VDRE). These transcription factors are able to bind to the MKP-1 promoter region to regulate the induction of MKP-1 gene. Once the gene has been transcribed into mRNA, proteins such as human antigen R (HuR), nuclear factor 90 (NF-90) and various micro RNAs (miR) are able to bind to the 3’ untranslated region specifically to the adenosine uridine rich areas, to modulate the stability of MKP-1 mRNA transcripts. MKP-1 can also be modified at the post translational level, serines can be phosphorylated, lysines can be acetylated and cysteines can be oxidised, causing MKP-1 protein activity, stability and degradation status to change.
Figure 1.5 Schematic representation of MKP-1 protein

The dual specificity phosphatase catalytic domain (DSPc, residues 173-311) and the key regulatory motifs and residues that post-translationally control MKP-1 are shown, including: the kinase interaction motif (KIM, residues 50-58); the DEF motif (residues 339-342) that mediates S296/S323 phosphorylation and proteasomal degradation; and S359/S364 involved in phosphorylation-mediated protein stability.
A The mechanism of glucocorticoid action when a glucocorticoid binds to the glucocorticoid receptor (GR), it then translocates to the nucleus and homodimerises. It then triggers the histone deacetylation and the binding to the glucocorticoid response elements (GRE). This stimulates the increase in anti-inflammatory transcription factors and hence anti-inflammatory molecules. 

B The various factors involved in glucocorticoid insensitivity. This is not an exhaustive list of every factor leading to glucocorticoid insensitivity it is more of a brief overview as to the complex network of molecules and their triggers, that could lead to glucocorticoid insensitivity.

Figure 1.6 Glucocorticoids mechanism of action and factors leading to glucocorticoid insensitivity

A. Mechanism of glucocorticoid action

B. Factors of glucocorticoid insensitivity

- Microbial agents
  - Viral and bacterial antigens

- Allergens
  - IL-2, IL-4, IL-13

- Oxidative stress
  - ROS from smoking and Obesity

- Immune mechanisms
  - Vitamin D₃
  - IL-10
  - IL-19
  - IL-17 Th17

- p38 MAPK
- GR translocation
- GR ligand binding affinity
- GRβ
- PP2A
- Transcription factors

- PI3Kδ pathway
- Histone deacetylase
- MKP-1

Glucocorticoid insensitivity
Chapter 2:

Materials and Methods
Chapter 2: 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 was 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-piperazinethanesulfonic acid), antibiotic-antimycotic liquid (containing 10,000 units/mL penicillin, 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 consisted of 0.1% (v/v) BSA (30% w/v solution, sterile) in DMEM.

A549 cells on the other hand cells were cultured in F-12K (Kaighn's) Medium (Invitrogen, Carlsbad, CA) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), and fetal bovine serum to a final concentration of 10%. 500 mL of A549 feeding media was prepared by adding 11 mL penicillin-streptomycin 50X liquid (Invitrogen, Carlsbad, CA: C/N 15070-063) and 55.5 mL of heat-inactivated (56°C for 30 minutes) fetal bovine serum (Interpath, VIC, Australia) to 500 mL of F-12K (Kaighn's) Medium ([Invitrogen: C/N 21127-022]).
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. ASM cells were dissected, purified and cultured as previously described by {Johnson, 1995 #2513}. To isolate the smooth muscle cells, the bronchi were first dissected from the surrounding lung tissue, followed by the removal of the epithelium layer. Once the translucent epithelial layer was removed the individual ASM tissue bundles were revealed and were then teased away from the structural layer (cartilage) of the bronchus. These smooth muscle bundles were then placed into 25cm² tissue culture flasks (Sarstedt, Nümbrecht Germany) with DMEM containing phenol red and supplemented with 2 mM L-Glutamine, 20 mM HEPES, 10% (v/v) heat inactivated fetal bovine serum (FBS) and 2% (v/v) 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% (v/v) 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 the cells were trypsinized (2 min at 37°C) the media was aspirated and the cells were washed two times with sterile phosphate buffered saline (PBS) (Ca²⁺/Mg²⁺-free) to remove all traces of FBS. The cells were then incubated at 37°C in a trypsin solution (0.5 g/l porcine trypsin and 0.2 g/l EDTA in Hank's balanced salt solution with phenol red) to complete the passaging step. The cell detachment 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 tryptic activity. Cell numbers were then
estimated on a haemocytometer using trypan blue solution. Cells were plated at a density of 1x10^6 cells/T175 flask (Sarstedt, Nümbrecht, Germany) and maintained at 5% CO₂ in air at 37°C, these cells were refed every week and passaged into further T175 flasks (split 1:3) every 2 weeks or when the flasks were deemed to be conflu ent. For all experiments in this thesis, smooth muscle cells were used between passage 4 and 8.

2.2.1.4 Passaging A549

Before the cells were trypsinized (2 min at 37°C) the media was aspirated and the cells were washed two times with sterile PBS (Ca^{2+}/Mg^{2+}-free) to remove all traces of FBS. The cells were then incubated at 37°C in a trypsin solution (0.5 g/L porcine trypsin and 0.2 g/L EDTA in Hank's balanced salt solution with phenol red) to complete the passaging step. The cell detachment 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 tryptic activity. Cells were split into 3 equal quantities once every 3-4 days (e.g. Monday and Thursday or Tuesday and Friday) and maintained at 5% (v/v) CO₂ in air at 37°C.

2.2.1.5 Plating and starvation of ASM cells

Cells were plated at a density of 1.0 x 10^4 cells/cm^2 according to the guidelines in Table 2.1 and grown for one week. Feeding media was changed approximately 72 h after plating. Before performing the experiments the cells were growth arrested. The feeding media was aspirated, the cells were washed twice with sterile PBS, and then growth arrest/starvation media was added for 48 h.
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>
</tr>
<tr>
<td>6-well</td>
<td>96,000 cells/well</td>
<td>3 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>

2.2.1.6 Plating and starvation of A549 cells

Cells were plated at a density of 250,000 cells/mL so that when 2 mL was added to 6 well dishes 500,000 cells/well was achieved according to the guidelines in Table 2.2. The cells were allowed to settle for 6-8 h and were then subsequently growth arrested before performing the experiments. The feeding media was aspirated, the cells were washed twice with sterile PBS, and then the growth arrest/starvation media was added for 14-16 h.

Table 2.2 Guidelines for experimental plating of A549 cells

<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>6,400 cells/well</td>
<td>0.1 mL</td>
</tr>
<tr>
<td>6-well</td>
<td>500,000 cells/well</td>
<td>2 mL</td>
</tr>
</tbody>
</table>

2.2.1.7 ASM cell characterization

To characterize ASM cells, after the first passage, the Respiratory Research Group performed 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). The passaged cells
were visually examined for the typical hill-and-valley morphology, where ASM cells were elongated, thin, spindle-shaped and showed concentric, oval nuclei.

2.2.2 Western blotting

2.2.2.1 Solutions

Cell lysis/sample buffer at 5x concentration was composed of TrisHCl 250 mM, DTT 500 mM, SDS 10% (w/v), bromophenol blue 0.5% (w/v), glycerol 50%, (v/v) pH 6.8 and was used to collect cell lysates. The separating gel (8% (w/v) if not otherwise specified) consisted of a 30% (v/v) acrylamide- 0.8% (v/v) 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% (v/v) acrylamide- 0.8% (v/v) bisacrylamide- solution TrisHCl (pH 6.8) 1 M, SDS 10% (w/v), TEMED and a 30% (w/v) APS solution. The electrophoresis buffer (pH 8.3) contained TRIS 25 mM, glycine 250 mM, and SDS 0.5 % (w/v).

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 was 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 Sample preparation and immunoblotting

According to cell needs and experimental plans, ASM cells were plated in 6 well plates (BD Bioscience, Franklin Lakes, NJ) and cultured until they reached confluence. After the cells were growth arrested for 48 hours, the cells were treated according to the experimental plans as indicated in the respective chapters. Cells were then washed two times with ice-cold PBS and scraped using 100μL of 1x sample buffer. The scraped lysates were collected in eppendorf tubes and boiled for 5 min. The samples were fractioned by size to detect specific proteins of interest, using a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-polyacrylamide 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 electro-transfer (100 V constant on the Power Pac 200, 1 hour). To check the uniformity and the overall effectiveness of the protein separation, 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-rabbit horseradish peroxidase-conjugated secondary antibodies (see Table 2.2) and visualized by enhanced chemiluminescence (PerkinElmer, Wellesley, MA). The blots were exposed to medical film (Fujifilm, Tokyo, Japan) and then developed with an x-ray processor (SRX-101A, Konica, Tokyo, Japan).
Table 2.3 Primary and secondary antibodies used for immunoblotting

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Buffer</th>
<th>Incubation</th>
<th>Company</th>
<th>C/N</th>
<th>% Acrylamide gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTP</td>
<td>Blocking buffer</td>
<td>Over night</td>
<td>Andy Clark</td>
<td>Sak21</td>
<td>12%</td>
</tr>
<tr>
<td>Phospho-TTP</td>
<td>Blocking buffer</td>
<td>Over night</td>
<td>Andy Clark</td>
<td>Ser 178 Ab</td>
<td>12%</td>
</tr>
<tr>
<td>Total p38 MAPK</td>
<td>Primary antibody dilution buffer</td>
<td>Over night</td>
<td>Cell signalling</td>
<td>9212</td>
<td>12%</td>
</tr>
<tr>
<td>Phospho-p38 MAPK</td>
<td>Primary antibody dilution buffer</td>
<td>Over night</td>
<td>Cell signalling</td>
<td>9211</td>
<td>12%</td>
</tr>
<tr>
<td>Total ERK</td>
<td>Primary antibody dilution buffer</td>
<td>Over night</td>
<td>Cell signalling</td>
<td>9102</td>
<td>12%</td>
</tr>
<tr>
<td>Phospho-ERK</td>
<td>Primary antibody dilution buffer</td>
<td>Over night</td>
<td>Cell signalling</td>
<td>9101</td>
<td>12%</td>
</tr>
<tr>
<td>MSK-1</td>
<td>Blocking buffer</td>
<td>Over night</td>
<td>Cell signalling</td>
<td>8%</td>
<td></td>
</tr>
<tr>
<td>Histone H3</td>
<td>Blocking buffer</td>
<td>Over night</td>
<td>Cell signalling</td>
<td>15%</td>
<td></td>
</tr>
<tr>
<td>MKP-1</td>
<td>Blocking buffer</td>
<td>Over night</td>
<td>Santa Cruz Biotechnology</td>
<td>G3013</td>
<td>12%</td>
</tr>
<tr>
<td>Phospho- MKP-1</td>
<td>Blocking buffer</td>
<td>Over night</td>
<td>Santa Cruz Biotechnology</td>
<td>12%</td>
<td></td>
</tr>
<tr>
<td>α-Tubulin</td>
<td>Blocking buffer</td>
<td>1 h RT</td>
<td>Santa Cruz Biotechnology</td>
<td>sc-32293</td>
<td>12%</td>
</tr>
</tbody>
</table>

Secondary antibody

| Anti-mouse IgG   | Blocking buffer | 1 h RT | Cell signalling | 7076 |
| Anti-rabbit IgG  | Blocking buffer | 1 h RT | Cell signalling | 7074 |

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/cm², 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. Extraction of total RNA was performed according to the Qiagen RNeasy® Mini Handbook (Qiagen, Valencia, CA) with slight modification. 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 1.5 mL microcentrifuge tubes. 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% (v/v) 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 centrifuged for 15 s >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 approximately 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. Therefore the RNA was considered to be intact.


2.2.3.2 Reverse transcription and polymerase chain reaction

After the 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 aliquoted into 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. The tubes were placed on ice and the reaction buffer, RNase inhibitor and dNTPs were added to the samples, after which 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 as follows: 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: 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.
### Table 2.4 Genes selected from applied biosystems with catalogue number

<table>
<thead>
<tr>
<th>Gene of interest</th>
<th>Catalogue Number</th>
<th>Identification number</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZFP36 (TTP)</td>
<td>4331182</td>
<td>Hs00185658_m1</td>
</tr>
<tr>
<td>DUSP1 (MKP-1)</td>
<td>4331182</td>
<td>Hs00610256_g1</td>
</tr>
<tr>
<td>IL-8</td>
<td>4331182</td>
<td>Hs00174103_m1</td>
</tr>
<tr>
<td>IL-6</td>
<td>4331182</td>
<td>Hs00174131_m1</td>
</tr>
<tr>
<td>18s</td>
<td>4331182</td>
<td>Lot 1409064</td>
</tr>
</tbody>
</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. The manufacturers’ lot-specific instruction/analysis Certificate was consulted for all recommended antibody dilutions. 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% (v/v) FBS, pH 7.0) and incubated for 1 h at room temperature (RT) to block all unbound sites and therefore prevent false positive results. After 3 other wash steps the standards and cell supernatant dilutions (prepared in 0.1% (v/v) 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 conjugate (SAv- HRP) in assay diluent, was added, in a 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 strepavidin-horse radish peroxidase (HRP), bound to the detection antibody, converts the added substrate into 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 (Microplate Reader, Model 680, Bio-rad, Hercules, CA).

For all ELISAs conducted during this study a protocol adapted from BD Biosciences protocol: was used: 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 ELISA Set (BD Bioscience, Franklin Lakes, NJ).

2.2.5 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:

Validation of MKP-1 Antibody
3 Chapter 3: Validation of MKP-1 Antibody

3.1 Introduction

Antibodies are widely used in research and play an integral part in the identification of target biomarkers in a variety of research assays such as enzyme linked immuno sorbent assay (ELISA), immunoprecipitation (IP), immunohistochemistry (IHC) and of particular importance for this study, western blotting (WB). We use an indirect method of western blotting where the primary antibody binds to the target while the HRP-tagged secondary antibody binds to the primary antibody and then the reaction is able to produce light in the presence of a luminol substrate. Both primary and secondary antibodies play a crucial role in target identification. It is estimated that there are over 180 companies producing thousands of antibodies worldwide, hence there is usually a commercially available antibody to identify most biological targets. There are many commercially available antibodies produced by many different companies, each of which has its own standards when it comes to antibody validation, and they all provide different levels of information about the efficacy of that particular antibody. The antibodies that are sold will not exclusively bind to the target protein alone and may also bind to a number of targets in a range (Saper, 2005; Couchman, 2009). Hence, the task of antibody validation often falls into the hands of the researchers who are going to use that antibody. The molecule of most interest to us is MKP-1. MKP-1 is a dual specificity phosphatase known to play a crucial role as an anti-inflammatory mediator and is therefore of interest to this work. There are many companies that produce MKP-1 antibodies and some even produce multiple batches of the same antibody raised in different animals.

WB is widely used to determine an antibody’s specificity and is an appropriate first validation step if the antibody recognizes the denatured antigen. The first indication that the antibody is
specific for the selected target would be to observe a single band at the known molecular weight of the target. There are more rigorous methods to test the specificity of antibody such as x-ray crystallography of the antigen antibody binding (Franklin et al., 2004), however this method is not readily available to all and use of western blotting is often sufficient. In our case we have a negative control and a positive control. The negative control is an unstimulated sample which is known not to produce MKP-1 under resting conditions and the positive control is a sample of the same cells stimulated with TNFα. TNFα has been shown to be a upregulator of MKP-1 (Manetsch et al., 2012b). Therefore if we can detect a single band at the known molecular weight with no presence of the protein in the negative control side, then we can state that the protein we detected is our target protein. To validate an antibody its specificity to a particular target has to be proved and this has to be reproducible. With validating antibodies it is important to note the origin of the antibodies as this can give us a clue as to the specificity. For example synthetic peptides are good as we know the amino acid structures to which they are able to bind, however they will not be able to recapture the 3D modifications that are inherently part of an endogenous antibody. Hence it is also important to know the assay on which the antibody will be used. Specificity of an antibody is also dependent on whether the antibody is polyclonal or monoclonal. Polyclonal antibodies are usually raised from the immunization of lab animals to a specific peptide of the target. These antibodies contain a pool of immunoglobulin molecules that can bind many sites on the target. Monoclonal antibodies are only able to bind one specific site on the target. Polyclonal antibodies are able to bind to multiple sites and they tend to amplify the signal and hence the sensitivity of the system can be increased. However, due to the multiple binding sites the antibody is also able to cross react and create some non-specific binding (Kurien et al., 2011). Due to this fact, polyclonal antibodies are known for batch to batch variability and need to be validated every time there is a new batch (Pozner-Moulis et al., 2007).

The protein we are interested in, MKP-1, is usually ordered from Santa Cruz Biotechnologies and is an “affinity purified rabbit polyclonal antibody raised against a peptide mapping near the
C-terminus of MKP-1 of human origin” (Santa Cruz provided data sheet). As such there are many batches of the same antibody and they all need to be validated as they could react differently with the target antigen.

This study aimed to validate new antibodies in comparison to a previously validated antibody (sc370 lot H1010). In order to get the best results, the conditions in which the antibody reacts to the antigen should be optimized for the specific antibody. The conditions known to affect antibody antigen sensitivity include pH, the temperature of antibody incubation, the concentrations of the reagents involved such as the primary and secondary antibody concentrations, the duration of the incubation and the type of blocking agent/antibody dilution agent used. These are all variables that need to be accounted for when trying to validate an antibody. They represent ways to manipulate the sensitivity of the system to thereby increase the specificity of the antibody of choice.

3.2 Materials and Methods

3.2.1 ASM cell culture

Human bronchi were 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. ASM cells were dissected, purified and cultured as previously described by Johnson et al. (Johnson et al., 1995c). A minimum of three different ASM primary cell lines were used for each experiment. Cells were stimulated with TNFα (10ng/mL) for 1 h.
3.2.2 Chemicals

TNFα was purchased from R&D Systems (Minneapolis, MN). Unless otherwise specified, all other chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO).

3.2.3 Western blotting

Western blotting was performed using polyclonal antibodies against MKP-1. MKP-1 was measured using rabbit polyclonal antibodies (3 different lots of C19 sc-370: Santa Cruz Biotechnology, Santa Cruz, CA), phosphor-MKP-1 (CST 2857: Cell Signalling Technology, Danvers, MA), (SA 4500676, SAB 2500331 Sigma-Aldrich St. Louis, MO). Primary antibodies were detected with donkey anti-goat or anti-rabbit horse radish peroxidase–conjugated secondary antibodies (Cell Signalling Technology) and visualized by enhanced chemiluminescence (PerkinElmer, Wellesley, MA).

3.3 Results

3.3.1 Validation of antibodies according to previous experimental conditions

Initially various existing antibodies were tested so that the detection of MKP-1 could be observed, and were tested the existing western blotting protocol. This protocol had been used successfully for the staining of all relevant antibodies in our previous experiments. The existing protocol called for the antibody to be incubated at room temperature for 1hr, and diluted at a concentration of 1:500 in 5% (w/v) skim milk buffer. Antibodies were either incubated for 1h or incubated overnight. The MKP-1 antibody (sc-370 Batch H1010), which we had been previously obtained from Santa Cruz Bio technology had been validated, however this particular antibody
was close to exhaustion and a replacement was needed. The antibody that had been previously validated and the new antibody that was ordered were both polyclonal antibodies and as such we expected a significant amount of batch to batch variability. In preparation for this we ordered 4 new antibodies to identify the best antibody for MKP-1 interaction. Antibodies 6 and 7, sc-370 D0413 and sc-370 G3013 respectively were received at later dates and so were not tested in Figure 3.1, but in Figure 3.3 and Figure 3.5 respectively. The seven antibodies used are listed below in table 3.1.

**Table 3.1 Potential MKP-1 Antibodies to be validated**

<table>
<thead>
<tr>
<th>Antibody Number</th>
<th>Primary Antibody Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Previously validated antibody sc-370 Lot H1010</td>
</tr>
<tr>
<td>2</td>
<td>sc-370 Lot D0313</td>
</tr>
<tr>
<td>3</td>
<td>Phospho-MKP-1 (CST 2857)</td>
</tr>
<tr>
<td>4</td>
<td>Anti-MKP-1 antibody raised in rabbit (Sigma 4500676)</td>
</tr>
<tr>
<td>5</td>
<td>Anti-DUSP-1 antibody raised in goat (Sigma SAB2500331)</td>
</tr>
<tr>
<td>6</td>
<td>sc-370 Lot D0413</td>
</tr>
<tr>
<td>7</td>
<td>sc-370 Lot G3013</td>
</tr>
</tbody>
</table>

The molecular weight of MKP-1 is 40kDa. In Figure 3.1A ASM cells were either unstimulated or treated with TNFα (10ng/mL). The unstimulated cells represented the negative control (n) as unstimulated cells do not produce MKP-1 and the cells treated with TNFα represented the positive control (p). Use of the existing protocol (1 h at RT with the primary antibody at a
Chapter 3

concentration of 1:500) with the validated antibody resulted in only one band. Since we did not see a positive band with the other four new antibodies it was probable that the new antibodies did not have the same high affinity for MKP-1 as the previously validated antibody sc-370 lot H1010. Hence the antibody affinity was tested and the experiment was repeated (see Figure 3.1A) except the antibodies were incubated overnight at room temperature (3.1B). The results indicated that none of the antibodies including the previously validated antibody demonstrated a positive band for MKP-1.

3.3.2 The effect of blocking agents on antibody sensitivity

The focus of our studies at this time was to observe the effect of phosphorylated MKP-1, as phosphorylated MKP-1 is more stable and might be active for longer when stabilised. Hence we focused on the phospho-MKP-1 antibody and tried to validate it. Before we checked the antibody we examined whether the buffer used to block non-specific proteins or the antibody dilution buffer had any impact on the antibody affinity. For this study, the antibody incubation time and temperature remained (i.e. The antibody was incubated at room temperature overnight). Blocking conditions need special attention when working with phosphosite-specific antibodies (Michalewski et al., 1999). The literature also states that nonfat (skim) milk in the appropriate buffer containing Tween-20 provides the most consistent results. However skim milk is a stringent blocker of non-specific binding sites and can sometimes bind to phosphosites, thereby nullifying the phospho antibody. This blocking can be reduced using a less stringent blocking agent such as bovine serum albumin. Therefore in this experiment we tested various combinations of blocking buffer and antibody dilution buffer. In Figure 3.2 ASM cells were either unstimulated or treated with TNFα under 5 different treatment conditions:
**Table 3.2 Treatment conditions of blocking agent and antibody dilution buffer**

<table>
<thead>
<tr>
<th>Label</th>
<th>Blocking Agent</th>
<th>Antibody Dilution Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5% Skim milk</td>
<td>5% BSA</td>
</tr>
<tr>
<td>2</td>
<td>5% Skim milk</td>
<td>5% Skim milk</td>
</tr>
<tr>
<td>3</td>
<td>2% Skim milk</td>
<td>2% Skim milk</td>
</tr>
<tr>
<td>4</td>
<td>2% BSA</td>
<td>2% BSA</td>
</tr>
<tr>
<td>5</td>
<td>5% BSA</td>
<td>5% BSA</td>
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</tbody>
</table>

Out of the five combinations listed, the best result was achieved with blot 5 where a lot more protein is present. Even though we detected more total protein, we were unable to identify phosphorylated MKP-1. Therefore this experiment yielded null results suggesting that the antibody affinity for the phosphosite on MKP-1 remained the same regardless of the blocking agent or the buffer in which the antibody was diluted.

### 3.3.3 Primary antibody concentration optimization

Once the blocking agent and the antibody dilution buffer had been ruled out as a reason for the lack of antibody antigen binding the next logical step was to increase the concentration of the antibody. The concentration was increased according to the manufacturers specifications on the upper limit 1:50. Before increasing the antibody concentration another antibody provided to us from Santa Cruz Bio-technologies was first tested, sc-370 lot D0413. This new antibody was blotted alongside the verified antibody sc370 lot H1010. In Figure 3.3A ASM cells were either unstimulated or treated with TNFα and these lysates were used to depict this comparison. We can see that there is a clear MKP-1 band from the previously validated antibody and no MKP-1 band from the new antibody at the experimental conditions similar to Figure 3.1. After observing
no MKP-1 band with the new antibody either we decided to change the antibody concentration. The concentration of antibody we had been using fell within the recommended range starting at a concentration of 1:500 at the lower limit and 1:50 at the upper limit. As our experiments had been unsuccessful at the lower limit of antibody concentration we decided to try the upper limit with the new antibody. The experimental conditions for Figure 3.3B are the same as for Figure 3.1 (primary antibody incubated at room temperature for 1h). From Figure 3.3B it can be seen that there is no MKP-1 band even in the presence of an antibody concentration of 1:50. This increase in concentration did however increase the number of non-specific bands as witnessed by the appearance of dark bands above and below where we would expect to see the MKP-1 band. The antibody at a concentration of 1:500 was used as a control. Once we saw no MKP-1 band at this concentration we decided to increase the length of primary antibody incubation as done in Figure 3.1B (overnight incubation at room temperature). This lead to the appearance of an MKP-1 band, which was visible between the two dark bands located above and below the MKP-1 band. Once the band was detected, the new experimental conditions were adopted. We also tested the antibody at a concentration of 1:100 and found that no MKP-1 band was observed however the non-specific bands were present.

### 3.3.4 Antibody validation using new experimental conditions

Once the sc-370 lot D0413 antibody and been validated it was then used as the control to test other antibodies. In Figure 3.4A the newly validated antibody was compared to sc-370 lot D0313 (used in experiment 1). Under the newly adopted experimental conditions we noticed that this antibody showed affinity for MKP-1 as well. In Figure 3.4B we tried to see if this new antibody would have a higher affinity to MKP-1 and we subsequently tested it at lower concentrations. As with other MKP-1 antibodies it was only effective at the highest concentration.
3.3.5 Secondary antibody concentration optimization

The next step was to try and capture as much of the MKP-1 signal as possible, which could be achieved by manipulating the concentration of the secondary antibody. All of our experiments were conducted with a secondary antibody concentration of 1:2000. Unlike the primary antibody, this concentration was at the high end of the concentration range according to the literature. Therefore, we tested the primary antibody with a secondary concentration of 1:2000, 1:5000 and 1:10000. This is illustrated in Figure 3.5A MDA-MB-231 cells were either untreated or stimulated with TNFα. Our results showed that the MKP-1 band is more intense when the secondary antibody concentration is lower. This is then confirmed in Figure 3.5B as we observed that a new antibody lot sc-370 lot G3013 was able to detect the MKP-1 signal at a lower concentration of 1:100.
Figure 3.1 Validation of antibodies according to previous experimental conditions.

Growth arrested ASM cells were stimulated with TNF\(\alpha\) (+) or left unstimulated (-). To identify which antibody is able to bind to MKP-1 protein the best, MKP-1 protein expression was measured using western blotting. Antibody 1 is sc-370 Lot H1010, Antibody 2 is sc-370 Lot D0313, Antibody 3 is Phospho-MKP-1 (CST 2857), Antibody 4 Anti-MKP-1 Antibody raised in rabbit (Sigma 4500676), Antibody 5 anti-DUSP-1 antibody raised in goat (Sigma SAB2500331). (A) The antibodies were incubated at room temperature for 1 h at a concentration of 1:500, arrowhead next to antibody 1 indicating a faint band in the positive control side. (B) The antibodies were incubated at room temperature overnight at a concentration of 1:500.
Figure 3.2 The effect of blocking agents on antibody sensitivity.

Growth arrested ASM cells were stimulated with TNFα (+) or left unstimulated (-). To identify the impact of blocking buffer and antibody dilution buffer on sensitivity of antigen antibody binding, phospho-MKP-1 antibody was blocked and diluted in different buffers. The following are the treatments represented as with the blocking agent and its percentage on the left / dilution agent and percentage on right (SM skim milk, BSA bovine serum albumin). 1: 5% skim milk / 5% bovine serum albumin (BSA), 2: 5% skim milk / 5% skim milk, 3: 2% skim milk / 2% skim milk, 4: 2% BSA / 2% BSA, 5: 5% BSA / 5% BSA. MKP-1 is not present in any of the treatments, there are however non-specific bands present under treatment conditions 4 and 5. All percentage values were determined as w/v.
Figure 3.3 The effect of primary antibody concentration on antibody sensitivity.

Growth arrested ASM cells were stimulated with TNFα (+) or left unstimulated (-). (A) Antibody 1 is sc-370 lot H1010 and antibody 6 is sc-370 lot D0413. The antibodies were incubated for 1 h at room temperature at a concentration of 1:500. (B) To examine the impact of primary antibody concentration on the sensitivity of the antigen antibody reaction antibody sc-370 lot D0413 was incubated for 1 h at room temperature at a concentration of 1:50 and a concentration of 1:500. (C) Antibody sc-370 D0413 was then incubated overnight at room temperature at concentrations of 1:50, 1:100 and 1:500. The arrowheads indicate the presence of MKP-1. There are no specific bands present in all but two blots.
Figure 3.4 Antibody validation using new experimental conditions.

Growth arrested ASM cells were stimulated with TNFα (+) or left unstimulated (-). Antibodies were incubated at room temperature overnight. (A) sc-370 D0413 was compared to sc-370 D0313 at a concentration of 1:50. (B) Optimization of sc-370 D0313 using the antibody at a concentration of 1:50, 1:100 and 1:500. The arrow heads indicates the presence of MKP-1. There are non-specific bands present in all but one blot.
Figure 3.5 The effect of secondary antibody concentration on antibody sensitivity.

Growth arrested MDA-MB-231 cells were stimulated with TNFα (+) or left unstimulated (-). To examine the impact of secondary antibody concentrations on the sensitivity of antibody antigen reaction sc-370 D0313 was incubated at room temperature overnight at a concentration of 1:50. (A) The intensity of WB staining was measured using secondary antibody concentrations of 1:2000, 1:5000 and 1:10000. (B) WB was used to validate the newest antibody G3013 at room temperature overnight at a primary antibody concentration of 1:50 and 1:100 and a secondary antibody concentration of 1:10000.
3.4 Discussion

By getting an antibody that is specific and sensitive to a target protein you can perform experiments to determine the protein’s interactions and intensity in a given molecular system. Before we can start to identify the nature of the target, we need to determine if a particular antibody has reproducible results and consistently binds to the correct target. Hence we need to validate the antibody. Antibody validation can be a multifaceted process, however, this process can be streamlined using western blotting as a first means of determining antibody affinity (Bordeaux et al., 2010). The easiest way to do this is through the use of appropriate controls. The idea is to be able to identify the target protein in the presence of a negative control. The ideal negative control for this experiment would be cells with the protein of interest knocked out, and the ideal positive control would be cells transfected with the protein of interest. This can however be a challenging exercise as these knockout cells or transfected cells are not always available. A simple solution to this is to use a cell line known to express the target protein only in the presence of a stimulant that way the unstimulated cells will become the negative control. In our experiments our protein of interest is MKP-1 and ASM cells are known to produce MKP-1 when stimulated with TNFα and not produce MKP-1 when unstimulated. Hence, to validate a particular antibody we needed to show that there is a single immunoreactive band at the expected molecular weight of MKP-1 in the positive control and a lack of signal at the equivalent weight in the negative control.

Our experiments show that commercially available antibodies are prone to batch to batch variability and usually have low affinity for the target protein. This is shown in Figure 3.1, which tested new antibodies at conditions that were suitable for one previously validated antibody sc-370 lot H1010 1 h primary antibody incubation at room temperature at a concentration of 1:500. This concentration is at the low end of the range recommended by the manufacturer, and to achieve the best results it is best to follow the data sheet provided by the
manufacturer. An antibody with high affinity only requires a short amount of incubation at a low concentration to bind to its antigen while an antibody with lower affinity might have to be incubated for a longer time at a lower temperature. Although the rate of reaction (antibody binding to antigen) is faster at room temperature, the antibody is also likely to degrade faster at this temperature. Along with the antibody, there is also a possibility that the blocking buffer might break down as it is made from skim milk. Hence one reason there is no signal could be that the antibody might be degrading, this is however unlikely as the previously validated antibody was intact at room temperature. When the 1 h room temperature incubation did not work, the experimental conditions were changed to increase the sensitivity. There are a number of factors that affect sensitivity (Reverberi and Reverberi, 2007) not limited to temperature, length of incubation, concentration of antibody and use of blocking agent. As our first point of change we decided to alter the length of incubation to increase the sensitivity. Therefore the samples were incubated with the same new antibodies at room temperature overnight rather than 1 h. The results however were negative even when the sensitivity was slightly increased. This suggested that the antibodies were either degrading after this longer incubation or that the antibodies were still not at a point to bind to the antigen specifically.

Another important step to consider is the use of the blocking agent with which to block non-specific proteins (Spinola and Cannon, 1985), thereby improving sensitivity. Optimizing the blocking agent is important as it maximizes the signal to noise ratio. The lack of a detectable MKP-1 specific band was attributed to an increase in the background noise and hence an experiment was performed to determine whether or not the blocking agent or the buffer used to dilute the antibody had an impact on the antigen antibody affinity. We used two blocking agents; skim milk and bovine serum albumin (BSA). BSA is a less stringent blocking agent and allows more proteins to be identified. We used this to determine whether the antibody was binding to the skim milk and hence interfering with the antibody antigen interaction, this was also
supplemented with different concentrations of blocking agents (2% (w/v) vs 5% (w/v) of either skim milk or BSA). The results indicated that the difference in blocking buffer did alter the amount of non-specific proteins present, however it did not appear to aid in the immunoreactivity of MKP-1. This suggested that the blocking buffer and the buffer used to dilute the antibody were not interfering with the antibody antigen interaction.

Before replacing the antibody, another method was employed to increase the sensitivity. This time the concentration of the antibody was increased in line with the recommended guidelines provided by the manufacturer. The upper concentration limit was 1:50, so this concentration was used in conjunction with an increased incubation time (overnight) to potentially attain the maximum sensitivity. At this concentration and this incubation time we reached the desired sensitivity to uncover the MKP-1 immunoreactive band. However, by increasing the sensitivity to such an extent there were a number of unspecific bands that were also uncovered by the antibody. Since the antibodies used were polyclonal in nature antibodies producing non-specific bands were unavoidable at this high concentration. The desired band of MKP-1 lies between two sets of non-specific bands which occur above and below it. These bands are known as non-specific as they are equally intense in the negative control sample as well as the positive control sample. While the MKP-1 band has only one intense band in the positive control side with nothing present on the negative control side. Once the antibody was validated in this manner, we tried to lower the concentration of the antibody as antibodies are expensive and need to be used frequently. This unfortunately proved to be unsuccessful and suggested that the antibodies provided by the manufacturer were of very low affinity as a high concentration along with increased incubation were required to facilitate the antibody antigen reaction. The high amount of non-specific binding present was due to the high primary antibody concentration however we were still using the same secondary antibody concentration as was used when we had a low primary antibody concentration. Hence when we optimized the primary antibody concentration
we proceeded to optimize the secondary antibody concentration to coincide with the change in conditions. We incubated the samples at secondary concentrations of 1:2000, 1:5000 and 1:10000. The results indicated that the sample showed the highest intensity MKP-1 band in the presence 1:10000 dilution. Since there is less secondary antibody compared with the primary the competition for the secondary is reduced and it is able to bind to the primary antibody more robustly. With the reduced secondary concentration we then re-tested the primary antibody at a lower concentration and found that the MKP-1 band was observed at a concentration of 1:100. This meant that the antibody would now last twice as long as before and we were able to find an adequate middle ground between resource consumption and positive experimental results. These results indicate allow us to settle on an experimental protocol that could be carried through the rest of the thesis. Antibody batch G3013 (at a concentration of 1:100 and it was incubated overnight at 4°C) was used predominantly.
Chapter 4:

Temporal regulation of cytokine mRNA expression by tristetraprolin: dynamic control by p38 MAPK and MKP-1.

This chapter was published in the American Journal of Physiology – Lung Cellular and Molecular Physiology in 2015. “Temporal regulation of cytokine mRNA expression by tristetraprolin: dynamic control by p38 MAPK and MKP-1”

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Chapter 4: Temporal regulation of cytokine mRNA expression by tristetraprolin: dynamic control by p38 MAPK and MKP-1

4.1 Introduction

Many chronic inflammatory diseases are a common consequence of overactive inflammatory signalling pathways, and utilising these pathways as potential drug targets represents a way to re-establish control and attenuate the severity of such chronic inflammatory diseases. Asthma is a chronic inflammatory disease, which is characterized by reversible airway obstruction, structural remodelling and airway hyper responsiveness. A plethora of pro-inflammatory cytokines have been implicated in the pathophysiology of asthma. The common therapies for asthma include glucocorticoids and β₂ agonists; however there is still a proportion of the population for whom this treatment is ineffective. Hence a need arises to find alternative anti-inflammatory strategies, like small molecule inhibitors of inflammatory cascades or by increasing activity of anti-inflammatory proteins. In order to achieve this goal a greater understanding of the regulatory networks that control cytokines are urgently required.

Our group along with others uncovered the importance of the anti-inflammatory proteins such as MKP-1 and TTP in the control of airway inflammation. MKP-1 is a MAPK deactivator with multiple modes of anti-inflammatory action. It is an endogenous protein that functions as a negative feedback effector repressing MAPK mediated pro-inflammatory signalling and cytokine secretion (Che et al., 2012; Manetsch et al., 2012a). It can also be upregulated by anti-asthma medications such as β₂-agonists and corticosteroids (Quante et al., 2008; Manetsch et al., 2012c; Manetsch et al., 2013; Che et al., 2014a; Rahman et al., 2014), serving as an important
pathway from which these front-line asthma medicines achieve part of their anti-inflammatory function.

This study reveals that a key member of the MAPK superfamily, p38 MAPK, is intrinsically involved in regulating the expression and activity of important anti-inflammatory molecules. Our previous studies had indicated that p38 MAPK activation was responsible for the expression of early-response genes such as MKP-1. Once made, the phosphatase activity of MKP-1 dephosphorylates p38 MAPK and acts as a negative feedback effector limiting the strength and duration of p38 MAPK signalling (Manetsch et al., 2012a). Another endogenous anti-inflammatory molecule of interest along this inflammatory pathway was identified as TTP. This study shows previously unexplored the dynamic interaction between p38 MAPK and MKP-1 controls the expression and activity of TTP to repress cytokines in ASM cells.

TTP is an immediate early response gene that functions to destabilize mRNA of many cytokines (Brooks and Blackshear, 2013), including those involved in asthma (Prabhala and Ammit, 2014). TTP confers this mRNA instability and degradation by binding the conserved adenosine/uridine-rich element present within the 3’-untranslated regions of many mRNA transcripts (Carballo et al., 1998; Lai et al., 1999a). Importantly, its function is controlled by p38 MAPK; its expression is p38 MAPK-dependent (Mahtani et al., 2001a) and its anti-inflammatory activity is controlled by p38 MAPK-mediated phosphorylation (phosphorylated – OFF; unphosphorylated – ON) (Mahtani et al., 2001a; King et al., 2009b).

The importance of TTP was first elucidated when TTP was knocked-out and the mice developed a pro-inflammatory phenotype due to the overexpression of the cytokine tumor necrosis factor (TNFα) resulting in cachexia, myeloid hyperplasia and a host of other inflammatory conditions (Taylor et al., 1996). Since that time, the role of TTP in inflammatory conditions such as arthritis has become firmly established, while its impact on regulation of asthmatic inflammation is
relatively under-explored. This was addressed within this study by utilizing human ASM cells; a cell type characterized as having an important immunomodulatory role perpetuating airway inflammation through the secretion of various pro-inflammatory cytokines, including IL-6 (Lazaar and Panettieri, 2005a). This study is the first to investigate the role and function of TTP in this pivotal airway cell type in asthmatic inflammation. By stimulating cells with TNFα the kinetics of TTP expression are able to be explored and its p38 MAPK-dependency can be confirmed. Notably, by controlling p38 MAPK phosphorylation status in a temporally-distinct manner, MKP-1 ensures that TTP is expressed and made functional at an exact time to repress cytokine expression. Together, p38 MAPK, MKP-1 and TTP form a regulatory network that exerts significant control on cytokine secretion in asthmatic inflammation through precise temporal signalling.

4.2 Material and Methods

4.2.1 ASM cell culture

Human bronchi were 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. ASM cells were dissected, purified and cultured as previously described by Johnson et al. (Johnson et al., 1995c). A minimum of three different ASM primary cell lines were used for each experiment.
4.2.2 Chemicals

TNFα was purchased from R&D Systems and celecoxib from Cayman Chemical Company. Unless otherwise specified, all other chemicals used in this study were purchased from Sigma-Aldrich.

4.2.3 ELISAs

Cell supernatants were collected and stored at -20°C for later analysis by ELISA. IL-6 ELISAs were performed according to the manufacturer’s instructions (BD Biosciences Pharmingen).

4.2.4 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 IL-6 (Hs00174131_m1), nucleotide-binding domain and leucine-rich repeat protein 3 (NLRP3: Hs00918082_m1), TNFR1 (TNFRSF1: Hs01042313_m1), TTP (Zfp36: Hs001856583_m1) and MKP1 (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.

4.2.5 Western blotting
Western blotting was performed using rabbit monoclonal or polyclonal antibodies against phosphorylated (Thr\textsuperscript{180}/Tyr\textsuperscript{182}) and total p38 MAPK (from Cell Signalling Technology). TTP was measured by Western blotting using rabbit antisera against TTP (Sak21). MKP-1 was measured using a rabbit polyclonal antibody (C19: Santa Cruz Biotechnology), compared to α-tubulin as the loading control (mouse monoclonal IgG\textsubscript{1}, clone DM 1A). Primary antibodies were detected with goat anti-mouse or anti-rabbit horse radish peroxidase–conjugated secondary antibodies (Cell Signalling Technology) and visualized by enhanced chemiluminescence (PerkinElmer).

4.2.6 TTP siRNA

ASM cells were transiently transfected using nucleofection with 1 μg TTP-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 previous publication (Quante et al., 2008). Briefly, ASM cells were transfected with the Nucleofector (Amaxa, Koln, Germany), using the basic kit for primary smooth muscle cells with the manufacturer's optimized protocol of P-024. ASM cells were plated for 16 h after transfection, before being growth-arrested for a further 24 h. Cells were then stimulated with TNFα (10 ng/ml) for the indicated times before TTP and IL-6 mRNA measurement by RT-PCR and IL-6 protein secretion measured by ELISA.

4.2.7 Statistical analysis
Statistical analysis was performed using regression, 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.

4.3 Results

4.3.1 TNFα-induced IL-6 secretion is due to biphasic IL-6 mRNA expression

In order to investigate the temporal regulation of IL-6 expression by TNFα, growth-arrested ASM cells were stimulated with TNFα (10 ng/ml) for up to 24 h, and the temporal profiles of IL-6 gene expression and subsequent protein secretion compared. Firstly, the inflammatory capacity of TNFα was confirmed by measuring levels of induced IL-6 in ASM cells (Quante et al., 2008), with significant amounts of IL-6 protein secretion observed by 24 h \( (P<0.05) \). This was followed by a detailed assessment of the temporal profile of IL-6 mRNA expression responsible for IL-6 secretion. Figure 4.1A demonstrates that TNFα-induced IL-6 mRNA has a biphasic expression profile. The peak in the first phase of TNFα-induced mRNA expression occurs at the 1 h time point, where a 54.5±11.5-fold increase in TNFα induced IL-6 mRNA can be observed \( (P<0.05) \). This initial increase in mRNA is followed by lower levels of mRNA expression until the 4 h time point where the levels of TNFα-induced IL-6 mRNA expressed are no longer significant. Following this nadir at 4 h, a secondary phase of IL-6 mRNA expression ensues, reaching a peak of 35.1±9.1-fold increase at 24 h \( (P<0.05) \).
4.3.2 The biphasic nature of TNFα-induced IL-6 mRNA expression was not due to COX-2-derived prostanoids, increased expression of NLRP3 inflammasome components, or upregulation of the cognate receptor for TNFα - TNFR1

To uncover the cell signalling mechanisms responsible for the biphasic nature of TNFα-induced IL-6 mRNA expression it was first examined whether prostanoid products of COX-2 might be involved as TNFα is known to induce COX-2 in this cell type (Belvisi et al., 1997; Pang and Knox, 1997; Alkhouri et al., 2014), and prostanoids, including PGE₂ and PGI₂, may increase IL-6 via cAMP-dependent means (Pang and Knox, 1997; Ammit et al., 2000). To investigate this possibility, growth-arrested ASM cells were pre-treated with the COX-2 selective inhibitor celecoxib for 1 h, prior to stimulation with TNFα for 24 h. Figure 4.2A illustrates the impact of celecoxib on TNFα-induced mRNA expression. Consistent with our earlier results, IL-6 mRNA is augmented after 24 h of TNFα stimulation; IL-6 mRNA levels are increased by 27.0±4.9-fold, compared to vehicle alone (P<0.05). However, celecoxib has no significant effect on IL-6 mRNA expression in the presence or absence of TNFα. Thus, as TNFα-induced IL-6 mRNA is unaffected by celecoxib our results indicate that IL-6 mRNA is upregulated in a prostanoid-independent manner. To then test whether components of the inflammasome, specifically NLRP3, might be involved, expression level of NLRP3 was measured after 24 h stimulation with TNFα. The results indicated no significant difference compared with vehicle-treated cells (Figure 4.2B). Finally, the potential auto-regulatory mechanism by which TNFα upregulates itself by controlling its cognate receptor TNFR-1 and its possible link to enhanced IL-6 expression was examined. As shown in Figure 4.2C, the levels of TNFR1 were not significantly upregulated after 24 h stimulation with TNFα with only a 1.4±0.2-fold increase in mRNA expression, compared to control. Taken together, these data suggest that the secondary phase of TNFα-induced IL-6 mRNA expression is independent of PGE₂, NLRP3 inflammasome and TNFR1 upregulation.
4.3.3  TNFα rapidly upregulates MKP-1 in a p38 MAPK-dependent manner

The study then examined whether the kinase p38 MAPK and its phosphatase MKP-1 might be involved in regulatory networks controlling cytokine expression. MKP-1 is an early-response gene that can be rapidly transcribed and expressed as a protein in a p38 MAPK-dependent manner (Manetsch et al., 2012a). In Figure 4.3A, the temporal profile of MKP-1 mRNA was established and demonstrated a rapid 10.1±1.8-fold upregulation of MKP-1 mRNA at the 1 h time-point (P<0.05), that then rapidly returns to basal levels. MKP-1 mRNA and protein expression is augmented via the p38 MAPK-mediated, but not ERK-mediated, pathway. This is shown in Figures 4.3B and 4.3C, where growth-arrested ASM cells were pre-treated for 30 min with either vehicle or pharmacological inhibitors of the p38 MAPK (SB203580) and ERK (PD98059) pathways, prior to stimulation with TNFα. TNFα-induced MKP-1 expression was then observed as early as 30 min, and was significantly attenuated by SB203580, but not PD98059 (Figure 4.3B: P<0.05). MKP-1 mRNA is rapidly translated into MKP-1 protein by 1 h (Figure 4.3C) and was similarly repressed when the p38 MAPK-mediated pathway was inhibited in accordance with our previous study (Manetsch et al., 2012a).

4.3.4  Temporal kinetics and p38 MAPK-dependence of TTP mRNA expression induced by TNFα

This is the first study to demonstrate the dynamic regulation of cytokine regulatory networks controlled by MKP-1 and p38 MAPK in ASM cells. This intricate regulation involves the destabilizing RNA-binding protein - TTP. This is demonstrated in Figure 4.4 where it can be seen that TNFα upregulates TTP mRNA expression in ASM cells and does so in a p38 MAPK-dependent manner. To explore the temporal kinetics of TNFα-induced TTP mRNA expression,
growth-arrested ASM cells were treated with TNFα and TTP mRNA was measured by RT-PCR at 0, 0.5, 1, 2, 4, 8, and 24 h. As shown in Figure 4.4A, TTP mRNA is significantly and rapidly increased by TNFα with 8.7±2.0-fold at the 1 h time point ($P<0.05$). To determine whether this augmentation is mediated via the p38 MAPK pathway, growth-arrested ASM cells were pre-treated with SB203580 and PD98059 before stimulation with TNFα for 30 min. The results show that TNFα-induced TTP mRNA is repressed in the presence of the p38 MAPK inhibitor, while unaffected by the ERK inhibitor (Figure 4.4B). Hence, this shows that TNFα-induced TTP mRNA expression in ASM cells is p38 MAPK-dependent.

### 4.3.5 TTP activity is temporally regulated by MKP-1 via control of p38 MAPK phosphorylation

Previous publications have shown that p38 MAPK is rapidly activated by TNFα (Quante et al., 2008; Manetsch et al., 2012a) and that upregulation of MKP-1 in a p38 MAPK-dependent manner gives rise to a negative feedback loop (Manetsch et al., 2012a). In this study it is shown for the first time, that in ASM cells, this intricate control of p38 MAPK phosphorylation by MKP-1, regulates cytokine secretion via modulation of TTP expression and activity. Figures 4.5A and 4.5B illustrate the temporal inter-relationship that exists between p38 MAPK, MKP-1 and TTP. This is shown by western blot analysis and the respective densitometry. It was observed that TNFα rapidly induces p38 MAPK phosphorylation by 0.25 h and 0.5 h. MKP-1 protein is expressed in response to an increase in p38 MAPK phosphorylation, with a peak of protein upregulation at 1 h. However, as levels of this MAPK phosphatase increase, there is a corresponding decrease in phospho-p38 MAPK. Then as MKP-1 degrades (being subject to proteasomal degradation), the restraint on p38 MAPK is lessened and phospho-p38 MAPK builds up again as best shown in densitometric analysis (Figure 4.5B). These data are in accord
with earlier studies (Manetsch et al., 2012a) and provide the first complete analysis of temporal inter-relationship between p38 MAPK phosphorylation and MKP-1.

To then explore the extent of TTP regulation conferred by phosphorylated p38 MAPK, TTP protein levels were measured in parallel to MKP-1 and phosphorylated p38 MAPK. TTP is detected using rabbit antisera against TTP (Sak21). Previous studies utilizing this antibody demonstrate two immunoreactive bands for TTP that are indicative of TTP phosphorylation status and thus activity; the upper immunoreactive band identified as the phosphorylated (inactive) form and the lower band identified as the unphosphorylated (active) form (Mahtani et al., 2001a; King et al., 2009b). As shown in Figure 4.5A, TNFα-induces TTP protein expression at 0.25 h and 0.5 h. Since TTP mRNA expression is p38-MAPK-dependent and protein translation occurs during peak p38 MAPK phosphorylation levels, the phosphorylated form of TTP predominates. By 1 h however, when p38 MAPK phosphorylation is switched off due to the action of MKP-1, the lower unphosphorylated TTP immunoreactive band appears (Figure 4.5B). Then, when MKP-1 subsides and p38 MAPK is no longer deactivated, both immunoreactive forms of TTP are present, switching to the phosphorylated, upper immunoreactive band further along the time course. Collectively, these results reveal a temporally co-ordinated regulatory mechanism involving phosphorylated p38 MAPK, MKP-1 and TTP.

4.3.6  TNFα-induced IL-6 mRNA expression is biphasic due to the temporal regulation of TTP activity by p38 MAPK

The temporally-specific control of p38 MAPK phosphorylation by MKP-1 that governs cytokine secretion via regulation of TTP expression and activity was next to be explored. Specifically it was proposed that the secondary phase of IL-6 mRNA expression occurs because MKP-1 is no
longer present to restrain p38 MAPK and consequently TTP, although expressed, is phosphorylated and therefore inactive. To test this experimentally, growth-arrested ASM cells were stimulated with TNFα and then treated with vehicle or the p38 MAPK inhibitor (SB203580) 3.5 h after TNFα. Cell lysates were then prepared 4, 8, and 24 h after TNFα stimulation for Western blotting (Figures 4.6A and 4.6B) and the impact on IL-6 mRNA expression and secreted IL-6 measured by RT-PCR and ELISA, respectively (Figures 4.6C and 4.6D). Figure 4.6A shows the protein expression profile of TTP and phosphorylated p38 MAPK. In the absence of SB203580, TNFα-induced p38 MAPK phosphorylation is sustained at 4-24 h and although overall expression levels of TTP are elevated, it is mainly the upper phosphorylated (inactive) form. Notably, after SB203580 treatment, p38 MAPK phosphorylation is reduced at 4 h and while the upper immunoreactive band is less intense, importantly, the lower unphosphorylated band prevails. As this unphosphorylated band is the active form of TTP, a significant inverse relationship between p38 MAPK phosphorylation versus TTP (% active) (i.e. % unphosphorylated TTP/total TTP) is observed when analysed by densitometry (Figure 4.6 C: \( r^2=0.59; \ P<0.0001 \)). Accordingly, by reducing p38 MAPK phosphorylation at 3.5 h (with SB203580), the TTP activity is increased and significantly \((P<0.05)\) represses TNFα-induced IL-6 mRNA expression (Figure 4.6C) and IL-6 secretion (Figure 4.6D). In this way, the molecular mechanisms responsible for the biphasic expression of IL-6 mRNA expression can be revealed and show how p38 MAPK controls TTP phosphorylation status (and therefore TTP activity) in a temporally-specific manner to regulate cytokine secretion in ASM cells.

4.3.7  Impact of TTP knockdown (by siRNA) on TNFα-induced IL-6 mRNA expression and protein secretion

Finally, the impact of specifically knocking down TTP with siRNA on TNFα-induced IL-6
mRNA expression and protein secretion was examined. Primary cultures of ASM were hard to transfect with conventional transfection techniques, thus a well established nucleofection technique was utilized to knockdown TTP (Quante et al., 2008). ASM cells were transiently transfected using nucleofection with scrambled control or TTP siRNA, growth-arrested, then stimulated with TNFα (10 ng/ml) for 0, 1, 4, 8 and 24 h. As shown in Figure 4.7A, the peak of TTP mRNA expression at 1 h was significantly reduced by TTP siRNA (P<0.05). As predicted by the difficult-to-transfect nature of ASM cells, the transfection efficiencies ranged from a low of 23.2% to a high of 77.2% knockdown; with the average being 53.5±5.8% (n=8 primary ASM cell cultures; results expressed as a percentage of TTP mRNA in cells nucleofected with siRNA, compared to scrambled control, after 1 h stimulation with TNFα). The results were focused on the primary cell cultures with ≥ 50% knockdown of TTP (n=6 primary ASM cell cultures), regression analysis revealed that there was a significant relationship between the % of TTP knockdown and the impact on % TNFα-induced IL-6 mRNA at 4 h (y=45.103+0.104x: r²=0.745; P<0.0267). Figures 4.7B and 4.7C show the impact of TTP knockdown (by siRNA) on TNFα-induced IL-6 mRNA expression and protein secretion in these ASM cell cultures. The kinetics of IL-6 mRNA expression in cells transfected with TTP siRNA differed when compared to those transfected with scrambled control, albeit non-significantly (Figure 4.7B) and TTP knockdown resulted in significantly greater IL-6 secretion after TNFα stimulation, compared to cells nucleofected with scrambled control (Figure 4.7C; P<0.05).
Figure 4.1 TNF α-induced IL-6 secretion is due to biphasic IL-6 mRNA expression.

Growth-arrested ASM cells were treated with TNFα (10 ng/ml) for 0, 1, 2, 4, 8, and 24 h. (A) IL-6 protein secretion was measured by ELISA and (B) IL-6 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 one-way ANOVA then Fisher’s post-hoc multiple comparison test, (where * denotes a significant effect of TNFα on IL-6 expression (P<0.05)). Data are mean±SEM values from n=12 primary ASM cell cultures.
Figure 4.2 The biphasic nature of TNFα-induced IL-6 mRNA expression was not due to COX-2-derived prostanoids, increased expression of NLRP3 inflammasome components, or upregulation of the cognate receptor for TNF α - TNFR1.

Growth-arrested ASM cells were treated with vehicle or TNFα (10 ng/ml) for 24 h. In (A) cells were pre-treated with vehicle or 10 µM celecoxib for 1 h. (A) IL-6, (B) NLRP3, (C) TNFR1 mRNA expression was quantified by real-time RT-PCR (results expressed as fold increase compared to vehicle-treated cells). Statistical analysis was performed using Student’s unpaired t test, where * denotes a significant effect of TNFα (P<0.05). Data are mean+SEM values from n=3 primary ASM cell cultures.
Figure 4.3 TNFα rapidly upregulates MKP-1 in a p38 MAPK-dependent manner.

(A) Growth-arrested ASM cells were treated with TNFα (10 ng/ml) for 0, 0.5, 1, 2, 4, 8, and 24 h and the temporal kinetics of MKP-1 mRNA expression quantified by real-time RT-PCR (results expressed as fold increase compared to vehicle-treated cells at 0 h). Statistical analysis was performed using one-way ANOVA then Fisher’s post-hoc multiple comparison test, where * denotes a significant effect of TNFα on MKP-1 expression (P<0.05). Data are mean ± SEM values from n=8 primary ASM cell cultures. (B, C) To confirm that TNFα-induced MKP-1 mRNA expression (B) and protein upregulation (C) was p38 MAPK-dependent, growth-arrested ASM cells were pretreated for 0.5 h with vehicle, 1 µM SB203580 or 10 µM PD98059, prior to stimulation with TNFα. (B) MKP-1 mRNA expression was quantified by real-time RT-PCR at 0.5 h (results expressed as % TNFα-induced MKP-1 mRNA expression). Statistical analysis was performed using Student's unpaired t test, where § denotes significant inhibition of TNFα-induced MKP-1 mRNA expression (P<0.05). Data are mean±SEM values from n=4 primary ASM cell cultures. (C) MKP-1 protein upregulation at 1 h was analysed by Western blotting (with α-tubulin as the loading control). Results are representative Western blots (from n=6 primary ASM cell lines).
**Figure 4.4 Temporal kinetics and p38 MAPK-dependence of TTP mRNA expression induced by TNFα**

(A) Growth-arrested ASM cells were treated with TNFα (10 ng/ml) for 0, 0.5, 1, 2, 4, 8, and 24 h and the temporal kinetics of TTP mRNA expression quantified by real-time RT-PCR (results expressed as fold increase compared to vehicle-treated cells at 0 h). Statistical analysis was performed using one-way ANOVA then Fisher’s post-hoc multiple comparison test, where * denotes a significant effect of TNFα on TTP expression (P<0.05). Data are mean+SEM values from n=8 primary ASM cell cultures.

(B) To demonstrate that TNFα-induced mRNA expression was p38 MAPK-dependent, growth-arrested ASM cells were pre-treated for 0.5 h with vehicle, 1 µM SB203580 or 10 µM PD98059, prior to stimulation with TNFα, and TTP mRNA expression was quantified by real-time RT-PCR at 30 min (results expressed as % TNFα-induced TTP mRNA expression). Statistical analysis was performed using Student’s unpaired t test, where § denotes significant inhibition of TNFα-induced TTP mRNA expression (P<0.05). Data are mean+SEM values from n=4 primary ASM cell cultures.
Figure 4.5 TTP activity is temporally regulated by MKP-1 via control of p38 MAPK phosphorylation.
Growth-arrested ASM cells were treated with TNFα (10 ng/ml) for 0, 0.25, 0.5, 1, 2, 4, 8, and 24 h and the temporal kinetics of p38 phosphorylation, MKP-1 and TTP protein upregulation compared by Western blotting (with α-tubulin as the loading control). Please note two bands of immunoreactivity for TTP: bands at higher molecular weight indicate phosphorylated TTP (inactive), while lower bands are unphosphorylated (active). (A) Results are representative Western blots, while (B) demonstrates densitometric analysis of TNFα-induced p38 MAPK phosphorylation compared to MKP-1 protein upregulation over time (data are mean±SEM values from n=4 primary ASM cell cultures).
A

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B

p38 MAPK phosphorylation (fold increase) vs TTP (% active)
Chapter 4

Figure 4.6 TNFα-induced IL-6 mRNA expression is biphasic due to the temporal regulation of TTP activity by p38 MAPK.

To show that p38 MAPK controls TTP phosphorylation status (and therefore TTP activity) in a temporally-specific manner to regulate cytokine secretion, growth-arrested ASM cells were stimulated with TNFα (10 ng/ml) and then treated with vehicle or the p38 MAPK inhibitor SB203580 (1 µM) 3.5 h after TNFα. (A, B) Lysates were prepared 4, 8, and 24 h after TNFα stimulation to analyse temporal kinetics of p38 phosphorylation and TTP protein upregulation (with α-tubulin as the loading control) by Western blotting. Results are (A) representative Western blots of n=4 primary cell cultures, while (B) demonstrates the negative correlation between p38 MAPK phosphorylation versus TTP (% active) (i.e. % unphosphorylated TTP/total TTP) analysed by densitometry (y=5.355-0.095x, r²=0.59: P<0.0001). (C, D) IL-6 mRNA expression and protein secretion were measured by RT-PCR and ELISA. Statistical analysis was performed using two-way ANOVA then Bonferroni’s post-test where § denotes significant inhibition of TNFα-induced IL-6 (P<0.05)). Data are mean±SEM values (C) n=3 and (D) n=7 primary ASM cell cultures.
Figure 4.7 Impact of TTP knockdown (by siRNA) on TNFα-induced IL-6 mRNA expression and protein secretion.

ASM cells were transiently transfected using nucleofection with scrambled control or TTP siRNA, growth-arrested, then treated for 0, 1, 4, 8 or 24 h with TNFα (10 ng/ml). (A) TTP and (B) IL-6 mRNA was measured by RT-PCR (results expressed as fold increase over 0 h, scrambled control) and (C) supernatants were removed for IL-6 protein measurement by ELISA (results expressed as % of TNFα-induced IL-6 secretion at 24 h, scrambled control). Statistical analysis was performed using two-way ANOVA then Bonferroni’s post-test where § denotes significant inhibition of TNFα-induced TTP mRNA, and * denotes significant increase in TNFα-induced IL-6 secretion (P<0.05)). Data are mean±SEM values (A) n=8 and (B, C) n=6 primary ASM cell cultures.
4.4 Discussion

To date, research on TTP has primarily focused on its role in rheumatoid arthritis, leaving investigations into the anti-inflammatory function of TTP in airway disease relatively unexplored. Importantly, a number of critical cytokines that drive inflammation in asthma are destabilized by TTP (Lai et al., 2006; Tudor et al., 2009). This includes IL-6 (Quante et al., 2008) and in this study the temporal profile of TNFα-induced IL-6 mRNA was investigated and its expression in ASM cells to reveal the important role played by TTP. Notably, TTP expression and activity is regulated by p38 MAPK and controlled in a temporally-distinct manner by MKP-1.

Investigations into ASM cells have revealed their immunomodulatory function outside their originally proposed roles, regulating airway bronchomotor tone. This led to the finding that when primary cultures of ASM cells were treated in vitro with pro-inflammatory stimulants (such as TNFα) they secreted numerous cell adhesion molecules and cytokines (Lazaar and Panettieri, 2005a), including IL-6 (Ammit et al., 2000; McKay et al., 2000). IL-6 has many pro-inflammatory functions relevant to inflammation in asthma. In vitro investigations utilizing primary cultures of ASM cells stimulated with TNFα have allowed us to delineate many of the molecular mechanisms responsible for IL-6 cytokine secretion in this pivotal immunomodulatory cell type.

Previously observed results that TNFα-induced IL-6 mRNA expression occurred in a biphasic manner were confirmed (Quante et al., 2008; Hirota et al., 2013), to date however, the cellular signalling regulatory networks responsible had not been delineated. Studies had shown that the NLRP3 inflammasome and its components are able to elicit an inflammatory response through the activation of caspase-1. This caspase-1 is able to cleave pro-forms of IL-1β and IL-18 into bio-active cytokines that can initiate or amplify diverse downstream signalling pathways (Im and Ammit, 2014). Along with the inflammasome, TNFα is also able to induce COX-2 expression,
and COX-2 products, including prostanoids such as PGE\(_2\) and PGI\(_2\), have been shown to play a role in the pathogenesis of asthma (Belvisi et al., 1997; Pang and Knox, 1997; Belvisi et al., 1998; Pang et al., 1998). These prostanoids are cAMP-elevating agents and can trigger transcriptional responses leading to cytokine secretion in a number of different cell types, including ASM cells (Ammit et al., 2000; Manetsch et al., 2013). Thus, due to their inflammatory capabilities, whether NLRP3 inflammasome and cAMP-regulating prostanoids were upregulated by TNF\(\alpha\) was examined in this study as the potential basis of the secondary phase of IL-6 mRNA upregulation. Moreover, the upregulation of tumor necrosis factor receptor 1 (TNFR1) was also explored to determine if TNF\(\alpha\) was involved in a positive feedback loop with its own receptor. However, the results showed that TNF\(\alpha\)-induced IL-6 mRNA was upregulated in an inflammasome- or COX-2-independent manner. The results also indicated that the TNFR1 receptor was not upregulated as a result of TNF\(\alpha\). However, as our earlier studies showed that TNF\(\alpha\) is able to induce IL-6 and since TNF\(\alpha\) is known to elicit its pro-inflammatory effects via the p38 MAPK pathway. It was intriguing to investigate the role of p38 MAPK in the temporal regulation of IL-6 mRNA expression. The current studies demonstrate that the dynamic interaction between p38 MAPK and MKP-1 regulates TTP expression and activity and thus exerts a significant impact on cytokine expression.

MKP-1 is the archetypal member of a family of dual specificities phosphatases, which can dephosphorylate, thereby deactivating, members of the MAPK family (Doddareddy et al., 2012). Studies have supported a role for MKP-1 as a negative feedback regulator of the p38 MAPK pathway and as a repressor of pro-remodelling functions of ASM cells (Issa et al., 2007b; Quante et al., 2008; Moutzouris et al., 2010; Manetsch et al., 2012a; Che et al., 2014a). MKP-1 has been widely explored; however its potential interaction with TTP is less well studied. This study confirmed that TNF\(\alpha\) increases the level of MKP-1 via a p38 MAPK-dependent pathway (Manetsch et al., 2012a). It is now shown that, like MKP-1, TTP gene expression is p38 MAPK-
mediated. The upregulation of TTP mRNA has been previously shown in human monocytes treated with lipopolysaccharide (Mahtani et al., 2001a) and A549 cells treated with IL-1β (King et al., 2009b). This study is the first to present this data in ASM cells. TTP activity (as indicated by upper and lower immunoreactive bands, in accordance with King et al. (King et al., 2009b)) is also temporally regulated by p38 MAPK. The results also indicated that TNFα increases TTP mRNA by as early as 30 minutes. At this time p38 MAPK has peak levels of phosphorylation and any translated TTP protein present is phosphorylated and therefore inactive (upper band). However, MKP-1 mRNA is also expressed at 30 min and MKP-1 protein rapidly produced. It then restrains p38 MAPK phosphorylation to allow greater levels of the active form of TTP protein to be present (lower band). Active TTP reduces cytokine expression and thus the initial peak of cytokine expression subsides. But MKP-1 upregulation after TNFα is also transitory and so when the restraint of MKP-1 phosphatase activity is removed, p38 MAPK levels build again over time. Correspondingly, TTP is less active and allows a secondary phase of cytokine expression.

Collectively, these studies provide evidence that the biphasic nature of the TNFα-induced IL-6 mRNA expression profile is due to the co-ordinated regulation of TTP expression and activity by p38 MAPK. As p38 MAPK activity is regulated by MKP-1, these results provide further support for the important anti-inflammatory role played by MKP-1 in asthma and airway remodelling and demonstrate that targeting these molecules represent potential anti-inflammatory therapies in the future.
Chapter 5:

Corticosteroid-induced MKP-1 represses pro-inflammatory cytokine secretion by enhancing TTP activity, not TTP mRNA and protein expression

As of the 4\textsuperscript{th} of January 2016, this chapter has been submitted to the Journal Cellular Physiology and is currently under review.

Prabhala P, Bunge K, Ge Q and Ammit AJ.
Chapter 5

Chapter 5: Corticosteroid-induced MKP-1 represses pro-inflammatory cytokine secretion by enhancing TTP activity, not TTP mRNA and protein expression

5.1 Introduction

Tristetraprolin (TTP) is an anti-inflammatory protein that promotes mRNA decay of many proteins, including pro-inflammatory cytokines that drive respiratory disease progression. Due to its nature as an immediate-early gene (Carballo et al., 1998) and critical anti-inflammatory molecule, it is not surprising that the expression and function of TTP is highly regulated and amenable to rapid control in a mechanism akin to an on-off molecular switch. Critically, its function is controlled by p38 MAPK: its expression is p38 MAPK-dependent (Mahtani et al., 2001a); and its mRNA destabilizing activity is controlled by p38 MAPK/MK2-mediated phosphorylation on two key serine residues (S52 and S178) preventing initiation of mRNA decay (Marchese et al., 2010a).

Given the critical role of p38 MAPK in TTP regulation, it might seem logical to attempt to block the p38 MAPK pathway to enhance TTP anti-inflammatory function. However this doesn’t work because it stops TTP mRNA from being expressed in the first place (Mahtani et al., 2001a). Data from the previous study suggests that a better approach towards repression of inflammation is to gain an in depth understanding of the temporal regulation of TTP expression and control of its anti-inflammatory activity by p38 MAPK. Understanding cytokine regulatory networks in this way will allow future development of novel pharmacotherapeutic approaches that repress pro-inflammatory cytokines while ensuring that vital, anti-inflammatory proteins necessary for disease resolution remain operational (reviewed in (Prabhala and Ammit, 2015)).
Harnessing the power of the endogenous MAPK deactivator - mitogen-activated protein kinase phosphatase 1 (MKP-1) – is one such approach. The phosphatase MKP-1 dephosphorylates p38 MAPK (Quante et al., 2008; Manetsch et al., 2012a) and it was recently demonstrated that the precise temporal regulation of p38 MAPK phosphorylation status by MKP-1 ensured that TTP was expressed and made functional at an exact time to repress cytokine expression in a pivotal airway cell type implicated in respiratory disease ASM cells (Prabhala et al., 2015b). MKP-1 is a corticosteroid-inducible gene (Clark, 2003b) and recent research has shown that dexamethasone (Quante et al., 2008; Manetsch et al., 2012a) and the clinically-used corticosteroid fluticasone propionate (Manetsch et al., 2013) upregulate MKP-1 in ASM cells. But to date, only a preventative protocol has been used in our in vitro model of asthmatic inflammation, i.e. added corticosteroids before stimulating cells with tumour necrosis factor (TNFα)(a pro-asthmatic cytokine found elevated in human disease (Broide et al., 1992a) and widely utilized in in vitro studies of asthmatic inflammation). The repressive action of dexamethasone when it is added after stimulation (therapeutic protocol) has not been explored. Nor has the p38 MAPK-mediated modulation of TTP by dexamethasone in this cell type. These studies will yield vital clues that may uncover how inflammation can be targeted in a corticosteroid-sparing or a non-steroidal manner. These highlighted gaps are addressed within this study and show that TTP is a highly adaptable molecule that can exert anti-inflammatory power even at low levels of expression. This is due to the fact that it is the activity, not overall amount of TTP, which is responsible for its anti-inflammatory mRNA destabilizing impact in ASM cells. Confirmatory results indicate that TTP exerts anti-inflammatory activity when in the unphosphorylated form and that p38 MAPK modulates the phosphorylation status of TTP. By regulating p38 MAPK activity, this switch is controlled by dexamethasone-induced MKP-1 in a temporally distinct manner.
5.2 Material and Methods

5.2.1 ASM cell culture

Human bronchi were 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. ASM cells were dissected, purified and cultured as previously described by Johnson et al. (Johnson et al., 1995c). A minimum of three different ASM primary cell lines were used for each experiment.

5.2.2 Chemicals

TNFα was purchased from R&D Systems (Minneapolis, MN). Unless otherwise specified, all other 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, 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 IL-6 (Hs00174131_m1), TTP (Zfp36: Hs001856583_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 quantified by delta delta Ct calculations.
5.2.4 ELISAs

Cell supernatants were collected and stored at -20°C for later analysis by ELISA. IL-6 ELISAs were performed according to the manufacturer’s instructions (BD Biosciences Pharmingen, San Diego, CA).

5.2.5 Western blotting

Western blotting was performed using rabbit monoclonal or polyclonal antibodies against phosphorylated (Thr\(^{180}\)/Tyr\(^{182}\)) and total p38 MAPK (from Cell Signalling Technology, Danvers, MA). TTP was measured by Western blotting using rabbit antisera against TTP (Sak21) (Mahtani et al., 2001a) (generously provided by Professor Andrew R. Clark, University of Birmingham, UK). MKP-1 was measured using a rabbit polyclonal antibody (C19: Santa Cruz Biotechnology, Santa Cruz, CA), compared to α-tubulin as the loading control (mouse monoclonal IgG\(_1\), clone DM 1A: Santa Cruz). Primary antibodies were detected with goat anti-mouse or anti-rabbit horse radish peroxidase–conjugated secondary antibodies (Cell Signalling Technology) and visualized by enhanced chemiluminescence (PerkinElmer, Wellesley, MA).

5.2.6 Statistical analysis

Statistical analysis was performed using Student's unpaired \(t\) test. \(P\) values < 0.05 were sufficient to reject the null hypothesis for all analyses.
5.3 Results

5.3.1 Temporal kinetics of TNFα-induced IL-6 mRNA expression and protein secretion: impact of treating with dexamethasone 1 h after TNFα stimulation

The Ammit group (Ammit et al., 2002; Quante et al., 2008) and others (McKay et al., 2000) have established that the corticosteroid dexamethasone significantly represses cytokine IL-6 secretion from ASM cells. But these publications have used a preventative protocol where ASM cells have been pre-treated with corticosteroids 1 h before cell stimulation with TNFα. Whether corticosteroids repress IL-6 when added after cell stimulation was unknown. To address this, we treated ASM under the following conditions: vehicle; dexamethasone at 1 h; TNFα; TNFα then dex at 1 h (time of TNFα stimulation designated as 0 h), and examined the impact on IL-6 mRNA expression and protein secretion (Figure 5.1). Under unstimulated conditions (vehicle), there is no induction of IL-6 mRNA and adding dexamethasone in the absence of TNFα (dex at 1 h) had no effect on expression. In accordance with our previous publications (Quante et al., 2008; Prabhala et al., 2015b), TNFα rapidly induces IL-6 mRNA expression with a peak at 1 h. Notably, dexamethasone added after TNFα stimulation (TNFα then dex at 1 h) did inhibit TNFα-induced IL-6 expression, with significantly less IL-6 mRNA expression demonstrated at 2, 4 and 8 h (Figure 5.1A: \( P<0.05 \)). Accordingly, the resultant IL-6 protein secretion was reduced, with significantly less IL-6 protein secretion demonstrated at 8 and 24 h in cells where dexamethasone was added 1 h after TNFα, compared to TNFα alone (Figure 5.1B: \( P<0.05 \))
5.3.2 Temporal kinetics of TNFα-induced MKP-1 mRNA expression: impact of treating with dexamethasone 1 h after TNFα stimulation

Dexamethasone induces MKP-1 expression in ASM cells (Quante et al., 2008; Manetsch et al., 2012c; Che et al., 2014a). Thus, it was of interest to examine the temporal kinetics of MKP-1 mRNA in this context. ASM cells were treated under the four experimental conditions as outlined above and the temporal kinetics of MKP-1 mRNA expression measured. As shown in Figure 5.2, adding dexamethasone at 1 h induced a significant 2.9±0.6-fold increase in MKP-1 mRNA expression at the 2 h time point that was sustained for up to 24 h (3.2±0.5-fold)(P<0.05). Vehicle alone was without effect. When cells were stimulated with TNFα, MKP-1 mRNA expression peaked at 1 h and then declined; confirming the temporal kinetics of MKP-1 mRNA expression induced by TNFα in this cell type (Prabhala et al., 2015b). The impact of treating cells with dexamethasone 1 h after TNFα stimulation is characterized by a sustained expression of MKP-1 mRNA at 4-24 h (P<0.05); that is, while TNFα induced MKP-1 mRNA expression is transient, the somewhat additive effects of dexamethasone ensure that MKP-1 mRNA expression exists at the later time points. We were intrigued to examine the consequence of this sustained expression of MKP-1 mRNA expression.

5.3.3 Treatment with dexamethasone 1 h after TNFα stimulation results in sustained MKP-1 protein upregulation: effects on p38 MAPK phosphorylation

MKP-1 is an immediate early gene that can be rapidly translated into protein (Sun et al., 1993). MKP-1 dephosphorylates members of the MAPK superfamily and in ASM cells we have shown that the extent and duration of p38 MAPK phosphorylation is controlled by MKP-1 (Manetsch et al., 2012a; Prabhala et al., 2015b). Thus it is likely that MKP-1 mRNA expressed as a result of adding dexamethasone after TNFα stimulation may exert similar repressive effects, although the
impact of this regulatory network after the peak of cytokine expression has been established is less clear. To address this, we compared the temporal kinetics of MKP-1 upregulation and impact on p38 MAPK phosphorylation in cells treated with TNFα, compared to TNFα then dexamethasone at 1 h (Figure 5.3). Western blotting was performed and results expressed as representative blots (Figure 5.3A (TNFα) & Figure 5.3B (TNFα then dex at 1 h)) and densitometric analysis (Figures 5.3C (TNFα) & Figure 5.3B (TNFα then dex at 1 h)). TNFα rapidly induces p38 MAPK phosphorylation at 0.25 and 0.5 h; this was similar under both treatment conditions as dexamethasone was not added until 1 h. At 1 h, TNFα induced a 12.1±2.4-fold increase in MKP-1 protein upregulation that declined to 2.8±0.7-fold by 8 h. In accordance with the sustained expression pattern for MKP-1 mRNA, TNFα-induced MKP-1 protein upregulation was also sustained when cells were treated with dexamethasone at 1 h (Figures 5.3B & 5.3C). MKP-1 protein peaked at 2 h (19.8±2.6-fold) and this significantly increased when compared to TNFα at the same time point (11.9±1.3-fold) (Figure 5.3C: \( P<0.05 \)). While the impact on p38 MAPK phosphorylation (Figures 5.3B & 5.3D) was less pronounced than that observed for MKP-1, the extent of TNFα-induced p38 MAPK phosphorylation appeared reduced (from 2-24 h) in cells treated with dexamethasone and this difference was significant at 2 h (Figure 5.3D: \( P<0.05 \)).

5.3.4 Treatment with dexamethasone 1 h after TNFα stimulation results in reduced TTP mRNA expression at 2 h

In Prabhala et al. (Prabhala et al., 2015b) we showed that IL-6 cytokine expression in ASM cells is temporally controlled by regulatory network between MKP-1, p38 MAPK and TTP. By controlling p38 MAPK phosphorylation, MKP-1 ensures that TTP is expressed and made functional in temporally-specific manner. Dexamethasone-induced MKP-1 will likely contribute
in a similar, TTP-dependent way. To confirm this assertion, we examined the impact of dexamethasone (at 1 h) on the temporal kinetics of TTP mRNA expression stimulated by TNFα. As shown in Figure 5.4, TNFα increased TTP mRNA expression in ASM cells with a peak at 1 h. TTP is a p38 MAPK-responsive gene (Mahtani et al., 2001a; Prabhala et al., 2015b), accordingly, it is interesting to note that at 2 h, the same time point where dexamethasone treatment increases MKP-1 protein upregulation (Figures 5.3B & 5.3C), mirrored by repressed p38 MAPK phosphorylation (Figures 5.3B & 5.3D), TTP mRNA expression is significantly repressed (Figure 5.4: \( P < 0.05 \)). Vehicle and dexamethasone-only controls had no significant effect on TTP mRNA expression (Figure 5.4).

5.3.5 Treatment with dexamethasone 1 h after TNFα stimulation increases abundance of the unphosphorylated (active form) of TTP, not TTP protein upregulation

Finally, we examined TTP protein upregulation and activity by Western blotting using the rabbit antisera against TTP - Sak21. TTP protein stability and activity is controlled by phosphorylation. TTP phospho-forms appear with different electrophoretic mobility on SDS-PAGE and can be detected by Sak21. When TTP is phosphorylated by MK2 the protein is stabilized (Tchen et al., 2004b; Brook et al., 2006a; Hitti et al., 2006b), but this phosphorylated form of TTP is inactive as an anti-inflammatory RNA destabilizing factor (Marchese et al., 2010a). In the unphosphorylated state, TTP is active and can induce mRNA decay however, this form of TTP is also subject to degradation by the ubiquitin-proteasome system (Brook et al., 2006a). Previous studies utilizing Sak21 (Mahtani et al., 2001a; King et al., 2009b; Rahman et al., 2015) Prabhala, 2015 #4220} reported that bands at higher molecular weight indicate phosphorylated TTP (inactive), while lower bands are unphosphorylated (active). Thus, it is critical to note that it is not just the amount of TTP protein present that is important (because this may be the inactive,
but stable, phosphorylated form), but the activity (estimated by unphosphorylated immunoreactive bands detected by Sak21). To explore this further in our experimental context; we have compared the temporal kinetics of TTP protein upregulation (and phosphorylation status) in cells treated with TNFα or TNFα then dexamethasone at 1 h (Figure 5.5). Results are expressed as representative blots (Figure 5.5A (TNFα) & Figure 5.5B (TNFα then dex at 1 h)) and densitometric analysis; where Figure 5C is total TTP protein upregulation (normalised to α-tubulin) and Figure 5.5D is TTP (% active) (i.e. % unphosphorylated TTP/total TTP) over time. TNFα upregulates TTP protein in a temporally-distinct manner (Figure 5.5C) and interestingly, it appears the total amount of TTP is reduced by dexamethasone (albeit non-significantly); this aligns with the mRNA data. But it is the activity, not the amount of TTP that dictates its anti-inflammatory function. Notably, at 1 h after TNFα stimulation, TTP is observed in both phosphorylated and unphosphorylated immunoreactive forms (Figures 5.5A & 5.5B). Densitometric analysis revealed that TTP (% active) was 43.3±2.3% (Figure 5.5D); these data concur with our earlier study (Prabhala et al., 2015b). Notably, the impact of dexamethasone aligned with its ability to increase MKP-1 and repress p38 MAPK phosphorylation at 2 h; as shown in Figure 5.5B, there is a region of immunoreactivity indicative of unphosphorylated phospho-forms at 2 h. This is shown in Figure 5.5D, where TTP (% active) was significantly greater in cells treated with dexamethasone (41.0±2.4%), compared to TNFα alone (24.9±5.4%) at the same time point (P<0.05). Taken together our study suggests that by increasing MKP-1, dexamethasone represses p38 MAPK to control TTP phosphorylation status (and therefore TTP activity) in a temporally-specific manner to regulate IL-6 cytokine secretion in ASM cells.
Figure 5.1 Temporal kinetics of TNFα-induced IL-6 mRNA expression and protein secretion: impact of treating with dexamethasone 1 h after TNFα stimulation.

Growth-arrested ASM cells were stimulated with TNFα (10 ng/ml) or vehicle for 0, 1, 2, 4, 8, and 24 h (time of TNFα stimulation designated as 0 h). To demonstrate the impact of adding dexamethasone 1 h after stimulation with TNFα, cells were treated with dexamethasone (100 nM), compared to vehicle controls. (A) IL-6 mRNA expression was quantified by real-time RT-PCR expression (results expressed as the percentage of TNFα-induced IL-6 mRNA at 1 h (peak of expression)); (B) IL-6 protein secretion measured by ELISA (results are expressed as the percentage of TNF-induced IL-6 protein at 24 h (peak of secretion)). Statistical analysis was performed using Student’s unpaired t test, where * denotes a significant effect of dexamethasone on TNFα-induced IL-6 (P<0.05)). Data are mean + SEM values from n=6 primary ASM cell cultures.
Figure 5.2 Temporal kinetics of TNFα-induced MKP-1 mRNA expression: impact of treating with dexamethasone 1 h after TNF stimulation.

Growth-arrested ASM cells were stimulated with TNFα (10 ng/ml) or vehicle for 0, 1, 2, 4, 8, and 24 h (time of TNFα stimulation designated as 0 h). To demonstrate the impact of adding dexamethasone 1 h after stimulation with TNFα, cells were treated with dexamethasone (100 nM), compared to vehicle controls. MKP-1 mRNA expression was quantified by real-time RT-PCR expression (results expressed as fold increase compared to 0 h). Statistical analysis was performed using Student's unpaired t test, where § denotes a significant effect of dexamethasone on MKP-1 mRNA expression, and * denotes a significant effect of dexamethasone on TNFα-induced MKP-1 mRNA expression (P<0.05)). Data are mean+SEM values from n=6 primary ASM cell cultures.
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TNFα then dex at 1 h
Figure 5.3 Treatment with dexamethasone 1 h after TNFα stimulation results in sustained MKP-1 protein upregulation: effects on p38 MAPK phosphorylation.

Growth-arrested ASM cells were stimulated with TNFα (10 ng/ml) or vehicle for 0, 0.25, 0.5, 1, 2, 4, 8, and 24 h (time of TNFα stimulation designated as 0 h). Dexamethasone (100 nM), or vehicle, was added 1 h after stimulation with TNFα and the temporal kinetics of MKP-1 upregulation and impact on p38 MAPK phosphorylation compared by Western blotting (with α-tubulin as the loading control). (A, B) Results are representative Western blots when cells were treated with: (A) TNFα (B) TNFα then dexamethasone at 1 h. (C, D) Demonstrates densitometric analysis of (C) MKP-1 protein upregulation (normalized to α-tubulin and expressed as fold increase compared to 0 h) and (D) p38 MAPK phosphorylation (normalized to total p38 MAPK and expressed as fold increase compared to 0 h) over time. Statistical analysis was performed using Student’s unpaired t test, where * denotes a significant effect of dexamethasone on (C) TNFα-induced MKP-1 protein upregulation or (D) p38 MAPK phosphorylation (P<0.05). Data are mean±SEM values from n=4 primary ASM cell cultures.
Figure 5.4 Treatment with dexamethasone 1 h after TNFα stimulation results in reduced TTP mRNA expression at 2 h.

Growth-arrested ASM cells were stimulated with TNFα (10 ng/ml) or vehicle for 0, 1, 2, 4, 8, and 24 h (time of TNFα stimulation designated as 0 h). To demonstrate the impact of adding dexamethasone 1 h after stimulation with TNFα, cells were treated with dexamethasone (100 nM), compared to vehicle controls. TTP mRNA expression was quantified by real-time RT-PCR expression (results expressed as fold increase compared to 0 h). Statistical analysis was performed using Student's unpaired t test, where * denotes a significant effect of dexamethasone on TNFα-induced TTP mRNA expression (P<0.05)). Data are mean+SEM values from n=6 primary ASM cell cultures.
Figure 5.5 Treatment with dexamethasone 1 h after TNFα stimulation increases abundance of the unphosphorylated (active form) of TTP, not TTP protein upregulation.

Growth-arrested ASM cells were stimulated with TNFα (10 ng/ml) or vehicle for 0, 0.25, 0.5, 1, 2, 4, 8, and 24 h (time of TNFα stimulation designated as 0 h). Dexamethasone (100 nM), or vehicle, was added 1 h after stimulation with TNFα and the temporal kinetics of TTP upregulation compared by Western blotting (with α-tubulin as the loading control). Please note two bands of immunoreactivity for TTP: bands at higher molecular weight indicate phosphorylated TTP (inactive), while lower bands are unphosphorylated (active). (A, B) Results are representative Western blots when cells were treated with: (A) TNFα, (B) TNFα then dexamethasone at 1 h. (C, D) demonstrates densitometric analysis of (C) TTP protein upregulation (normalized to α-tubulin and expressed as fold increase compared to 0 h) and (D) TTP (% active) (i.e. % unphosphorylated TTP/total TTP) over time. Data are mean ± SEM values from n=4 primary ASM cell cultures. 
5.4 Discussion

This study underscores the importance of the MKP-1/p38 MAPK/TTP regulatory network in the control of cytokine expression in ASM cells. *In vivo* in MKP-1 and TTP transgenic mice (Ross et al., 2015; Smallie et al., 2015) and *in vitro* in cell types apart from ASM (King et al., 2009a; Shah et al., 2014), MKP-1 has been shown to deactivate p38 MAPK and co-operate with TTP to orchestrate cytokine expression. Our previous study in ASM cells (Prabhala et al., 2015b) showed that TTP acts in a negative feedback manner to temporally regulate cytokine expression via MKP-1/p38 MAPK dynamic control. Although corticosteroids have been shown to increase MKP-1 and deactivate MAPKs in ASM cells (Issa et al., 2007c; Quante et al., 2008), a direct examination of the expression and activity of TTP, and its regulation by dexamethasone-induced MKP-1 has not been explored.

We addressed this herein and confirm that dexamethasone-induced MKP-1 mRNA expression and protein upregulation in ASM cells. We commonly pre-treat ASM cells with corticosteroids for 1 h (Quante et al., 2008; Che et al., 2014b; Rahman et al., 2014), so in this study, treating cells with dexamethasone 1 h after stimulation with TNFα shifted the time-course of MKP-1 expression. In this way, dexamethasone-induced MKP-1 protein and TNFα-induced MKP-1 repressed p38 MAPK phosphorylation at 2 h in an additive manner. Accordingly, as the phosphorylation status of TTP is regulated by p38 MAPK, there was a shift from phosphorylated (inactive) to unphosphorylated (active) at this time point. We propose that this active TTP then represses IL-6 mRNA expression and protein secretion.

This is the first study to demonstrate that dexamethasone has no effect on TTP expression and protein upregulation in ASM cells and indicates that cell type differences exist. In A549 pulmonary epithelial cells, Smoak & Cidlowski (Smoak and Cidlowski, 2006) showed that 100 nM dexamethasone induced TTP expression by 4-5-fold from 2-8 h. This was confirmed by
King et al., where dexamethasone (albeit at 10-fold higher concentration, i.e. 1 µM) induced a 3-4-fold induction in TTP mRNA expression after 1 h of treatment that was sustained for up to 18 h (King et al., 2009b). Why differences exist in ASM cells is unclear at present, although perhaps the relative expression of MKP-1 vs. TTP in each cell type may play a compensatory role (Rahman and Ammit, unpublished data).

Regardless of the relative expression of each of the players in this regulatory network, the emerging view is that MKP-1/p38 MAPK and TTP co-operate in a coordinated fashion to orchestrate cytokine expression. These molecules are amenable to manipulation and represent legitimate targets for pharmacotherapeutic intervention. But perhaps most notably, our study has revealed that the timing of the intervention is important. As outlined (Prabhala and Ammit, 2015) TTP is directly regulated by p38 MAPK in a number of ways, but as its expression is p38 MAPK-mediated, p38 MAPK inhibitors would result in reduced expression of this critical anti-inflammatory protein in this first place. This is not ideal. Instead, what we have shown is that by adding dexamethasone after stimulation had commenced, MKP-1 induction occurs and protein is expressed at 2 h; this then reduced p38 MAPK phosphorylation and consequently repressed TTP mRNA expression at this time point. TTP is a highly adaptable molecule that can exert anti-inflammatory power even at very low levels of expression. This is because p38 MAPK also controls the switch between active and inactive TTP phospho-forms. Put simply, the anti-inflammatory function of TTP is dictated by the phosphorylation status of TTP at S52 and S178 (phosphorylated – OFF; unphosphorylated – ON) (Mahtani et al., 2001a). This is detected in our study as an increase in the TTP (% active) at 2 h. Also notable from our study is the predominance of the higher molecular weight, immunoreactive band for TTP; this equates to the inactive phosphorylated form of TTP. By confirming that TTP exerts anti-inflammatory activity when in the unphosphorylated form; results are in accord with recent publication that states “that
TTP is most evident when it is least active and most active when it is least evident” (Smallie et al., 2015).

Collectively, our study suggests that corticosteroids increase TTP activity, not expression, via MKP-1 in a p38 MAPK-mediated manner. Excitingly, as the phospho-states of TTP are amenable to pharmacological manipulation, our study reveals a number of potential sites for intervention and demonstrates that switching TTP on to repress cytokines in airway inflammation is a feasible strategy towards combating respiratory disease.
Chapter 6: Molecular Modelling of MKP-1
6 Chapter 6: Molecular modelling of MKP-1

6.1 Introduction

MKP-1 is the prototypical member of a family of dual specificity phosphatases that act to dephosphorylate MAPKs. MAPKs have been shown to be key signalling molecules in propagating and driving inflammation. Hence MKP-1 acts as an anti-inflammatory molecule during normal physiology. MKP-1 is however only transiently active and is switched off soon after its induction, thereby leading to a chronic inflammatory state in the presence of unregulated MAPKs. Hence finding a method through which the longevity and activation of MKP-1 could be increased would be beneficial in an inflammatory context.

MKP-1 is able to act as both a substrate and a target of MKPs. Out of the three MAPKs relevant to respiratory inflammation, it has been shown to have the greatest effect on p38 MAPK. However this MAPK is also able to invoke MKP-1 upregulation in an auto regulatory manner. Similarly ERK MAPK is able to act on MKP-1 to recruit it into the proteasome and paradoxically also increase its stability. Since ERK is a crucial regulator of MKP-1 its binding sites on MKP-1 would be of great interest for possible pharmacological intervention. ERK is able to bind to MKP-1 at specific sites where a serine residue is immediately followed by a proline residue. This specific genetic alignment occurs at four different locations; ser 296, ser 323, ser 359 and ser 364. Through the use of point mutation and truncation studies it was shown that ERK binding ser 296 and ser 323 was responsible for the proteasomal degradation whereas ERK binding ser 359 and ser 364 was responsible for MKP-1 stability (Lin et al., 2003; Lin and Yang, 2006; Crowell et al., 2014).

The authors were able to show that the stability of MKP-1 and MKP-2 was reduced in the presence of an ERK pathway inhibitor, confirming that in the absence of ERK, MKP-1 protein stability was reduced, however the survival of MKP-1 was increased since there was less ERK to bind MKP-1.
and recruit it into the proteasome. Hence finding suitable molecules that competitively bind this site (ser 296) would be a method to protect MKP-1 from the proteasomal machinery and allow it to remain present within the cells enabling it to exert its anti-inflammatory action for longer. There are many ways to increase the levels of MKP-1; one such method involves the use of proteasomal inhibitors. The use of proteasome inhibitors and their effects on cytokine secretion has been previously shown (Brondello et al., 1999; Moutzouris et al., 2010). The findings from these works, suggests that in the presence of a proteasome inhibitor MKP-1 is increased and cytokine secretion is attenuated. However, the use of a proteasome inhibitor as a therapeutic target poses a problem as the proteasome degrades many proteins and blocking it may cause an unhealthy augmentation of proteins that should have been endogenously degraded as part of normal airway physiology. Therefore instead of targeting the proteasome it is more therapeutically feasible to target MKP-1 itself. One way to target MKP-1 is through the use of small compounds that are able to bind to highly specific areas.

Structure based drug discovery involves designing and optimising drugs based on the identification of the structural characteristics of protein targets, in this case the ERK binding sites on MKP-1. The goal of this type of discovery project is to identify lead compounds to then test in vitro. In this study digitalised representations of crystal structures are attained using homology modelling (Evers and Klebe, 2004). The homology model of the MKP-1 binding region was created based on the delineated crystal structure of MKP-2. In an ideal situation the crystal structure of MKP-1 would have been available and it could have been accurately used to identify small lead compounds. Since the crystal structure of MKP-1 is still unresolved we had to use a homology model. Unlike MKP-1 the crystal structure of MKP-2 has been resolved through the use of x-ray crystallography. Due to the conserved nature of the sequences across all the MKPs we can create a reasonably accurate homology model that is structurally close the real target. This is especially true near the catalytic region (close to the DEF motif). Hence we generated a homology model of MKP-1 based on the
crystal structure of MKP-2 to recreate the ERK binding pockets. Finding these compounds by computational means represents the first step in the road to finding a novel pharmacotherapy.
6.2 Materials and Methods

6.2.1 ASM cell culture

Human bronchi were 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. ASM cells were dissected, purified and cultured as previously described by Johnson et al. (Johnson et al., 1995c). Three different ASM primary cell lines were used to test ZINC compound 1 (ZINC 38867909) while only one cell line was used to test ZINC compound 4 (ZINC 1991893).

6.2.2 Chemicals

TNFα was purchased from R&D Systems (Minneapolis, MN). Unless otherwise specified, all other chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO).

6.2.3 ELISAs

Cell supernatants were collected and stored at -20°C for later analysis by ELISA. IL-6 and IL-8 ELISAs were performed according to the manufacturer’s instructions (BD Biosciences Pharmingen, San Diego, CA).

6.2.4 Western blotting

Western blotting was performed using rabbit monoclonal or polyclonal antibodies against phosphorylated (Thr\textsuperscript{180}/Tyr\textsuperscript{182}) and total p38 MAPK, phosphorylated and total ERK MAPK (from
Cell Signalling Technology, Danvers, MA). MKP-1 was measured using a rabbit polyclonal antibody (C19: Santa Cruz Biotechnology, Santa Cruz, CA), compared to α-tubulin as the loading control (mouse monoclonal IgG1, clone DM 1A: Santa Cruz). Primary antibodies were detected with goat anti-mouse or anti-rabbit horse radish peroxidase–conjugated secondary antibodies (Cell Signalling Technology) and visualized by enhanced chemiluminescence (PerkinElmer, Wellesley, MA).

### 6.2.5 Creating the Homology Model

Corresponding the homology model of MKP-1 catalytic domain was synthesised using the ‘prime’ suite in Maestro (prime, version 2.2, Schrodinger, LLC, New York, NY) and the crystal structure of MKP-2 catalytic domain was attained from the protein data bank (PDB code 3EZ2). The MKP-1 sequence has an accession code NP_004408 and was aligned to the MKP-2 structure.

### 6.2.5.1 Protocol to MKP-1 homology modelling using Maestro

1. Open the ‘Prime’ suite located within the Applications tab
2. Go into homology modelling and then on use the ‘structure prediction wizard’ application
3. Find the MKP-1 accession code online, can be found in PubMed (NCBI protein bank). Once the code has been located, save the file as a text or fasta sequence
4. Upload this file into the homology modelling (HM) suite opened using the appropriate tab, i.e. if your importing the text file then copy paste the text, if you want to import the file then click on the ‘file’ button. Once the code has been imported, hit next at the bottom right
5. Find the structure on which you want to base your new structure, in this case we used MKP-2 which shares an 86% sequence similarity with MKP-1 near the catalytic region as our reference structure. To find the MKP-2 structure go to the ‘protein database website’ (pdb). Save the structure as a pdb file.

6. In the HM suite import the reference structure (pdb file) into the open window. Once the homolog has been imported you can press the next button. Alternatively in step 6 you can apply the blast homology search function to find homologs of MKP-1 that may be available.

7. Tick the clustalW box and hit ‘run’ to apply secondary structures, then hit ‘align’ followed by the ‘globally conserved residues’ setting. > ‘next’

8. At the top of the panel the overlapping residues from the accession code for MKP-1 and the residues for MKP-2 should appear. Using the knowledge based model building method, build the model (press the button ‘build’).

6.2.5.2 Preparing the model for docking

1. To prepare the model for docking first hydrogens need to be added, assigned bond orders and disulphide bonds. In the ‘protein preparation wizard’ application in the workflows tab, set alignment to selected entry and tick the boxes to add hydrogens, assign bond orders and add disulphide bonds.

2. Inside the ‘refine’ tab within the protein preparation wizard application, make sure the OPLS 5 is selected and the waters are eliminated. Then hit minimise
6.2.5.3 Receptor grid generation

1. Open the ‘glide’ suite within the applications tab in the maestro workspace. Then open the receptor grid generation application.

2. Select the ‘centroid selected residues’ box and then proceed to click the ‘specify the residue’ button. A small box will appear as a pop up and you can select the residue that is required from the bar at the bottom. There is a bar at the bottom that shows all the residues of the new homology model and you can select the residue you need. You can customise the size of the compounds you want to bind to your model by adjusting the ‘compound docking scale’. In this case we selected ser 318 the equivalent of ser 296.

3. When generating the grid no other parameters need to be changed. Press start and a grid will be generated and saved in the Schrodinger file.

4. Once the grid has been generated, go back to the ‘glide’ suite and enter the ligand docking application.

5. Search a chemical compound library that you want to run through your docking site, in this case we used a zinc database ((Irwin et al., 2005), which was downloaded from the internet as an .sdf file.

6. Under the ligand tab enter the library that you searched in the previous step (step 5)

7. Under the settings tab enter the receptor grid that you generated in step 3

8. ‘Start’ the process using the standard screening process, which is quicker and finds a number of compounds from the database that would bind to the docking site you specified.
9. This will generate a list of compounds with a glide docking score, the more negative the score the better the binding. A score of -6 and below represents a good match with a match of -10 being very good.

10. From this list you can then select the compounds with the lowest glide scores and process them individually through an extra precision docking. This would be the same as repeating steps 5-9 with individual compounds which can be downloaded from the database website in this case we went back to the ZINC database, searched and found the individual .sdf files for each of the compounds. This enabled us to find potential compounds that had excellent specificity.

From the ZINC library we identified 9 suitable compounds that were able to bind to ser 318, Table 6.1. We then went about the process of locating these compounds. Out of the nine theoretical matches we were only able to source two ZINC compound 4 1991893 and ZINC compound 1 ZINC 38867909. Commercial preparations of the molecules, ZINC 1991893 and ZINC 38867909 were sourced from the chemical manufacturer Ambinter in association with Greenpharma (Orleans, France). The other compounds were not commercially available from any major chemical supplier or were unable to be synthesised within a feasible budget or time frame.

6.3 Results

6.3.1 The homology model

The MKP-1 model was aligned via the conserved backbone and side chain residues present within both MKP-1 and MKP-2. The non-conserved residues were then predicted and conformed in a way to maximise stability. This model was then prepared for docking using the protein preparation
wizard which adds hydrogens, assigns bond orders and adds disulphide bonds. The final structure was then minimised again using the ‘imperf minimisation’ by selecting hydrogens only so that heavy atoms were untouched so as to minimise changes to structure. Once the model of the catalytic domain was prepared, it was ready for docking studies. The model we generated is shown in Figure 6.2 where we see a ribbon depiction of the protein secondary structure. Within this model we can see the location of the molecule of interest, ser 318, the DSPc region and the DEF motif. The model was generated using the sequence alignment of MKP-1 to MKP-2. The overlapping regions are represented in black and are most notable in the catalytic domain and less so in the early portion of the two sequences. The early portion does have some sequence similarity which is likely the KIM domain. Once the MKP-1 model had been elucidated then the compound binding could be attempted.

6.3.2 Docking

Compound docking was performed using the glide program within the maestro suite. To accurately screen for the compounds that could bind to the catalytic domain a receptor grid was generated centred around ser 296, which in MKP-2 was ser 318. When the model was created ser 318 was the region that contained the serine followed by a proline sequence that we see at the site where MKP-1 can be phosphorylated, the difference between ser 296 and ser 318 is 22 amino acids. Once the grid was generated a host compounds from an online database (ZINC chemical database) were passed through this receptor grid in a sequential screening process. All structures were already prepared and therefore no compound preparation was required for this library. The first screening was a standard precision screening from which the top 5-10% hits were then processed through an extra high precision screening resulting in a list of 30 molecules with the potential to bind the MKP-1 catalytic domain. From the 30 molecules we identified the best 9 to be used for in vitro testing and
those nine have been displayed in more detail below. Table 6.1 outlines the results of the compound docking study with the best nine listed from 1-9. The glide scores indicate the interaction between compound and the site. In Figures 6.3 - 6.11 we can see the result of the homology model and the compound together. In each Figure, (A) represented a view of the binding pocket within 7 Angstroms of the compound, which was to show the potential binding that could take place between the compound and its target (ser 318). In (B) we see the compounds themselves and (C) we see the location of the overall binding of MKP-1 homology model to the compound with respect to the target. Due to preliminary nature of this study we decided to test some of the identified molecules. However due to the manufacturer’s constraints and the unavailability of other compounds, only two of the compounds were sourced from the chemical manufacturer within a reasonable budget. The two compounds were, compound 1 (ZINC 38867909) and compound 4 (ZINC 19911893). These compounds will be first tested for efficacy using a concentration gradient and its subsequent effect on MKP-1 protein level. This data would then be supplemented by cytokine expression data to give a representation of the possible effects of increased MKP-1 on inflammatory mediators. This would then allow us to determine the molecules that had the best in vitro efficacy and give us an avenue to pursue further studies.

6.3.3 MKP-1 protein level is increased in the presence of compound 4 ZINC 19911893 and in the presence of compound 1 ZINC 38867909.

Based on the homology model, which was generated to increase the viability of MKP-1 by thwarting the effect of the proteasome, we aimed to find a molecule which could bind to MKP-1 and prevent it from being destroyed. In this proof of principle study, we tested the ZINC compound herein referred to as compound 4 and ZINC compound 38867909 referred to as compound 1. The compounds were tested under four different treatment conditions TNFα, 1 μM compound + TNFα,
10 μM compound + TNFα and 100 μM compound + TNFα. Figure 6.12A-D represents the results from the four different concentrations of compound 4 respectively while Figure 6.12E-H represents compound 1. Figure 6.13 are representative densitometric analysis of the above conditions.

TNFα was used to stimulate the ASM to mimic the inflammatory process under normal physiological conditions. In the presence of TNFα alone the transient nature of MKP-1 can be visualised. It has a peak expression at the 1 h time point followed by a swift drop back to basal levels by the 4 h time point (Prabhala et al., 2015a). We confirmed this result in Figure 6.12A and Figure 6.13A. All the MKP-1, phosphorylated p38 MAPK and phosphorylated ERK protein results are based on growth arrested ASM cells which have been pre-treated with either compound or vehicle for half an hour followed by stimulation with TNFα for 0, 0.25, 0.5, 1, 2, 4, 8 and 24 h to determine the viability and the kinetics of MKP-1 and its substrate molecules p38 MAPK and ERK MAPK.

Figure 6.13B indicates that MKP-1 longevity is increased as slowly drops down to a low at the 8 h time point as opposed to the 4 h time point as per the TNFα control. Interestingly this low point is still higher than the level of MKP-1 seen at 0 h in the same figure. This result suggests that in the presence compound 4 at a concentration of 1 μM the kinetics of MKP-1 longevity are increased. This upregulation of MKP-1 is limited to the 1 μM concentration of compound 4. Figure 6.13C and D show an MKP-1 expression profile similar to what is observed in the time matched TNFα control (Figure 6.13A). The results from compound 1 indicate that compared to time matched controls, there is an increase in the MKP-1 visualised do not appear to be as robust, however there is an increase at the 100 μM concentration of compound 1 Figure 6.12G and Figure 6.13G.

In addition to MKP-1 the representative kinetics of phosphorylated p38 MAPK and phosphorylated ERK were also shown. The only change in the expression pattern of ERK occurs in Figure 6.12B which is also the same concentration at which MKP-1 is affected. At this concentration of
compound 4, ERK does not appear to have a reduction in intensity at the 8 h time point unlike in Figure 6.12A. P38 MAPK phosphorylation has a different expression pattern based on the concentration of the compound used. Since p38 MAPK is a substrate for MKP-1 it is an easy way to check the activity of MKP-1. The data shows that p38 MAPK phosphorylation pattern changes in the presence of different compound concentrations. The impact of MKP-1 on p38 MAPK phosphorylation is most visibly seen in compound 4 at the 1 μM concentration, Figure 6.12B. At this concentration we can clearly see a drop in the phosphorylation of p38 MAPK at the 8 h and 24 h time points, which corresponds to the elevated level of MKP-1 at these time points. These kinetic expression profiles are further highlighted by densitometric analysis of the representative blots in figures 6.13B. These figures emphasise the connection that exists between MKP-1 and p38 MAPK which can be witnessed in any blot in Figure 6.13E-G, where it is noticeably evident at the 24 h time point that p38 MAPK phosphorylation begins to rise again once MKP-1 has dropped to its lowest level. In the presence of 100 μM compound 1 Figure 6.12H, we would expect to see a decrease in the level of phosphorylated p38 MAPK however, we see an increase instead at the 24 h time point instead.

From our previous work we showed that MKP-1 is induced by p38 MAPK and then it is able to dephosphorylate p38 MAPK. Importantly when the MKP-1 levels reduce at the later points along the time course there is a rebound increase of p38 MAPK phosphorylation. This data taken together suggests that compound 4 at a concentration of 1 μM and compound 1 at a concentration of 100μM are able to control this rebound in p38 MAPK and hence they should have an effect in reducing cytokine expression at this time point due to the presence of MKP-1
6.3.4 TNFα induced cytokine IL-8 is reduced in the presence of ZINC19911893 at 24 h

From our previous experiment we showed that MKP-1 levels are elevated at later time points which causes a subsequent reduction in p38 MAPK phosphorylation, hence we would expect to see a reduction in the level of cytokine expression. This is because we have a repression in p38 MAPK which is a chief instigator of inflammation. Growth arrested ASM cells were pre-treated with either compound or vehicle for half an hour followed by stimulation with TNFα for 0, 0.25, 0.5, 1, 2, 4, 8 and 24 h. The results are only shown for the 24 h time point as IL-8 cytokine expression is the highest at this time point. Based on our data from the previous experiment we know that 1 μM concentration of compound 4 shows the potential to impact the MKP-1, p38 MAPK axis. If this were indeed true then we should see a drop in cytokines levels at this concentration. This is what we presumably see in Figure 6.14B where we can see that the trend in IL-8 protein secretion is heading below the level of the untreated cells. From our results we also showed a downward trend in the amount of IL-6 cytokine expressed in the presence of compound 1 at a concentration of 100 μM. It is interesting to note that compound 4 might have an impact on IL-8 while compound 1 might be more selective and only impact IL-6. Hence the data suggests that the TNFα may be able to induce undegraded MKP-1 and this MKP-1 might be able to reduce cytokine concentration at 24 h.
**Figure 6.1 Sequence similarity between MKP-1 and MKP-2.**

The black represents the overlapping region between MKP-1 and MKP-2. Across the first 180 amino acids the sequence similarity between MKP-1 and MKP-2 is not very high with only a few areas of overlapping amino acids, these early conserved sequences code for the KIM domain as indicated. The highly conserved region is highlighted by the arrow. This area of the sequence is the basis of the model presented in Figure 6.2. The two amino acids of interest in the MKP-1 sequence are ser 296 and ser 32, which are highlighted with the black box. The corresponding amino acids on MKP-2 are ser 318 and ser 345. Ser 345 was not resolved in the due to a lack of sequence similarity.
Figure 6.2 Homology Model of MKP-1.

Colours indicate the residue position, starting from red and finishing at purple. This is an image of the homology model of MKP-1 based on the crystal structure of MKP-2. MKP-1 and MKP-2 share the greatest sequence near the catalytic region. The ribbon structure depicts this catalytic region. Ser 318 is the corresponding amino acid of ser 296 in MKP-2. The DEF motif identified at the top of figure 6.2, is where we would expect to see ser 323. The DSPc region identified represents the active site of MKP-1, where MAPKs bind.
Table 6.1 Structures of the virtual hits attained after extra precision compound docking

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Figure 6.3 Zinc 38867909.

The three images show the nature of the molecule and how it can interact with its target. The binding of this compound to the homology model is shown in detail in (A). The image consists of all the residues within 7 Angstroms of the compound, and we can see that the ser 318 residue (highlighted by the circle) is close to the bonding pocket and that this compound close enough to hydrogen bond to it. (B) is an image of the compound present with all its atoms, Yellow represents sulfur, grey is carbon, red is oxygen and white is hydrogen. (C) represents the 3 dimensional view of the relationship and relative location of the compound to the whole protein.
Figure 6.4 Zinc 71781212.

The three images show the nature of the molecule and how it can interact with its target. The binding of this compound to the homology model is shown in detail in (A). The image consists of all the residues within 7 Angstroms of the compound, and we can see that the ser 318 residue (highlighted by the circle) is close to the bonding pocket and that this compound close enough to hydrogen bond to it. (B) is an image of the compound present with all its atoms, Blue represents nitrogen, grey is carbon, red is oxygen and white is hydrogen. (C) represents the 3 dimensional view of the relationship and relative location of the compound to the whole protein.
Figure 6.5 Zinc 01575552.

The three images show the nature of the molecule and how it can interact with its target. The binding of this compound to the homology model is shown in detail in (A). The image consists of all the residues within 7 Angstroms of the compound, and we can see that the ser 318 residue (highlighted by the circle) is close to the bonding pocket and that this compound close enough to hydrogen bond to it. (B) is an image of the compound present with all its atoms, Blue represents nitrogen, grey is carbon, red is oxygen and white is hydrogen. (C) represents the 3 dimensional view of the relationship and relative location of the compound to the whole protein.
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A

Compound 4
ZINC19911893

Ser 318
The three images show the nature of the molecule and how it can interact with its target. The binding of this compound to the homology model is shown in detail in (A). The image consists of all the residues within 7 Angstroms of the compound, and we can see that the ser 318 residue (highlighted by the circle) is close to the bonding pocket and that this compound close enough to hydrogen bond to it. (B) is an image of the compound present with all its atoms, Blue represents nitrogen, grey is carbon, yellow is sulfur, red is oxygen and white is hydrogen. (C) represents the 3 dimensional view of the relationship and relative location of the compound to the whole protein.
The three images show the nature of the molecule and how it can interact with its target. The binding of this compound to the homology model is shown in detail in (A). The image consists of all the residues within 7 Angstroms of the compound, and we can see that the ser 318 residue (highlighted by the circle) is close to the bonding pocket and that this compound close enough to hydrogen bond to it. (B) is an image of the compound present with all its atoms, Blue represents nitrogen, grey is carbon, red is oxygen and white is hydrogen. (C) represents the 3 dimensional view of the relationship and relative location of the compound to the whole protein.

**Figure 6.7 Zinc 01575556.**
Figure 6.8 Zinc 02570838.

The three images show the nature of the molecule and how it can interact with its target. The binding of this compound to the homology model is shown in detail in (A). The image consists of all the residues within 7 Angstroms of the compound, and we can see that the ser 318 residue (highlighted by the circle) is close to the bonding pocket and that this compound close enough to hydrogen bond to it. (B) is an image of the compound present with all its atoms. Blue represents nitrogen, grey is carbon, red is oxygen and white is hydrogen. (C) represents the 3 dimensional view of the relationship and relative location of the compound to the whole protein.
Figure 6.9 Zinc 38200471.
The three images show the nature of the molecule and how it can interact with its target. The binding of this compound to the homology model is shown in detail in (A). The image consists of all the residues within 7 Angstroms of the compound, and we can see that the ser 318 residue (highlighted by the circle) is close to the bonding pocket and that this compound close enough to hydrogen bond to it. (B) is an image of the compound present with all its atoms, Blue represents nitrogen, grey is carbon, red is oxygen and white is hydrogen. (C) represents the 3 dimensional view of the relationship and relative location of the compound to the whole protein.
Chapter 6
Figure 6.10 Zinc 38200473.

The three images show the nature of the molecule and how it can interact with its target. The binding of this compound to the homology model is shown in detail in (A). The image consists of all the residues within 7 Angstroms of the compound, and we can see that the ser 318 residue (highlighted by the circle) is close to the bonding pocket and that this compound close enough to hydrogen bond to it. (B) is an image of the compound present with all its atoms, Blue represents nitrogen, grey is carbon, red is oxygen and white is hydrogen. (C) represents the 3 dimensional view of the relationship and relative location of the compound to the whole protein.
Figure 6.11 Zinc 01575555.

The three images show the nature of the molecule and how it can interact with its target. The binding of this compound to the homology model is shown in detail in (A). The image consists of all the residues within 7 Angstroms of the compound, and we can see that the ser 318 residue (highlighted by the circle) is close to the bonding pocket and that this compound close enough to hydrogen bond to it. (B) is an image of the compound present with all its atoms. Blue represents nitrogen, grey is carbon, red is oxygen and white is hydrogen. (C) represents the 3 dimensional view of the relationship and relative location of the compound to the whole protein.
Chapter 6

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1 μM ZINC 19911893 + TNFα

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10 μM ZINC 19911893 + TNFα

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100 μM ZINC 19911893 + TNFα

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TNFα

E

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phosphorylated p38

p38

phosphorylated -ERK

ERK

MKP-1

α- tubulin

1 μM ZINC 38867909 + TNFα

F

0 h 0.25 h 0.5 h 1 h 2 h 4 h 8 h 24 h

phosphorylated p38

p38

phosphorylated -ERK

ERK

MKP-1

α- tubulin
Figure 6.12 Treatment with ZINC 19911893 and ZINC 38867909 and then with TNFa stimulation results in sustained MKP-1 protein upregulation: effects on p38 MAPK phosphorylation.

Growth-arrested ASM cells were stimulated with TNFa (10 ng/ml) or vehicle for 0, 0.25, 0.5, 1, 2, 4, 8, and 24 h (time of TNFa stimulation designated as 0 h). ZINC 19911893 (A-D) and ZINC 38867909 (E-H) (100 μM, 10 μM and 1 μM), or vehicle, were added half an h before stimulation with TNFa and the temporal kinetics of MKP-1 upregulation and impact on p38 MAPK phosphorylation compared by Western blotting (with α-tubulin as the loading control). (A, B, C, D, E, F, G, H) Results are representative Western blots when cells were treated with: (A, E) TNFa, (B, F) 100 μM respective compound + TNFa, (C, G) 10 μM compound + TNFa, (D, H) 1 μM compound + TNFa.
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C

\[ \text{p38 MAPK phosphorylation in the presence of 10 \, \mu M ZINC 19911893 (fold increase)} \]

\[ \text{MKP-1 upregulation in the presence of 10 \, \mu M ZINC 19911893 (fold difference)} \]

Time after TNF\(\alpha\) stimulation

D

\[ \text{p38 MAPK phosphorylation in the presence of 100 \, \mu M ZINC 19911893 (fold increase)} \]

\[ \text{MKP-1 upregulation in the presence of 100 \, \mu M ZINC 19911893 (fold difference)} \]

Time after TNF\(\alpha\) stimulation
Growth-arrested ASM cells were stimulated with TNFα (10 ng/ml) or vehicle for 0, 0.25, 0.5, 1, 2, 4, 8, and 24 h (time of TNFα stimulation designated as 0 h). ZINC 19911893 (A-D) and ZINC 38867909 (E-H) (100 μM, 10 μM and 1 μM), or vehicle, were added half an h before stimulation with TNFα. Figure 6.13 demonstrates densitometric analysis of MKP-1 protein upregulation (normalized to α-tubulin and expressed as fold increase compared to 0 h) and p38 MAPK phosphorylation (normalized to total p38 MAPK and expressed as fold increase compared to 0 h) over time.
Figure 6.14 TNFα induced IL-8 is reduced in the presence of lower concentrations of ZINC 19911893 and 38867909.

Growth-arrested ASM cells were stimulated with TNFα (10 ng/ml) or vehicle for 0, 0.25, 0.5, 1, 2, 4, 8, and 24 h (time of TNFα stimulation designated as 0 h). ZINC 19911893 (A-D) and ZINC 38867909 (E-H) (100 μM, 10 μM and 1 μM), or vehicle, were added half an h before stimulation with TNFα. Cell supernatants were collected and analysed using ELISA to determine the concentrations of cytokines (A) IL-6 and (B) IL-8. The results are from one cell line.
6.4 Discussion

MKP-1 is an important anti-inflammatory mediator which regulates the strength and duration of MAPK activation. However, MKP-1 is only active transiently and hence is subject to degradation via the proteasomal machinery. This degradation is stimulated via the binding of ERK MAPK to MKP-1 at the site of ser 296 and ser 323. Once bound it subsequently recruits MKP-1 into the proteasome. Hence we aimed to target the ERK binding pocket close to ser 296 through the use of a small compound. We suggested that in the presence of a small compound bound to the ERK binding pocket that we could prevent ERK from binding to MKP-1 at serine 296 / 323 and potentially protect MKP-1 from the proteasomal machinery. Since proteasome inhibitors had already been investigated we aimed to use computational techniques to uncover some potential small compounds that were able to bind to MKP-1 at the desired location. Before we could try and identify compounds that could bind to MKP-1 we needed to generate a model of MKP-1. We used the crystal structure of MKP-2 as a template to create a homology model of MKP-1 since they share a lot sequence similarity. The model that we generated is constrained to the conserved region of the MKP-1 and MKP-1 gene sequence. MKP-1 and MKP-2 do not share high sequence similarity everywhere and hence extending the model beyond the conserved area would be highly inaccurate.

Virtual screenings have many benefits as we are able to access massive chemical compound libraries and screen them through our docking site to ascertain molecules with high affinity. The chemical compound library we decided to use for our experiment was the ZINC database (Irwin et al., 2005) as all the molecules found on that database are commercially available and provided by the department of pharmaceutical chemistry at the University of California, San Francisco (UCSF). The results of the screening through the glide suite on the Maestro program were numerous; the standard precision screening (quickly resulted in close to 1000 hits). The highest ranked (top 30) (based on affinity binding) were then grouped according to structural similarity, to attain the highest chance of success. There are however limitations to virtual screenings which can include the
existence of alternate protein and compound confirmations not seen in the crystal structure, and hence building a model based on this could lead to false positives. In order to dispel the false positives the compounds with the highest affinity and structural similarity were placed through an extra precision screening, which is a more powerful and discriminating calculation designed to incorporate penalties for unfavourable interactions and hence provide better positional and conformational specification of the compounds. The second screening provided many molecules that had a high affinity score (glide score) and a good conformational fit.

As mentioned above there are four serine locations to which ERK can bind. Two of these sites ser 296 and ser 323 are more relevant to this study as they are responsible for MKP-1 degradation. It is unfortunately not possible to modulate both ser 296 and ser 323 with one small compound as the residues are further apart than the size of one small compound. Hence, for the purpose of this study we focused on one serine; ser 296, which is the equivalent to serine 318 in MKP-2 To then test these compounds further we utilized human ASM cells; a cell type characterized as having an important immunomodulatory role perpetuating airway inflammation through the secretion of various pro-inflammatory cytokines (Lazaar and Panettieri, 2005a). Notably these compounds at certain concentrations are able to increase MKP-1 longevity and we were able to see a small change in the p38 MAPK phosphorylation status in a temporally-distinct manner.

Since the model created is based on MKP-2 we had to first identify the equivalent serine residue pattern present within the MKP-2 genetic sequence. The genetic sequences for MKP-1 and MKP-2 are 22 base pairs apart (Figure 6.1) therefore the desired serine on MKP-1, ser 296, is equivalent to ser 318 on MKP-2. Once we identified potential binding pocket we then aimed to use the model and identify potential lead compounds to investigate further. As a result of the computational modelling
we had identified 9 suitable compounds that could bind to the ser 318 site. The 9 compounds were also chosen because they had the highest affinity and hence the highest probability to work *in vitro*. The 9 compounds are represented in Figures 6.3 to 6.11, it is interesting to note that all the compounds have multiple sites with oxygen atoms suggesting that hydrogen bonding is the likely process through which these compounds bind to the target site. In order to truly ascertain which of these molecules was the best we would have to do *in vitro* testing. Out of the 9 compounds identified we managed to source two of them for further analysis and we then tested them in ASM cells. Compound 1 was tested in three cell lines and compound 4 was tested in one cell line.

*In vitro* analysis of the compounds revealed interesting results, notably that compound 1 was not as effective as compound 4, we can say this as compound 4 appears to be effective at a lower concentration (1 μM) than compound 1 which seems to be effective at a higher concentration (100 μM). From our previous paper (Prabhala et al., 2015a) we had published a temporal profile of the interaction between MKP-1 and phosphorylated p38 MAPK. This gave us some insight in what to ascertain from this experiment. We showed that MKP-1 was directly responsible for the decrease of phosphorylated p38 MAPK and that in the absence of MKP-1 we get a rebound of phosphorylated p38 MAPK which leads to an increase in cytokines at the 8 h and 24 h time points. Under normal stimulation with TNFα we have previously shown that MKP-1 upregulation is transitional and is immediately switched off after a robust upregulation at 1 h. Hence we decided to observe what would happen if we increased MKP-1 beyond its normal regulation. Modulating MKP-1 has been studied before through the use of proteasome inhibitors and through the use of truncated MKP-1 splice variant studies (Lin et al., 2008; Moutzouris et al., 2010). In the presence of the proteasome inhibitor there was a definite increase in the amount of MKP-1, which led to the reduction of various cytokines, especially IL-8. However this did raise the question that what else was being upregulated by inhibiting the proteasome. Hence a better strategy would be to target MKP-1 itself, and hence we decided to model MKP-1 and find a more specific way to manipulate the MKP-1 p38
MAPK axis in our favour. Compound 4 was more effective in addressing the aim of the study which was to keep MKP-1 viable and active well past its normal regulation. As indicated in Figure 6.12B and G we can see that MKP-1 band is present well past the normal 2 h time point all the way up to the 24 h time point. As discussed above MKP-1 has four locations to which ERK can bind, one at ser 296 / 323 and the other at ser 359 / 364. These four serines cause MKP-1 to undergo totally different processes. At ser 296 / 323 we know that ERK binding recruits the proteasome and causes degradation, at ser 359 ERK binding causes MKP-1 phosphorylation and increases it stability. Since we are unable to create a small compound to bind both the close serines at the same time it is difficult to directly state which site is more important for ERK binding. It is also difficult to tell whether the increase in MKP-1 is due to the presence of more ERK available or whether the compound we generated is blocking the proteasome effectively. If the ser 296 ERK binding site is being blocked then there will be more ERK available and it is likely that this excess ERK is available to stabilise MKP-1, thereby causing an increase in MKP-1. By stimulating ASM cells with TNFα we demonstrate the kinetics of MKP-1 and phosphorylated p38 MAPK in the presence of various concentrations of the identified compounds. We used three different concentrations of compound 4 100 μM, 10 μM and 1 μM. Interestingly the results indicate that MKP-1 was increased in the presence of the cells treated with 1 μM compound 4. This prolonged MKP-1 in the presence of the compounds ensures the TNFα induced cytokine expression through phosphorylated p38 MAPK is able to be attenuated. The cells were not as responsive to 10 μM or 100 μM concentrations suggesting that the binding pocket of MKP-1 is susceptible to saturation if the compound being used has a particular chemical sub group that is able to interact with the binding pocket, however we can’t state this with certainty. When the cells were treated with 100 μM compound 4 there was a lesser increase in the amount of MKP-1. Surprisingly there was no real concentration gradient present for either of the compounds. The most intense MKP-1 bands noticed
in the presence of compound 4 were at 1μM (Figure 6.12 B) and highest intensity bands for compound 1 noticed at the 100μM concentration (Figure 6.12 D).

Collectively this study shows that the homology model was an effective method in determining compounds that could be synthesised to target a specific location rather than a non-specific location. In this case targeting a protein to keep it from degrading rather than target the proteasome itself. The study also showed that compounds that were identified to bind to the specific location may bind to the binding pocket differently, and may cause different conformational changes. Our study also suggests that compound 4 and compound 1 are able to increase the longevity and activity of MKP-1 and thereby reduce the impact of p38 MAPK in the process of inflammatory signal transduction. Interestingly this provides an alternative pathway for target specific pharmacological intervention. This study shows that modulating the temporal kinetics of MKP-1 by supressing its degradation, is a way to exert a level of control on the regulatory network that leads to cytokine secretion in asthmatic inflammation.
Chapter 7
General Discussion
7 Chapter 7: General Discussion

7.1 Overview

Chronic inflammatory diseases, such as asthma and chronic obstructive pulmonary disease (COPD), are clinically and socioeconomically important diseases globally. Asthma is a chronic inflammatory condition that affects about 300 million people worldwide and about 2 million people in Australia. These diseases are set to increase in prevalence over the coming years. Currently the mainstay of anti-inflammatory therapy in respiratory diseases is corticosteroids in combination with β2-agonists. This corticosteroid based therapy has proven clinical efficacy and is usually enough to treat the conditions in mild to moderate patients, however these therapies have proven ineffective in patients with severe disease progression. It is these patients that disproportionately account toward the global economic burden of these diseases. Since these diseases are set to increase in prevalence, research into these diseases will have to keep pace, to match the increase in demand, otherwise risk becoming an even bigger challenge to the global disease burden. Despite a fundamental understanding that inflammation drives the disease progression, the underlying cause is still relatively unknown, and the gaps in the knowledge need to be addressed in order to tackle this growing problem.

In an effort to add to the knowledge of this growing problem, our group have focused in on asthma to potentially find an alternative anti-inflammatory strategies to combat this disease as effective anti-inflammatory strategies are currently unknown. Asthma is a disease state that is characterised by reversible airway obstruction, airway hype responsiveness and structural remodelling. There are numerous structural and molecular changes that occur in an asthmatic phenotype, out of which the most noticeable molecular changes include over recruitment of
inflammatory cells that result due to over active inflammatory signalling pathways. These molecular changes coincide with structural changes that can be seen to the basement membrane, which becomes thicker and to smooth muscle cells which proliferate and increase in mass. This increase in airway smooth mass is important as smooth muscle cells have been recently identified as key immunomodulatory cells affecting the pathophysiology of asthma. Some key immunomodulatory functions of ASM cells may be to increase the amount of cytokines present (Panettieri, 2002 #2204; Panettieri, 2003 #2481 during inflammatory asthma and to provide signals to increase pro inflammatory gene transcription. Therefore understanding the molecular mechanisms responsible for cytokine secretion may allow us to develop novel strategies to repress inflammation in the future. p38 MAPK has emerged as an important signalling molecule driving airway inflammation.

7.2 Approach

We focused our research on the effects of asthma via ASM cells. The primary ASM cell lines were established by (Johnson et al., 1995b) from human bronchi obtained in accordance with procedures approved by the Sydney South West Area Health Service and the Human Research Ethics Committee of the University of Sydney from patients undergoing surgical resection for carcinoma or lung transplant donors.

We propose that an alternative anti-inflammatory strategy to target airway inflammation may be to target the p38 MAPK inflammatory signalling cascade in order to find a potential anti-inflammatory molecule that can supress the action of p38 MAPK and hence supress downstream signalling like MKP-1. MKP-1 is a dual specificity phosphatase that is able to dephosphorylate and hence supress p38 MAPK signalling in a temporally specific manner. However, MKP-1 is only active for a short time and is rapidly degraded thereby allowing p38 MAPK to propagate
inflammatory responses without regulation. Along with being able to suppress inflammatory signals by deactivating p38 MAPK, MKP-1 is also able to activate another anti-inflammatory molecule downstream of p38 MAPK, tristetraprolin. Tristetraprolin is an mRNA destabilizing, RNA-binding protein that enhances the decay of mRNAs, including those encoding proteins implicated in chronic respiratory diseases. We suggest that understanding the molecular mechanism of TTP expression and its temporal regulation will guide future development of novel anti-inflammatory pharmacotherapeutic approaches to combat respiratory disease.

Hence the aim of this study is to gain an understanding over the precise temporal kinetics of MKP-1 and its far reaching effects on the p38 MAPK signalling pathway. This aim was broken down and addressed into 4 separate projects.

7.3 Establish suitable experimental conditions in which to test MKP-1 protein

- MKP-1 antibody is essential to the study and produces the best results when diluted a blocking buffer containing 5% skim milk
- MKP-1 antibody produces the best results at a concentration ratio of 1 part antibody to 100 parts blocking buffer
- MKP-1 antibody produces the best results when a secondary anti body concentration of 1 part to 10000 blocking buffer is used.

In this study we strived to establish consistent and reproducible results. This is essential to any study and hence we went about the process of validating MKP-1 antibodies so that we could use them to measure the interaction of MKP-1 to other inflammatory and anti-inflammatory molecules. The challenge to overcome was to detect an MKP-1 band for gel-electrophoresis. We received our antibodies from one manufacturer, however these antibodies were polyclonal in
nature and were raised in animals hence there was expected batch to batch variability. This variability was overcome by increasing the sensitivity of the antibody by increasing its concentration. This enabled us to optimize the conditions for antibody use and we could then use this antibody with confidence and proceed with our experiments testing the impact of MKP-1.

7.4 **Identify the temporal profile of MKP-1 and its effect on cytokine mRNA via p38 MAPK and tristetraprolin**

Before we could identify a possible way to harness the power of the endogenous anti-inflammatory molecules that can control inflammation like MKP-1 and TTP we first had to examine their potential inter connectivity and the specific signalling pathway through which they act. Once we could confirm that, we could then ascertain the precise temporal nature of that relationship and use this information to progress this study to potentially find an alternative anti-inflammatory strategy.

- TNFα induced IL-6 secretion is due to biphasic IL-6 mRNA expression
- TTP activity is temporally regulated by MKP-1 via control of p38 MAPK phosphorylation
- TNFα induced IL-6 mRNA expression is biphasic due to the temporal regulation of TTP activity by p38 MAPK
- Absence of TTP due to TTP siRNA increases IL-6 protein secretion

Previous works had shown the importance of TTP as an anti-inflammatory molecule (Taylor, 1996 #27; Lai, 1999 #30; Lai, 2000 #28), and its role in p38 MAPK signalling (Mahtani et al., 2001b; Zhu et al., 2001). In this study however, we investigate the precise p38 MAPK-mediated regulatory interaction of two anti-inflammatory proteins, mitogen-activated protein kinase phosphatase 1 (MKP-1) and tristetraprolin (TTP), in the context of asthmatic inflammation. In
In this context the knowledge on the role of TTP and MKP-1 has been underexplored with little information available about the temporal regulation of these key anti-inflammatory modulators. The eventual goal of this study was to provide information about the precise temporal regulation of this signalling pathway and enhance our knowledge to assist in the creation of an anti-inflammatory therapy that can be used to treat asthmatic patients. Similar to other studies we used IL-6 as our model of inflammation to elucidate the intricacies of this signalling pathway (Quante et al., 2008). We were able to show that TTP is p38 MAPK dependent in ASM cells and that the phosphorylation state of p38 MAPK is able to determine the phosphorylation status of TTP. King et al. showed that this change in TTP phosphorylation status is responsible for its anti-inflammatory activity, hence we showed that MKP-1 is able to control the phosphorylation status of TTP by controlling the phosphorylation status of p38 MAPK. Since MKP-1 is only active transiently we emphasised its impact on p38 MAPK phosphorylation and TTP phosphorylation status when it is active early in its temporal profile. Then we also showed the p38 MAPK dependence of TTP, later on in its temporal profile through the use of a p38 MAPK inhibitor. Hence, we were able to show a potential regulatory network that exists between MKP-1, p38 MAPK and TTP. Our eventual goal is to create a potential anti-inflammatory therapy, and hence this knowledge about the peaks and troughs of mediator signalling allows us to tailor a potential anti-inflammatory therapy to be most efficient.

7.5 **Examine the role of dexamethasone in cytokine suppression through its ability to induce MKP-1 and thereby alter TTP activity without altering its mRNA expression or its protein secretion.**

From the previous study we determined the precise control exerted on p38 MAPK and TTP phosphorylation by MKP-1. However, we noted above that the use of a p38 MAPK inhibitor is
detrimental to normal physiology and hence targeting this molecule has not proven to be effective. Hence one alternative strategy is to use a drug to alter the temporal profile of MKP-1 and thereby alter the profile of p38 MAPK and TTP phosphorylation. Since we established the role of TTP activity, and how it is able to be controlled via the MKP-1 and p38 MAPK, we aimed to determine the outcome of manipulating this system with a drug known to act in part through the activation of MKP-1. Dexamethasone was used as this drug as it serves a multifaceted role; firstly it served as a conduit of asthma treatment and secondly it had been previously shown to attenuate cytokine expression partly through the induction of the MKP-1 gene (Clark, 2003a). Using the information from the previous chapter, in this study we took a more therapeutic approach to understand the precise temporal relationship between p38 MAPK, MKP-1 and TTP by changing the treatment protocol to stimulate the cells after the cytokines had been induced via TNFα.

- TNFα induced IL-6 secretion is reduced in the presence of dexamethasone added 1 h after TNFα.
- MKP-1 mRNA and protein are upregulated in the presence of dexamethasone treatment after TNFα stimulation.
- Dexamethasone treatment after TNFα stimulation reduced TTP mRNA at 2 h
- Treatment with dexamethasone after TNFα stimulation increased percentage of unphosphorylated TTP (active form).

Our previous results focused on elucidating the precise temporal regulation of key pro and anti-inflammatory mediators involved in asthmatic inflammation in ASM cells. In this study we altered this temporal regulation through the use of a corticosteroid to potentially increase the activity of MKP-1 and to thereby repress the activity of p38 MAPK. From the literature we know that corticosteroids are the mainstay in asthma medication, and are able to act in part through MKP-1. Most of the literature however focuses on dexamethasone treatment in a
preventative fashion (Broide et al., 1992b), treatment added before the cytokines were induced. This protocol was then confirmed in studies examining the pathways that potentially imbue corticosteroids with their anti-inflammatory properties ((Quante et al., 2008; Manetsch et al., 2012d; Manetsch et al., 2013). In the presence of this new protocol, cytokine levels were reduced, the level of MKP-1 was elevated and the duration of active TTP was shown to be extended, therefore confirming that dexamethasone was able to successfully alter the temporal profile of one key regulator, MKP-1, and impact the other key mediators, p38 MAPK and TTP, in a favourable way. Hence confirming the previously established work and finding a way to alter the temporal profile of anti-inflammatory mediators. This study represented another step forward in an attempt to find an affective anti-inflammatory therapy. Corticosteroid treatment was a previously established therapy and we showed that its addition in a curative fashion, after cytokine induction, also relied on the MKP-1, p38 MAPK and TTP regulatory network. We also showed that this network was able to be manipulated and could aid in the development of an anti-inflammatory therapeutic with multifaceted effects on the inflammatory pathway.

7.6 **Identify potential alternative anti-inflammatory therapy through the use of *in silico* modelling of MKP-1 catalytic region.**

Since we established the regulatory network that exists between MKP-1, p38 MAPK and TTP and that this network can be manipulated in order to deliver anti-inflammatory effects we aimed to use method of *in silico* modelling to find compound that could keep MKP-1 viable for longer and thereby keep p38 MAPK dephosphorylated when necessary. This in turn would keep TTP dephosphorylated and active when necessary. Although corticosteroids are able to work in part via MKP-1 they are also subject to regulation via other pathways like the PGE₂ pathway. There is evidence to suggest that this pathway causes corticosteroid insensitivity (Rumzhum et al.,
Hence we aimed to find an alternative anti-inflammatory strategy.

- Created a homology of the MKP-1 catalytic region based on the crystal structure of MKP-2
- Identified compounds that could bind MKP-1 at a specific site to prevent its degradation
- Refined the compounds to match the site as best as possible through extra precision docking

We identified at total 9 compounds after the extra precision docking analysis. Out of the 9 compounds we were only able to source 2, which we tested as a proof of principle to show their ability to control MKP-1 levels and thereby affect p38 MAPK phosphorylation. Due to the nature of the compounds we were able to show that they were effective at different concentrations. These differences in effectiveness could be due to structural differences. Different structures could represent different levels of hydrogen bonding and would account for the difference in concentrations. Hence, we were able to provide insight into potential new anti-inflammatory compounds that could work in concert with the previously established regulatory network to potentially treat inflammation.

### 7.7 Conclusion and future direction

Inflammation is interesting, as it is a natural function of the body to maintain homeostasis. Asthma and other respiratory diseases represent an overactive but natural inflammatory response to expel unwanted particles from the airways. As part of this natural homeostasis there are anti-inflammatory negative feedback loops mediated by anti-inflammatory modulators. These modulators are only active transiently and are effectively switched off leading to unregulated
inflammation. Hence the most efficient option to abate inflammation would be to enhance the molecules already in place to control it. This study aimed to tackle this problem and to enhance the capabilities of these endogenous anti-inflammatory molecules and thereby provide information to aid in the design and identification of novel anti-inflammatory therapies.

This study represents only the first step in the overall goal of finding a new anti-inflammatory therapy. Our current approach to the above studies is mainly focused on normal ASM cells, and the immediate next step would be to test this regulatory network and its efficacy in asthmatic ASM cells. Since asthma is a heterogeneous disease and has different phenotypes it would be beneficial to explore this regulatory network in ASM cells from mild, moderate and severe (characterised based on FEV₁ response) asthmatics as well as from non-eosinophilic asthmatics. This would enable us to differentiate whether this regulatory network is ubiquitous to all phenotypes of asthma or whether it is selectively regulated. This will also enable us to determine whether this pathway remains active under the different phenotypes, since we know that severe asthmatics have some degree of glucocorticoid insensitivity, it could be possible that endogenous anti-inflammatory proteins also lose their effectiveness in a similar manner, in the presence of constant inflammatory assault. This could then be followed up by appropriate whole body models of inflammation which can be replicated in animals. The ovalbumin challenge animal model represents an appropriate vehicle to study asthma, and can be studied in an acute and chronic setup. This would be valuable information in order to progress the therapy to clinical trials. As a first step we built on existing knowledge about the regulation of inflammation via the p38 MAPK pathway to provide more specific knowledge as to its regulation via endogenous anti-inflammatory feedback loops provided by MKP-1 and TTP. The knowledge we ascertained will enable us to get closer to our eventual goal of identifying an alternative anti-inflammatory strategy to the existing therapies. The studies above show that, targeting MKP-1 could represent a middle ground between blocking p38 MAPK and causing unwanted side effects and targeting
the highly specific TTP and potentially keeping it active. MKP-1 is an adequate middle ground as it is able to control p38 MAPK phosphorylation and thereby TTP phosphorylation in a preventative and a curative method. As a proof of principle we have shown that MKP-1 is able to be increased in the presence of small compounds specifically designed to block its degradation via the proteasome. Designing these small compounds would have been aided by the presence of the crystal structure of MKP-1, as a limitation of this study is that a homology modelling is only a best estimate of MKP-1. In our present study we looked at one possible site of phosphorylation at ser 296, and the impact of blocking this one site. However, since there are two sites which are both thought to impact MKP-1 survival, it would be interesting to generate another receptor grid model and find small compounds that are able to bind ser 323 and test these compounds similarly to the compounds tested in chapter 6. This data could then be combined with studies concurrently testing the impact of blocking both ser sites with separate compounds on MKP-1 stability and survival. This data could then be adequately complimented by data from creating point mutations at these sites and see if the results are comparable. This would give us intimate knowledge about the importance of the ser sites and how they are connected, along with knowledge about MKP-1 stability and activity. We would predict that an increase in MKP-1 will affect p38 MAPK phosphorylation status and thereby affect TTP. This appears to be the way toward the future with a recent publication focusing on the temporal regulation and activity of p38 MAPK on individual cells at specific time points. They were able to show that p38 MAPK activation oscillated at the later time points slightly but significantly above basal levels, and that this oscillation was controlled by MKP-1 (Tomida et al., 2012; Tomida et al., 2015). In our study we focused on collecting all the cells at once and hence the trend is slightly normalised and we are unable to see the same level of oscillation. In our model we see that all the oscillations generated a slight increase in the amount of p38 MAPK levels (Figure 4.5B). Using the data from both studies it would be valuable to determine if this oscillation extends to all the members of the regulatory pathway examined in this study.
MKP-1 as an anti-inflammatory molecule presents an interesting opportunity to study the extent to which we could manipulate the p38 MAPK regulatory pathway before we see deleterious effects. MKP-1 was able to be truncated and its subsequent activity checked at some of its key structural sites (Crowell et al., 2014). This would allow us to confirm the role played by all the MAPKs involved in MKP-1 signalling. From the genetic sequence of MKP-1 and from the information above we know that it is susceptible to post translational modifications such as oxidation, acetylation and phosphorylation. Oxidation is particularly interesting as MKP-1 has a cysteine residue present within its catalytic region (needed to bind and deactivate the MAPKs), and since cysteine residues can be oxidised it would allow us to explore the role of oxidative stress on MKP-1. This could be examined as above and the MKP-1 regulatory network could be tested on ASM cells from smokers or from COPD patients since highly oxidative environments are present under both states.

This would also allow us to further study the compounds identified via homology modelling and to differentiate upregulation due to MKP-1 stability and upregulation due to the compound. Hence by studying these compounds further in the context of asthmatic inflammation we can take advantage of endogenous anti-inflammatory feedback regulators and find more efficient anti-inflammatory therapeutics.
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