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Enhancing the Power of Endogenous Protein Phosphatase 2A to Combat Asthmatic Inflammation: Role for MAPKs and Tristetraprolin

A thesis submitted for the degree of
Doctor of Philosophy

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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 co-supervision of Professor Philip Hansbro, School of Biomedical Science and Pharmacy, the University of Newcastle, Australia and Dr. Nicole M. Verrills, School of Biomedical Sciences and Pharmacy, Faculty of Health, 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.

Md. Mostafizur Rahman

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Publications

1. **Md. Mostafizur Rahman**, Pavan Prabhala, Nowshin N. Rumzhum, Brijeshkumar S. Patel, Thomas Wickop, Nicole M. Verrill and Alaina J. Ammit TLR2 ligation induces corticosteroid insensitivity in A549 lung epithelial cells: anti-inflammatory impact of PP2A activators. (Under review in *The International Journal of Biochemistry & Cell Biology*)

2. **Md. Mostafizur Rahman**, Nowshin N. Rumzhum, Jonathan C. Morris, Andrew R. Clark, Nicole M. Verrills and Alaina J. Ammit. Activating Protein Phosphatase 2A (PP2A) Enhances Tristetraprolin (TTP) Anti-Inflammatory Function in A549 Lung Epithelial Cells. *Cell Signal.* 2016 Apr;28(4):325-34.

3. Brijeshkumar S. Patel[*], **Md. Mostafiz Rahman[*]**, Nowshin N. Rumzhum, Brian G. Oliver, Nicole M. Verrills and Alaina J. Ammit Theophylline represses IL-8 secretion from airway smooth muscle cells in a manner independent of PDE inhibition: novel role for theophylline as an activator of PP2A. *Am J Respir Cell Mol Biol*. 2015 Nov 17. [Epub ahead of print]**. (*authors contributed equally)**.

4. Nowshin N. Rumzhum, **Md. Mostafizur Rahman**, Oliver BG, Ammit AJ. Sphingosine 1-phosphate Increases COX-2 Expression and PGE2 Secretion: Effects on β2-adrenergic Receptor Desensitization. *Am J Respir Cell Mol Biol*. First published online 22 Jun 2015 as DOI: 10.1165/rcmb.2014-0443OC.

5. **Md. Mostafizur Rahman**, Nowshin N. Rumzhum, Jonathan C. Morris, Andrew R. Clark, Nicole M. Verrills and Alaina J. Ammit. Basal protein phosphatase 2A activity restrains cytokine expression: role for MAPKs and tristetraprolin. *Sci. Rep*. 2015, 5, 10063; doi: 10.1038/srep10063.

6. Pavan Prabhala, Kristin Bunge, **Md. Mostafizur Rahman**, Qi Ge, Andrew R. Clark and Alaina J. AmmitTemporal regulation of cytokine mRNA expression by tristetraprolin: 5 dynamic control by p38 MAPK and MKP-1. *Am J Physiol Lung Cell Mol Physiol*. 2015 May 1;308(9):L973-80

7. **Md. Mostafizur Rahman**, Hatem Alkhouri, Francesca Tang, Wenchi Che, Qi Ge and Alaina J. Ammit. Sphingosine 1-phosphate induces neutrophil chemoattractant IL-8: repression by steroids. *PLoS One*. 2014 Mar 19;9(3):e92466.

8. Hatem Alkhouri, Nowshin N. Rumzhum, **Md. Mostafizur Rahman**, Meghan FitzPatrick, Monique de Pedro, Brian G. Oliver, Jane E. Bourke and Alaina J. Ammit**.** TLR2 activation causes tachyphylaxis to β2-agonists *in vitro* and *ex vivo*: modeling bacterial exacerbation. *Allergy*. 2014; 69: 1215–1222.

9. Jeremy A Hirota*, Hanna Im*, **Md Mostafizur Rahman**, Nowshin N Rumzhum, Melanie Manetsch, Chris Pascoe, Kristin Bunge, Hatem Alkhouri, Brian G Oliver and Alaina J Ammit (2013) The nucleotide-binding domain and leucine-rich repeat protein-3

inflammasome is not activated in airway smooth muscle upon toll-like receptor-2 ligation. *Am J Resp Cell Mol Biol.* 2013 Oct; 49(4):517-24. **(*joint first authors).**

10. Melanie Manetsch, **Md. Mostafizur Rahman**, Brijeshkumar S. Patel, Emma E. Ramsay, Nowshin N. Rumzhum, Hatem Alkhouri, Qi Ge and Alaina J. Ammit: Long-Acting b2-Agonists Increase Fluticasone Propionate-Induced Mitogen-Activated Protein Kinase Phosphatase 1 (MKP-1) in Airway Smooth Muscle Cells. *PLoS ONE*. 8(3) 2013: e59635. doi: 10.1371/ journal.pone. 0059635.

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Poster Presentations

1. **Brijeshkumar S. Patel***, Md. Mostafizur Rahman*, Nowshin N. Rumzhum, Brian G. Oliver, Nicole M. Verrills and Alaina J. Ammit Theophylline Represses IL-8 Secretion from ASM Cells Independently of PDE Inhibition: Novel Role as a PP2A Activator. American Thoracic Society (ATS) International Conference, May 13-May 18, 2016, San Francisco, California USA. **(*authors contributed equally)**.

2. **Md. Mostafizur Rahman**, Nowshin N. Rumzhum, Andrew R. Clark, Nicole M. Verrills and Alaina J. Ammit. Basal protein phosphatase 2A activity restrains cytokine expression in A549 lung epithelial cells: role for MAPKs and tristetraprolin. American Thoracic Society (ATS) International Conference, May 14-May 20, 2015, Denver, Colorado. USA.

3. **Nowshin N. Rumzhum,** Md. Mostafizur Rahman**,** Brian G. Oliver and Alaina J. Ammit. Sphingosine 1-phosphate Increases Cyclo-oxygenase 2 in Airway Smooth Muscle Cells and May Desensitize the β2-adrenergic Receptor in a Prostaglandin E2-dependent Manner. American Thoracic Society (ATS) International Conference, May 14-May 20, 2015, Denver, Colorado. USA.

4. **Pavan Prabhala**, Kristin Bunge, Md. Mostafizur Rahman, Qi Ge, Andrew R. Clark and Alaina J. p38 Mitogen Activated Protein Kinase (MAPK) and Mitogen Activated Protein Kinase Phosphatase 1 (MKP-1) Mediated Regulation of Tristetraprolin (TTP) and its Effects on the Temporal Regulation of Cytokine mRNA. American Thoracic Society (ATS) International Conference, May 14-May 20, 2015, Denver, Colorado. USA.

5. **Nowshin N. Rumzhum,** Hatem Alkhouri, Md. Mostafizur Rahman, Meaghan FitzPatrick, Monique de Pedro, Brian G. Oliver, Jane E. Bourke and Alaina J. Ammit. TLR2 activation causes tachyphylaxis to β_2-agonists *in vitro* and *ex vivo:* modeling bacterial exacerbation. Abstract submitted for 22nd Australian Society for Medical Research (ASMR) NSW Annual Scientific Meeting 2014. Sydney, Australia.

6. **Md. Mostafizur Rahman**, Nowshin N. Rumzhum**,** Nicole M. Verrills and Alaina J. Ammit: Protein Phosphatase 2A (PP2A) May Control Tristetrapolin (TTP) Anti-Inflammatory Function in Respiratory Epithelial Cells**.** Abstract submitted for Australian Society for Medical Research (ASMR) NSW Annual Scientific Meeting 3 June, 2013. Australian Technology Park, Redfern. Australia.

7. **Md. Mostafizur Rahman**, Hanna Im, Nowshin N. Rumzhum and Alaina J. Ammit: Possible Role of TLR2-Induced Activation of Inflammasome in Airway Smooth Muscle Cytokine Secretion. NewCastle Asthma Meeting-2012 (NAMe8 2012), 11~12 October 2012. Hunter Medical Research Institute, Australia. (Prize money has been awarded for this poster)

8. Hanna Im, Jeremy Hirota, Md. Mostafizur Rahman, Nowshin Nowaz Rumzhum, Melanie Manetsch, Chris Pascoe, Brian Oliver and **Alaina J. Ammit:** The NLRP3 Inflammasome is not Activated in Airway Smooth Muscle upon TLR2 Ligation. European Respiratory Society Annual Congress 7~11 September, 2013. Barcelona, Spain.

9. Melanie Manetsch, Md. Mostafizur Rahman, Brijeshkumar Patel, Emma Ramsay, Nowshin Nowaz Rumzhum, Hatem Alkhouri, Qi Ge and **Alaina J. Ammit:** Long-acting β2-agonists Increase Fluticasone Propionate-induced Mitogen-activated Protein Kinase Phosphatase 1 (MKP-1) in Airway Smooth Muscle Cells**.** European Respiratory Society Annual Congress 7~11 September, 2013. Barcelona, Spain.

Oral Presentations

1. Md. Mostafizur Rahman, Nowshin N. Rumzhum, Nicole M. Verrills and **Alaina J. Ammit**. Boosting the power of endogenous phosphatases to reduce inflammation in asthma. ComBio 2014, 28 September-2 October 2014. National Convention Centre, Canberra, ACT, Australia. (This work was presented by Ammit AJ as an invited speaker).

2. **Md. Mostafizur Rahman**, Nowshin N. Rumzhum, Nicole M. Verrills and Alaina J. Ammit. Basal protein phosphatase 2a and its anti-inflammatory function in respiratory epithelial cells. American Association for Pharmaceutical Scientists (AAPS) symposium on 29th August 2014. Faculty of Pharmacy. University of Sydney. Australia.

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4. **Md. Mostafizur Rahman**, Nowshin N. Rumzhum, Nicole M. Verrills and Alaina J. Ammit: Protein phosphatase 2A (PP2A) may control tristetrapolin (TTP) anti-inflammatory function in respiratory epithelial cells. Faculty Postgraduate Conference-2013, 4 December 2013, Faculty of Pharmacy, Sydney University.

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May Allah bless and help us all to render something for the cause of the mankind

Table of Contents

List of Figures

List of Tables

Abbreviations

AAL(S)	2-amino-4-(4-heptyloxyphenyl)-2-methylbutanol
AC	adenylyl cyclase
AHR	airway hyper-responsiveness
AMP	adenosine monophosphate
ANOVA	analysis of variance
AP-1	activator protein-1
API	asthma predictive indices
APS	ammonium persulfate
ARE	adenylate + uridylate-rich elements
ASC	apoptosis-associated speck-like protein containing a CARD
ASM	airway smooth muscle
BAL	bronchoalveolar lavage
BALF	bronchoalveolar lavage fluid
BLP	bacterial pathogen-producing lipoproteins
BSA	bovine serum albumin
°C	degrees celsius
cAMP	cyclic adenosine monophosphate
CARD	c-terminal caspase-recruitment domain
CD4	cluster of differentiation4
cDNA	complementary deoxyribonucleic acid
C/EBP	CCAAT/enhancer binding protein

COPD	chronic obstructive pulmonary disease
COX	cyclooxygenase
CRE	cAMP response elements
CREB	cAMP response-element binding protein
CXCL	chemokine (C-X-C motif) ligand
DAMP	damage associated molecular patterns
DMEM	dulbecco's modified eagle's medium
DNA	deoxyribonucleic acid
DP	D prostanoid
DTT	dithiothreitol
DUSPs	dual-specificity phosphatases
ECM	extracellular matrix
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinases
FDA	food and drug administration
FeNO	exhalted nitric oxide levels
FEV1	forced expiratory volume in 1 second
FTY720	2-amino-2-[2-(4-octylphenyl)]-1,3-propanediol hydrochloride
GDP	guanosine-5'-diphosphate
GINA	global initiative for asthma
GM-CSF	granulocyte macrophage colony-stimulating factor
GPCR	G protein-coupled receptors

GR	glucocorticoid receptor
GROα	growth regulated oncogene-alpha
GTP	guanosine-5'-triphosphate
h	hour
HBEC	human bronchial epithelial cell
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
H_20_2	hydrogen peroxide
HRP	horse radish peroxydase
HsP	heat shock protein
HTSMC	human tracheal smooth muscle cell
HuR	human Antigen-R
IBD	inflammatory bowel disease
ICS	inhaled-corticosteroids
IFN	interferon
IgG	immunoglobulin G
IgE	immunoglobulin E
IKKi	IκB kinase I
IL	interleukin
JNK	c-Jun N-terminal kinase and
kDa	kilodalton
KIM	kinase interaction motif
LABA	long acting β_2-agonist
LP	lipopeptide

LPS	lipopolysaccharide
LRR	leucine-rich repeat domain
M	molar
MAPK	mitogen-activated protein kinases
MDC	macrophage-derived chemokine
min	minute
MIP	macrophage inflammatory protein
miRNA	microRNA
MK2	MAPK-activated protein kinase 2
MKP-1	mitogen-activated protein kinase phosphatase 1
mL	millilitre
mm	millimetre
mM	millimolar
MMP2	matrix metalloproteinases-2
mRNA	messenger ribonucleic acid
MS	multiple sclerosis
MSK1	mitogen and stress-activated kinase1
NF-κB	nuclear factor kappa B
NF-IL6	nuclear factor for interleukin-6 expression
ng	nanogram
NHTBE	normal human tracheobronchial epithelial cells
NLRP	NACHT, LRR and PYD domains-containing protein
nm	nanometre

nM	nanomolar
NO	nitric oxide
NSAID	non-steroidal anti-inflammatory drug
OA	okadaic acid
O/N	over night
Pam3CSK4	N-Palmitoyl-S-[2,3-bis-(palmitoyloxy)-(2R,S)-propyl]-(R)-cysteinyl-seryl-(lysyl)-3-lysine
PAMPs	pathogen-associated molecular patterns
PBS	phosphate-buffered saline
PDE4	phosphodiesterase inhibitor 4
PG	prostaglandin
PKA	protein kinase A
PP	protein phosphatase
PP2A	protein phosphatase 2A
PPRE	peroxisome proliferator response element
PROP	purification of reversibly oxidized proteins
RANTES	regulated on activation, normal T cell expressed and secreted
RNA	ribonucleic acid
ROS	reactive oxygen species
RSV	respiratory syncytial virus
RV	rhinovirus
rpm	rotation per minute
RT	room temperature

S1P	sphingosine-1-phosphate
SABA	short-acting β_2-agonists
SDS	sodium dodecyl sulphate
Ser	serine
sec	second
SEM	standard error of the mean
siRNA	small interfering RNA
SRE	sterol response element
sTNF	soluble tumour necrosis factor
TACE	TNF alpha converting enzyme
TARC	thymus and activation-regulated chemokines
TBS (-T)	tris-buffered saline solution (with Tween 20)
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF	transforming growth factor
Th	T-helper17
Thr	threonine
TIA-1	T-cell intracellular antigen 1
TIR	toll/IL-1 receptor
TLR	toll-like receptor
TNFα	tumour necrosis factor α
TNF-R1	TNF receptor 1
TNF-R2	TNF receptor 2
TRAF	tumour-necrosis factor receptor-associated factor

TRIS	tris-(hydroxymethyl)-aminomethane
TTP	tristetraprolin
TX	thromboxane
Tyr	tyrosine
USP	ubiquitin-specific processing protease
3'-UTR	3'-untranslated region
UV	ultraviolet
V	volt
VEGF	vascular endothelial growth factor
WHO	world health organization
ZFP36	zinc finger protein 36
µg	microgram
µL	microliter
µM	micromolar

Abstract

Asthma is a chronic airway disease driven by inflammation, and thus corticosteroids are the mainstay of anti-inflammatory therapy to combat the disease. Typically asthma management involves long term use of corticosteroids, which often leads to severe side effects. Besides, though corticosteroids have a proven clinical efficacy in asthma, many asthmatic inflammatory conditions (e.g. infection, exacerbation) are not responsive to them. Therefore, alternative anti-inflammatory strategies are urgently needed and enhancing the function of endogenous phosphatases, especially protein phosphatase 2A (PP2A), offer great promise. PP2A is a master controller of multiple inflammatory signalling pathways driven by mitogen activated protein kinases (MAPKs) and is a target in asthma. Therefore in our present studies we aim to explore the role of PP2A in the context of asthmatic inflammation.

Inflammation is the key player in asthma pathogenesis, exacerbation and, most importantly, airway remodelling. Multiple inflammatory mediators are involved in this phenomenon and Sphingosine-1-phosphate (S1P) is one of them. S1P is found in increased amounts in asthmatics' airways and can regulate airway smooth muscle functions associated with asthmatic inflammation and remodelling, including cytokine secretion. To date, however, whether S1P induces secretion of an important chemokine responsible for neutrophilia in airway inflammation – interleukin 8 (IL-8) – was unexplored. In chapter 3, we therefore aim to investigate whether S1P induces IL-8 gene expression and secretion to enhance neutrophil chemotaxis *in vitro*, as well as examine the molecular mechanisms responsible for repression by the corticosteroid dexamethasone. Our studies show that S1P upregulates IL-8 secretion and enhances neutrophil chemotaxis *in vitro.* The corticosteroid dexamethasone significantly represses IL-8 mRNA

expression and protein secretion in a concentration and time-dependent manner. Additionally, we reveal that S1P-induced IL-8 secretion is p38 MAPK- and extracellular signal-regulated kinase (ERK)-dependent and that these key phosphoproteins act on the downstream effector mitogen- and stress-activated kinase 1 (MSK1) to control secretion of the neutrophil chemoattractant cytokine IL-8. The functional relevance of this *in vitro* data is demonstrated by neutrophil chemotaxis assays where S1P-induced effects can be significantly attenuated by pre-treatment with dexamethasone, pharmacological inhibition of p38 MAPK- or ERK-mediated pathways, or by knocking down MSK-1 with small interfering RNA (siRNA).

As such PP2A controls multiple signalling pathways driven by MAPKs and dephosphorylate a critical anti-inflammatory protein; tristetraprolin (TTP). Hence in chapter 4, we aim to explore the role of basal protein PP2A in retaining MAPKs and the involvement of TTP. Both in A549 and BEAS-2B lung epithelial cells, we show that inhibition of basal PP2A activity by okadaic acid (OA) releases restraint on MAPKs and thereby increase MAPK-mediated pro-asthmatic cytokines, including interleukin 6 (IL-6) and IL-8. Notably, PP2A inhibition also impacts on the anti-inflammatory protein – TTP, a destabilizing RNA binding protein regulated at multiple levels by p38 MAPK. Although PP2A inhibition increases TTP mRNA expression, resultant TTP protein builds up in the hyperphosphorylated inactive form. Thus, when PP2A activity is repressed, pro-inflammatory cytokines increase and anti-inflammatory proteins are rendered inactive. Importantly, these effects can be reversed by the PP2A activators 2-amino-2-[2-(4-octylphenyl)]-1,3-propanediol hydrochloride (FTY720) and 2-amino-4-(4-heptyloxyphenyl)-2-methylbutanol (AAL(S)), or more specifically by overexpression of the PP2A catalytic subunit (PP2A-C). Moreover, PP2A plays an important role in cytokine expression in cells stimulated with tumour necrosis factor α (TNFα); as inhibition of PP2A with OA or PP2A-C siRNA results

in significant increases in cytokine production. These data reveal the molecular mechanisms of PP2A regulation and highlight the potential of boosting the power of endogenous phosphatases as novel anti-inflammatory strategies to combat asthmatic inflammation.

It is well studied that TTP is regulated by phosphorylation and that PP2A is responsible for dephosphorylating (and hence activating) TTP, amongst other targets. However, their relationship and underlying molecular mechanisms are not yet clear. Therefore in chapter 5, we aim to explore the dynamic relationship between PP2A and TTP. In this study we demonstrate that PP2A activators such as FTY720 and AAL(S) significantly increase TNFα-induced PP2A activity and inhibit mRNA expression and protein secretion of IL-8 and IL-6; two key pro-inflammatory cytokines implicated in respiratory disease and TTP targets. The effect of PP2A activators is not via an increase in TNFα-induced TTP mRNA expression. Instead we propose here that PP2A activators affect the dynamic equilibrium of TTP; shifting the equilibrium from phosphorylated (inactive) towards unphosphorylated (active) TTP. We reveal a link between PP2A activation and TTP anti-inflammatory function by showing that specific knockdown of TTP with siRNA reversed repression of TNFα-induced IL-8 and IL-6 mRNA expression and protein secretion by the PP2A activator FTY720.

Corticosteroids have proven efficacy in chronic respiratory diseases, though these medicines can lose their efficacy during respiratory infection resulting in disease exacerbation. In our study it appears that PP2A has great promise as an alternative anti-inflammatory strategy in asthmatic inflammation. We therefore aim to check whether PP2A is capable of acting as an alternative anti-inflammatory molecule during corticosteroid insensitive condition. In line with our aim, in Chapter 6, we explore the role of PP2A activators in corticosteroid insensitivity using respiratory epithelial cells. To demonstrate the impact of PP2A activators in corticosteroid insensitive

conditions, we pre-treated cells with the synthetic bacterial lipoprotein N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2R,S)-propyl]-(R)-cysteinyl-seryl-(lysyl)3-lysine (Pam3CSK4 - a TLR2 ligand) to mimic bacterial (both +ve and –ve) infection and TNFα to simulate inflammation. Under these conditions, Pam3CSK4 induces corticosteroid insensitivity; demonstrated by substantially reduced ability of the corticosteroid dexamethasone to repress TNFα-induced IL-6 secretion. We then explored the molecular mechanism responsible and found that corticosteroid insensitivity induced by bacterial mimics was not due to altered translocation of the glucocorticoid receptor alpha (GRα) into the nucleus. Moreover, Pam3CSK4 did not affect corticosteroid-induced upregulation of anti-inflammatory MAPK deactivating phosphatase - MKP-1. However, Pam3CSK4 can induce oxidative stress and we show that a proportion of the MKP-1 produced in response to corticosteroid in the context of TLR2 ligation was rendered inactive by oxidation. Thus to combat inflammation in the context of bacterial exacerbation we sought to discover effective strategies that bypassed this road-block. We show for the first time that a known (FTY720) and novel (theophylline) activators of the phosphatase PP2A can serve as non-steroidal anti-inflammatory alternatives and/or corticosteroid-sparing approaches in respiratory inflammation where corticosteroid insensitivity exists.

To get further confirmation for our above studies (mostly performed in immortalized cancer cell line, A549), in Chapter 7 we aimed to perform some key experiments using another immortalized normal cell (BEAS-2B) and primary airway smooth muscle (ASM) cell. However due to individual cell type variability, we fail to demonstrate a clear picture. Nonetheless, these experiments will certainly improve our knowledge and will lead to future investigations.

In conclusion, studies performed in this thesis provide a greater understanding of the role of some widely used inflammatory mediators such as TNFα, S1P and Pam3CSK in asthmatic

inflammation and most importantly the anti-inflammatory impact of one important endogenous protein phosphatase, PP2A. Our studies partly revealed the underlying molecular mechanisms of endogenous PP2A and its relationship with the critical anti-inflammatory protein, TTP in the context of asthmatic inflammation in respiratory epithelial cells. Besides, these studies clearly demonstrate that PP2A activators can enhance the anti-inflammatory function of TTP and work even in corticosteroid insensitive conditions. Hence we presume that PP2A activators may have implications as alternative anti-inflammatory strategies to combat inflammation in respiratory disease.

Chapter 1
General Introduction

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1.1 Asthma

Asthma is a common chronic disease of the airway characterized by variable and recurring symptoms, reversible airflow obstruction, bronchial hyperresponsiveness, and an underlying inflammation. The prevalence of asthma has risen drastically in the last two decades, with a worldwide impact on health care systems. Asthma is the 14th most important disorder in the world in terms of the extent and duration of disability. According to the World Health Organization (WHO) asthma is one of the major public health concerns as it occurs in people of all ages and is an increasingly prevalent chronic disease in all parts of the world. It is a cause of substantial burden and often associated with a reduced quality of life. The burden of asthma is greatest for children aged 10-14 and the elderly aged 75-79. The number of people suffering from asthma in the world is as high as 334 million worldwide (Asher and Pearce, 2014), with 250,000 annual deaths attributed to this disease (Masoli et al., 2004). Hence its social and economic impacts are very significant. Historically, most people affected by asthma are in low and middle-income countries, and its prevalence is estimated to be increasing fastest in those countries. However, the increase in asthma prevalence in developed countries seen at the end of the last century has raised concern for the considerable burden of this disease on society as well as individuals.

Asthma research has received much attention in the last couple of decades; hence our understanding of the processes regarding this disease is in progress. In addition, many issues concerning the fundamental molecular mechanisms of airway diseases including asthma and

other inflammatory airway diseases are also evolving. However, we have much to do to discover effective therapeutic approaches to control the process causing this disease. Therefore for better control and management, asthma research still warrants rigorous investigation.

1.1.1 Characteristics of asthma

Asthma is a chronic inflammatory disease that affects people of all ages. Overactive inflammatory signalling pathways are often associated with asthmatic conditions. Hence inflammation plays the most critical role in the pathophysiology of asthma that triggers airway muscles tightening, swelling and production of high amounts of sticky mucous. These changes are ultimately responsible for narrowing the airway causing difficulty in breathing. Moreover, severity of asthma is characterized by airway inflammation, airway hyper-responsiveness (AHR) and airway remodeling.

Asthma symptoms commonly consist of coughing, wheezing, chest tightness and shortness of breath. These symptoms are usually worse at night and in the early morning or in response to exercise or cold air. Most importantly, all asthmatics are not equally responsive to asthma triggers. Some patients may hardly experience asthma symptoms in response to asthma triggers, whereas others may experience distinct and continual symptoms.

1.1.2 Asthma phenotype

Although asthma has been considered as a single disease for many years, recent studies reveal its heterogeneity (Wenzel, 2012). Exploring this heterogeneity has improved our understanding and we now know that asthma comprises of several phenotypes. Hence asthma can be considered as a heterogeneous syndrome made up of a number of disease phenotypes characterized by their

underlying pathophysiology into "asthma endotypes" (Anderson, 2008; Lötvall et al., 2011). Asthma phenotypes (as shown in Table 1.1) allow us to address its complexities; however to date very little has been known about the onset as well as the stability of asthma phenotypes. Therefore exploring asthma phenotypes demands further study to combat it effectively.

Table 1.1 Asthma phenotypes and their clinical features

Asthma Phenotype	Clinical characteristics
Early-onset allergic	Allergic symptoms and other diseases
Late-onset eosinophilic	Sinusitis; less allergic
Exercise-induced	Mild; intermittent with exercise
Obesity-related	Women are primarily affected; very symptomatic; airway hyperresponsiveness less clear
Neutrophilic	Low FEV1; more air trapping
Aspirin-sensitive	Nasal polyposis, aspirin sensitivity
API-positive preschool wheezer*	Family history of asthma

*API, asthma predictive indices

1.2 Pathophysiology of asthma

Asthma pathophysiology is complex and characterised by intermittent airflow obstruction, airway hyperresponsiveness and inflammation (Adcock and Lane, 2003). The most common signs and symptoms of asthma include intermittent dyspnoea, breathlessness, cough (typically in the early morning or at night) and wheezing (especially expiratory wheezing). Asthma syndrome is recognized by recurrent airflow limitation, which is caused by a variety of changes in the airway, including bronchoconstriction, airway hyperresponsiveness, swelling and airway remodelling, as shown in Figure 1.1 and described below-

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

b) **Airway hyperresponsiveness:** Airway hyperresponsiveness is an important feature of asthma which is exaggerated by bronchoconstriction caused by a wide range of stimuli; including allergic and nonallergic stimuli (Skloot et al., 1995). The extent of clinical severity of asthma depends on airway hyperresponsiveness. Several mechanisms influence airway hyperresponsiveness including inflammation, dysfunctional neuro-regulation and structural changes. However, inflammation is considered the major factor in determining the extent of airway hyperresponsiveness in asthma.

c) **Airway swelling:** Due to progressive inflammation and persistent disease conditions other factors (such as edema, inflammation, mucus hypersecretion and mucus plugs formation) further interfere in airflow. These factors cause airway structural changes and remodelling, which are not responsive to usual treatment options.

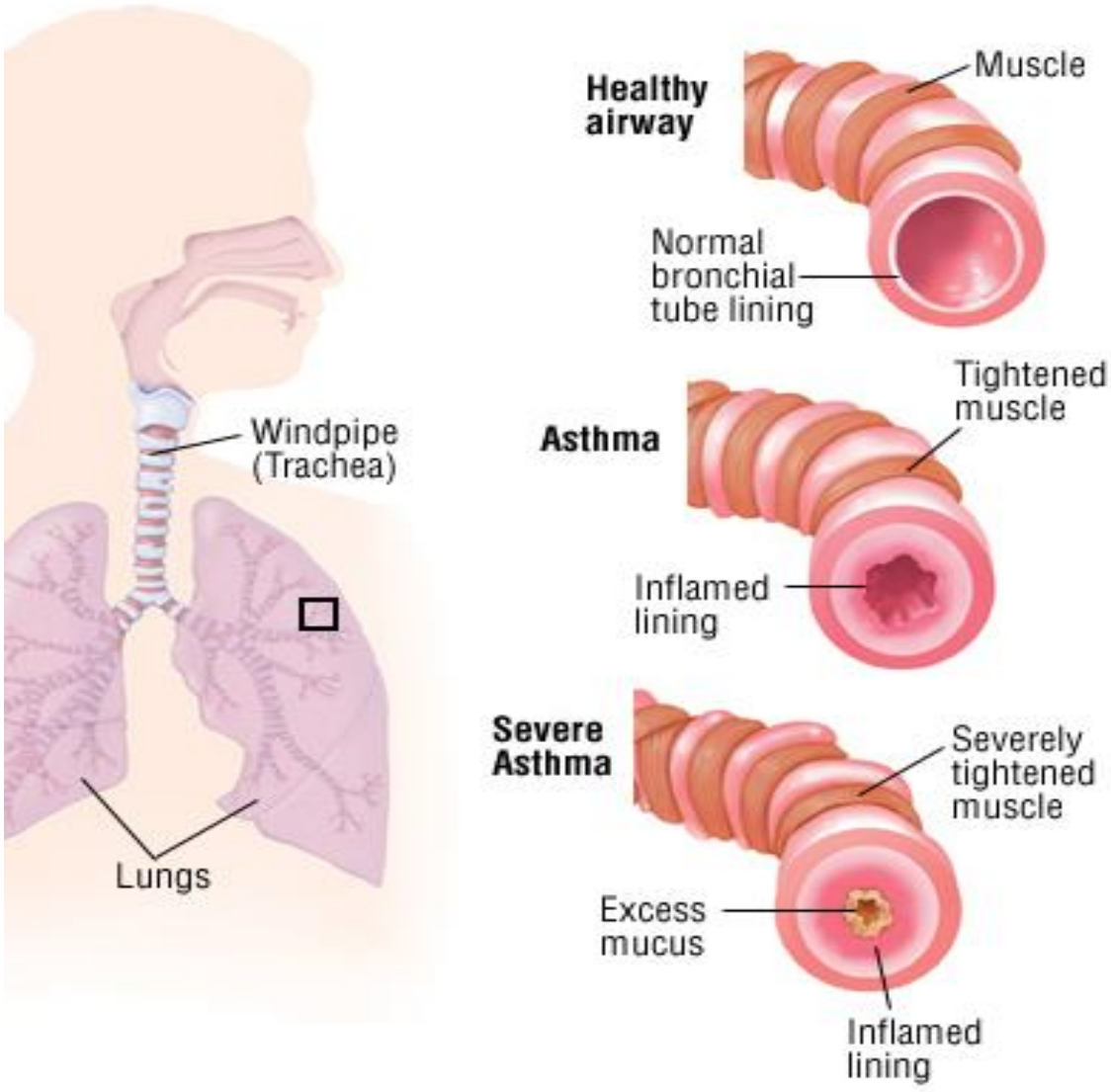


Figure 1.1 Pathophysiology of healthy and asthmatic airway
(http://www.drugs.com/health-guide/asthma.html)

d) **Airway remodelling:** Airway remodelling is characterised by a gradual loss of lung functions and most importantly this codition cannot be prevented by or be fully reversed using existing asthma therapies. In addition, airway remodelling causes activation of a wide range of structural cells (Elias et al., 1999) with subsequent permanent changes in the airway, which ultimately leads to airflow limitation and airway sensitivity and, most

importantly, patients become less responsive to therapy. Asthma severity depends on the structural changes of the airway, which includes airway wall thickening, subepithelial fibrosis, increases in myocite muscle mass, airway smooth muscle hypertrophy and hyperplasia, blood vessel proliferation and dilation, mucus hypersecretion, epithelial hypertrophy and mucus metaplasia (Elias et al., 1999).

1.3 Causative factors of asthma pathogenesis

Asthma pathogenesis is a complex process and a wide range of factors may lead to its development. These include host factors such as innate immunity, genetics, age and sex, and environmental factors (including allergens exposure), respiratory infections, pollutions, smoke (especially tobacco smoke) and stress (Moffatt et al., 2007; Postma et al., 2011; Olin and Wechsler, 2014). Key factors associated with asthma pathogenesis (as shown in Figure 1.2) are as follows:

Genetics: Although the link between inheritable components and asthma expression is well-studied, genetic involvement in asthma development is as yet unclear (Holgate, 1999; Ober, 2005). Studies revealed that a number of genes are responsible (either directly involved or linked) for asthma pathogenesis; however the complexity of their involvement in asthma pathogenesis demands more research. Hence, recently much attention has focused on the identification of the role of genetics in IgE production, airway hyperresponsiveness, regulation of inflammatory mediators (cytokines, chemokines, and growth factors) and how genetic variations regulate the response to therapy.

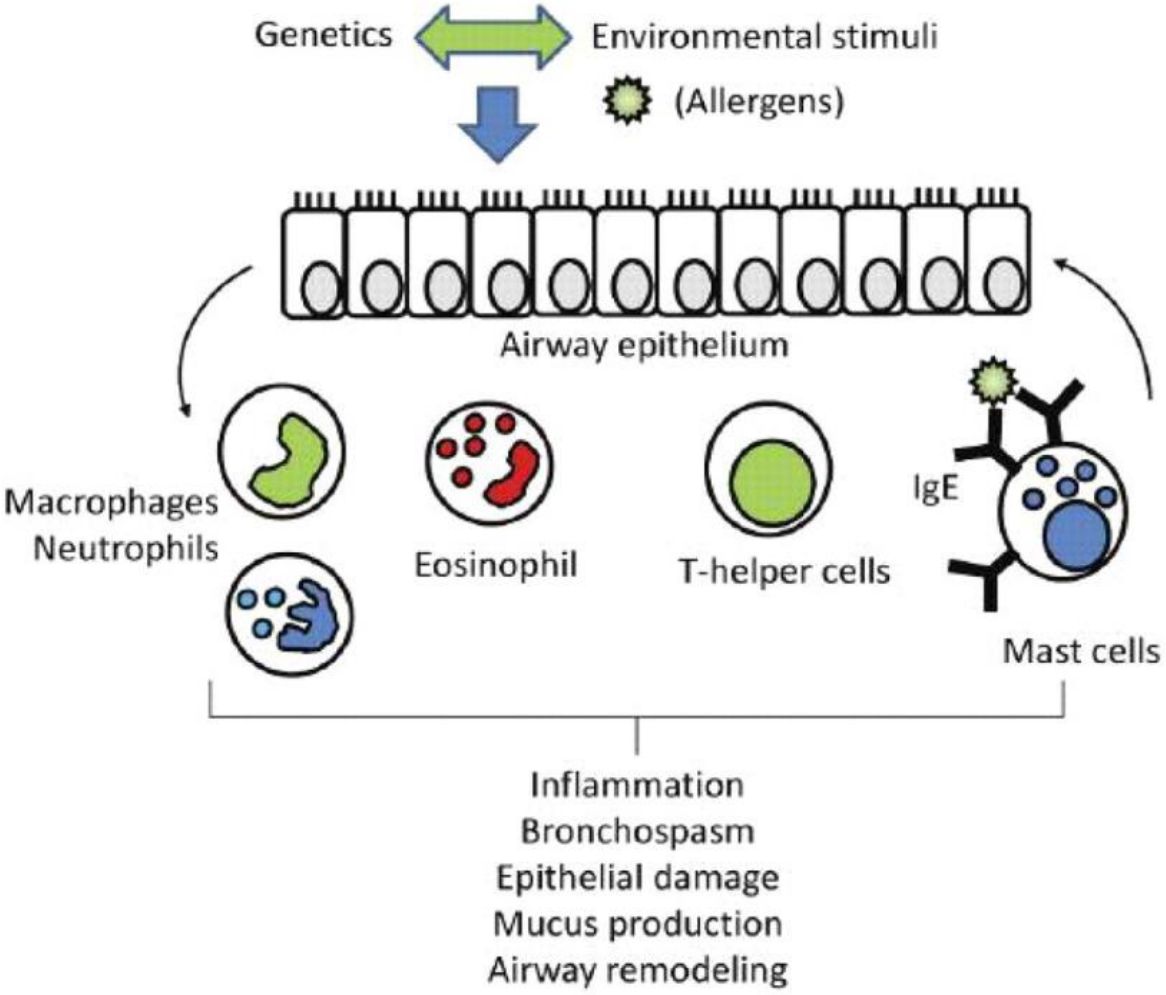


Figure 1.2 Factors involved in asthma pathogenesis (Adcock and Lane, 2003)

Allergens: Allergens are among the most important environmental factors responsible for the development of persistent symptoms and possibly the severity of asthma. However, their role in the development of asthma pathogenesis is yet to be explored. Though studies have revealed that animal (such as dog and cat) danders are associated with asthma development, under certain conditions exposure to animals in early life may boost the immune system to protect against the development of asthma. However, the reasons behind these diverse outcomes warrant more research.

Respiratory infection: Respiratory infection is another important environmental factor which has major influences in asthma pathogenesis. Infection is closely associated with airway hyperreactivity and asthma exacerbation both in children and adults (Busse, 1990). *Respiratory syncytial virus* (RSV), *rhinovirus* (RV), *influenza* and *parainfluenza viruses*, *coronavirus*, *enterovirus*, and *adenovirus* are most commonly involved in asthma pathogenesis and exacerbation (Tan, 2005; Pelaia et al., 2006). Currently it is also believed that some atypical bacteria such as chlamydia pneumonia and mycoplasma pneumonia are involved in the pathogenesis of asthma (Blasi et al., 2001; Pelaia et al., 2006).

Other factors: Other factors associated with the increased risk of asthma pathogenesis include smoke (especially from tobacco), occupation, diet and air pollution. To date the association between allergens/respiratory infections and asthma onset are clearly established and it is evident that gene-by-environmental interactions play a crutial role in the development of airway inflammation and eventual development of asthma characteristics. (Malo et al., 2004). However, the link between other factors and asthma pathogenesis is still unclear.

1.4 Airway structural cells in asthma pathogenesis

Airway structural cells such as airway smooth muscle, epithelial cells, myofibroblasts and fibroblasts play a significant role in asthma pathogenesis (Erle and Sheppard, 2014). Among all these structural cells airway smooth muscle and epithelial cells are critical for asthma pathogenesis. Structural cells are not only important to maintain the integrity of the bronchial wall but also they significantly contribute inflammatory responses mediated by various inflammatory stimuli including cytokines, chemokines, growth factors and inflammatory mediators. Interestingly, structural cells are also sources of these inflammatory molecules

(Levine, 1995; Boxall et al., 2006). Further, the most common asthma characteristics such as airway remodelling and hyperresponsiveness are driven by these structural cells. Hence in our study we focused on two important structural cells (ASM and epithelial cells) for better understanding the molecular mechanism of asthmatic inflammation and eventually to find effective therapeutic targets.

1.4.1 Airway smooth muscle cells

Asthma is a heterogeneous multicellular disease associated with airway hyperresponsiveness, inflammation and remodelling (Busse and Lemanske, 2001; Denis et al., 2001; Girodet et al., 2010). Structural cells play the key role in the development of asthma characteristics. Among all the structural cells, airway smooth muscle cells play the most crucial role in asthma pathophysiology(Zuyderduyn et al., 2008; Black and Roth, 2009), as shown in Figure 1.3. Airway smooth muscle cells are considered to be the key effectors. They cause excessive narrowing of the airway during severe asthmatic conditions that may lead to excessive shortness of breath, respiratory failure, and eventually even death (Erle and Sheppard, 2014). Increased ASM mass (Lambert et al., 1993) and surrounding tissue mediated altered load on ASM cells are also considered as important causes of airway narrowing (Brown et al., 1995). Previously it was thought that airway contraction is the major role of ASM and hence research focused mainly on its contractile properties. However, mounting evidence clearly demonstrates that ASM cells have proinflammatory and immunomodulatory functions (Hirst, 2003; Damera et al., 2009). ASM cells synthesize and express a wide range of cell surface molecules, integrins, costimulatory molecules, and Toll-like receptors; thereby ASM cells play an active role in asthmatic inflammation (Ozier et al., 2011) as shown in Figure 1.3.

The most common feature of asthmatic inflammation is nonspecific airway hyperresponsiveness, which is mediated via a number of direct (histamine, methacholine) or indirect (allergen, chemical) stimuli. Several studies demonstrate that direct stimuli activate ASM cell membrane receptors, whereas indirect mediators stimulate ASM cells via one or more intermediate cells and thereby cause airway hyperresponsiveness and ultimately airway contraction (O'Byrne and Inman, 2003; Anderson, 2010). ASM cell remodeling represents the airway remodeling, which is the key feature of asthma (Bara et al., 2010). A growing body of evidence demonstrates that ASM cells are also involved in promoting other bronchial remodeling including alteration of other structural cells including epithelial cells (Coutts et al., 2001). Most importantly, current asthma medications are insensitive to ASM remodeling, though they are effective in treating acute airway narrowing and inflammation (Girodet et al., 2011). Recent studies suggest that bronchial smooth muscle is infiltrated by inflammatory cells including mast cells, which may contribute to airway remodelling (Begueret et al., 2007). Further, several studies have shown that ASM cells amplify inflammatory responses and subsequently express a wide range of cytokines including IL-6 (Hollins et al., 2008) and IL-8 (Fong et al., 2000), which are known to be implicated in asthmatic inflammation. ASM cells play multifaceted roles in asthma pathogenesis and hence targeting these diverse functions is a major challenge for future asthma control and management.

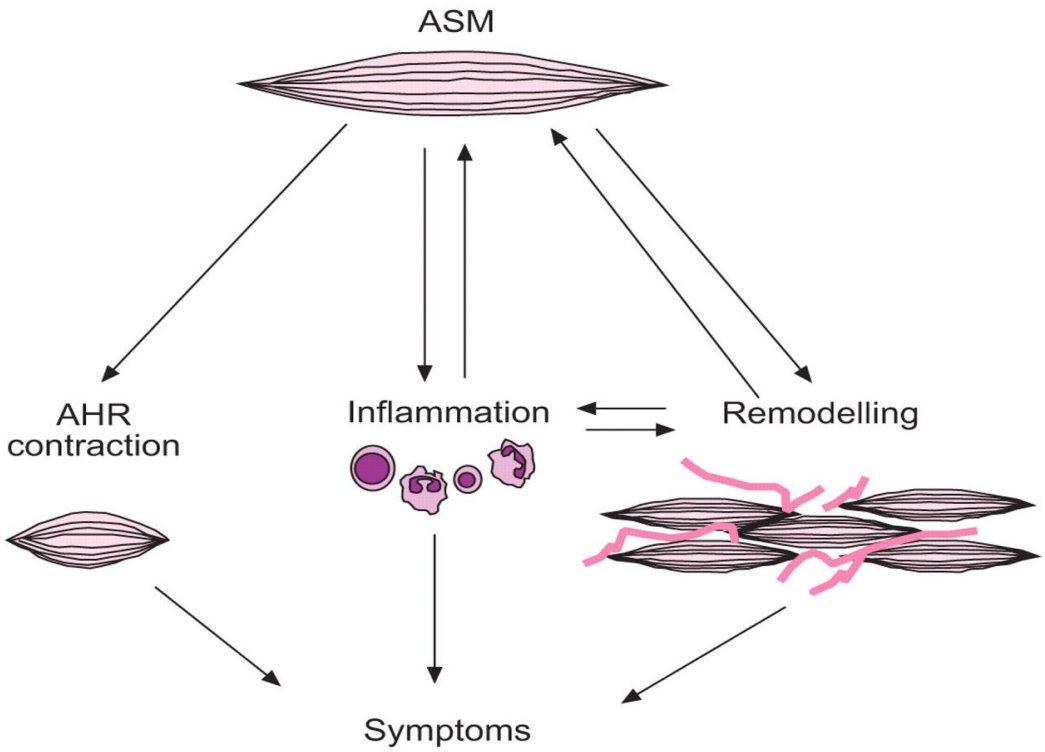


Figure 1.3 Contribution of airway smooth muscle cells in asthma pathogenesis (Chung, 2013)

1.4.2 Epithelial cells

Airway epithelial cells form the first line barrier against mechanical stress, oxidant stress, allergens, pollutants, infectious agents, and leakage of endogenous solutes. They also play important roles in mucociliary clearance and signaling. Epithelial cells express various types of pattern recognition receptors, including Toll-like receptor 4 (TLR4), thereby enabling responses to allergic and infectious stimuli (Salazar and Ghaemmaghami, 2013). In asthmatic conditions, epithelial cells derive cytokines and chemokines (Lambrecht and Hammad, 2012). The key function of the airway epithelial cells was thought to be the formation of a physical barrier. However, with the advantages of research it is known that it constitutes the interface between the internal milieu and the external environment as well as a primary target for inhaled respiratory

drugs. By secreting enormous numbers of molecules and mediators it responds to the changes in the external environment. Therefore, the epithelium has a unique role to translate gene–environment interactions (Knight and Holgate, 2003). Mounting evidence supports that the respiratory epithelium of asthmatics is abnormal and very susceptible to injury compared to normal epithelium. Furthermore, being more susceptible to damage, normal repair processes are compromised. Persistent mucosal injury can also occur due to failure of proper growth and differentiation of airway epithelial cells (Polito and Proud, 1998; Knight and Holgate, 2003).

1.5 Asthma management

Inflammation plays the key role in asthma pathogenesis; hence anti-inflammatory strategy is still one of the most important treatment options in asthma. Inflammation is responsible for airway swelling and mucus production and thereby it makes the airway more sensitive to asthma triggers. Anti-inflammatory medicines are the first line medicines, which help to stop this process and prevent asthma attacks.

The key treatment option (details are shown in Figure 1.4) for asthma is steroid or corticosteroid and other anti-inflammatory drugs (leukotriene modifiers and immunomodulators). Both of these drugs work by reducing inflammation, swelling, and mucus production in the airways and help to control asthma and prevent asthma attack.

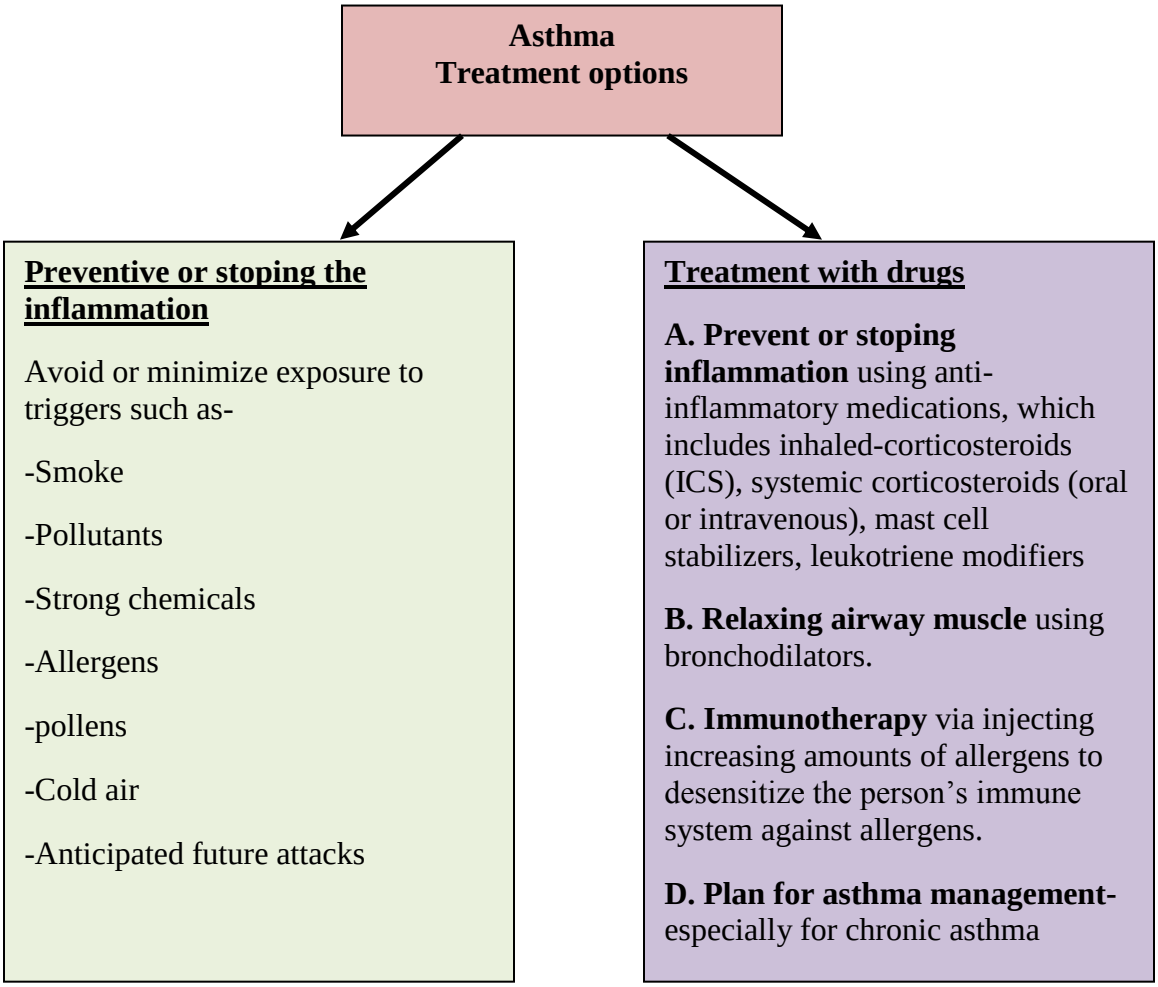


Figure 1.4 Treatment options for asthma

Asthma is a chronic disease and most importantly to date there is no drug to cure asthma, hence asthma patients have no choice other than long term use of corticosteroids, which often leads to unwanted side effects including skin and muscle atrophy, disturbed wound healing, osteoporosis, growth failure and delayed puberty (children), glaucoma and cataract, steroid psychoses, depression, adrenal insufficiency; and hypogonadism, hyperglycaemia, hypertension,

dyslipidaemia, peptic ulcers, upper gastrointestinal bleeding, pancreatitis and severe immunosuppression effects (Schäcke et al., 2002).

Moreover, a small number of asthmatics (<5 %) are not responsive to existing corticosteroid therapy (Adcock and Lane, 2003), which is a major drawback of anti-inflammatory strategy. Several studies clearly demonstrate that corticosteroid insensitivity can range from relative corticosteroid insensitivity to steroid resistance, as seen in severe asthma (reviewed in (Ammit, 2013; Chung, 2013)).

Despite, remarkable progress in anti-inflammatory strategies, severe side effects and corticosteroid insensitivity are the major challenges in this field. To meet these challenges, we urgently require alternative anti-inflammatory strategy with less or minimal side effects and are capable to work even corticosteroid insensitivity exist. Enhancing the function of endogenous phosphatases, especially PP2A, offer great promise.

1.6 Asthma exacerbation

Asthma is a highly prevalent chronic disease, which affects millions of people around the globe. Asthma exacerbation, an acute or subacute episode of progressive worsening of symptoms further worsens the situation and increases the social burden. In addition, asthma exacerbation is one of the significant causes of morbidity and drives the majority of the asthma care cost (Dougherty and Fahy, 2009). Asthma exacerbation is usually associated with the release of pro-inflammatory cytokines, chemokines and recruitment of inflammatory cells driven by a number of factors; particularly viral infection. Respiratory viral (especially rhinoviruses) infections cause most of the asthma exacerbations both in children and adults. Other factors involved in asthma exacerbation are bacterial infection, allergens, cigarette smoke, environmental pollutants,

occupational irritants, medication, and intrinsic factors such as deficient epithelial cell production of the anti-viral type I interferons (IFN-α and IFN-β) (Wark and Gibson, 2006). During asthma exacerbation lung functions sharply decline and elicit asthma symptoms including shortness of breath, coughing, wheezing, and chest tightness. Severe asthma exacerbation is one of the major causes of death from asthma and this number increases during winter, when the chance of viral infection is very high. In addition, the frequency of asthma exacerbation is also triggered by respiratory viral infections. Though the frequency of asthma exacerbation can be reduced by a proper action plan, infection associated exacerbations are very difficult to control (Wilson and Silverman, 1990; Doull et al., 1997; Harrison et al., 2004). Therefore more research is warranted in the context of asthma exacerbation for better understanding and management of this disease.

1.7 Inflammation and asthma exacerbation

A number of factors are responsible for the development of asthma. Inflammation appears to be a common factor that drives to the most severe asthmatic responses. Hence, inflammation is considered as a dominant feature in the pathophysiology of asthma (Adcock and Lane, 2003). Interactions of various cell types and inflammatory mediators with the airways is the major cause of airway inflammation, which eventually results in the pathophysiological features of asthma including bronchial inflammation, airflow limitation and subsequent recurrent episodes of coughing, wheezeing and shortness of breath (shown in Figure 1.5). The underlying molecular mechanisms by which these interactive events take place and direct clinical asthma features are yet to be uncovered. However, studies reveal that airway inflammation is a consistent and common feature of all asthma phenotypes. More importantly, the extent of airway inflammation

is independent of asthma severity, persistence and duration. Notably, the inflammation does not alter the cellular profile and response of the structural cells in asthmatics.

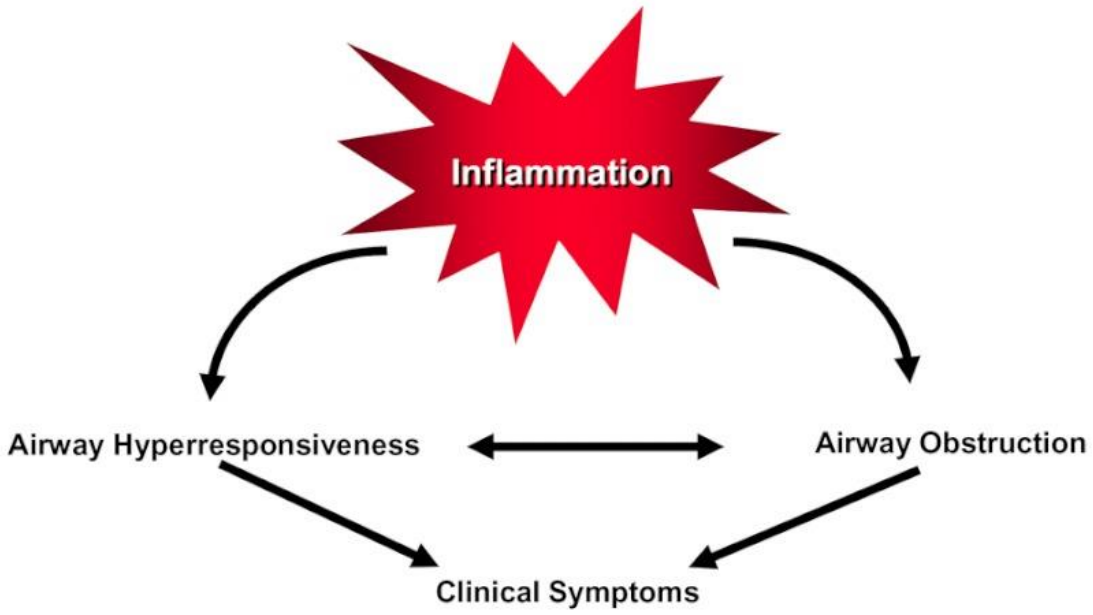


Figure 1.5 The interaction between airway inflammation and the clinical symptoms of asthma (Guidelines for the Diagnosis and Management of Asthma. Bethesda (MD): National Heart, Lung, and Blood Institute (US); 2007 Aug. Section 3)

Airways inflammation involves activated mast cells, eosinophils, neutrophils, T lymphocytes and macrophages, which is an established feature of asthma, and the key target for treatment (Louis et al., 2000). Though it is known that many inflammatory cells are involved in asthma (shown in Table 1.2), precise role of each cell is not certain yet. However it is evident that multiple inflammatory cells are liable for the complexity of asthma pathophysiology and some cells are predominant in asthmatic inflammation (Busse and Lemanske, 2001).

Table 1.2 Inflammatory cells and their role in asthmatic inflammation

Inflammatory cells	Role in asthma
Mast cells	-Mucosal mast cells activation releases bronchoconstrictor mediators, such as histamine, cysteinyl-leukotrienes, prostaglandin D_2 (Boyce, 2003). -Airway hyperresponsiveness may be linked with the increased number of mast cells in airway smooth muscle (Liesker et al., 2007). -Mast cells regulate the airway environment and promote inflammation even though limited exposure to allergens via releasing huge number of cytokines (Amin, 2012).
Eosinophils	-Most asthmatic patient's airway contains increased numbers of eosinophils (Chu and Martin). -Greater the number of eosinophils greater the severity of asthma (Walford and Doherty, 2014). -Eosinophils contain inflammatory enzymes, generate leukotrienes, and express a wide variety of pro-inflammatory cytokines (Walford and Doherty, 2014).
Neutrophils	-Airways and sputum of severe asthmatic patient contain increased number of neutrophils (Fahy, 2009). -Neutrophils may be the determinant factor of activation and alteration of lung function and response to corticosteroid (Fahy et al., 1995).
Dendritic cells or Antigen presenting cells.	-Stimulate TH2 cells production via increasing interaction with allergen (Kuipers and Lambrecht, 2004).
Macrophages	-Activated by allergen and release inflammatory mediators, cytokines and amplify inflammatory responses (Peters-Golden, 2004).
TH_2-lymphocytes	-Modulate airway inflammation and control asthma by producing a number of cytokines, such as L-4, IL-5, IL-9 and IL-13 (Barnes, 2001)

1.8 Inflammatory mediators and MAPKs

Asthma is a complex chronic inflammatory disease of the airway involving activation of a number of inflammatory cells such as mast cells, dendritic cells or antigen-presenting cells, eosinophils, neutrophils, macrophages, and TH_2-lymphocytes as well as structural cells. These inflammatory cells produce a wide range of inflammatory mediators and cytokines that exacerbate typical asthma symptoms such as bronchoconstriction, mucosal edema, mucus secretion and bronchial hyperresponsiveness (Barnes, 1996).

Numerous mediators provoke inflammatory response from respiratory structural cells. The cross-talk between the cells and the mediators are the principle driving force for the inflammatory response observed in respiratory diseases like asthma. In the following sections we will overview some key inflammatory mediators and stimuli we have used in this study and then we will discuss briefly their signalling molecules involved in asthmatic inflammation

1.8.1 Inflammatory mediators

There are a wide range of inflammatory mediators responsible for initiating/accelerating inflammation via triggering inflammation associated signaling pathways. Some important inflammatory mediators are described below:

1.8.1.1 Cytokines

There are significant number of pro-inflammatory cytokines (such as eotaxin, GM-CSF, IFNγ, IL-4, IL-5, IL-8, IL-6, IL-10, IL-12, IL-13, IL-17A, IL-17F, IL-18 and TNFα), which direct and modify the inflammatory response and determine the severity of asthma. They also play roles in eosinophil infiltration, mast cell activation, mucosal hypersecretion, epithelial shedding,

allergen-specific IgE production and bronchial muscle contraction. Among all these cytokines IL-6 and IL-8 are the two important cytokines which are largely implicated in asthmatic inflammation and are regulated by MAPKs family members such as p38 MAPK, ERK and JNK (Amrani et al., 2001; Henness et al., 2006; Quante et al., 2008; Moutzouris et al., 2010).

1.8.1.1.1 IL-6

IL-6 is one of the major cytokines implicated in asthmatic inflammation. It is a small glycoprotein produced by numerous cells including mast cells, macrophages, dendritic cells, neutrophils, B cells and a small amount by some cluster of differentiation 4 (CD4) effector Th cells. A wide variety of stimuli such as UV irradiation, reactive oxygen species (ROS), microbial products, viruses, or other proinflammatory cytokines can induce IL-6 secretion. Several studies reported elevated serum level of IL-6 in a wide number of inflammatory diseases including asthma (Yokoyama et al., 1995; Kishimoto, 2010), hence IL-6 is considered as a common marker of inflammatory diseases including asthma. However, with the progress of research it is evident that IL-6 is not a simple pro-inflammatory cytokine or just the end product of lung pathology but a critical factor in the pathogenesis of certain inflammatory diseases including asthma and chronic obstructive pulmonary disease (COPD) (He et al., 2009).

The role of IL-6 in asthmatic inflammation is well-established. However, conflicting results obtained from the most commonly used ovalbumin (OVA) mouse model require further studies to ascertain its role in asthmatic inflammation. Allergic airway inflammation potentiates the complexity of IL-6 involvement in the airway diseases. Using this model initial studies reported increased level of lung Th2 cytokines including IL-4, IL-5 and IL-13, eosinophilia and eotaxin level in IL-6 deficient mouse; however the same study also showed a decreased level of Th2

cytokines and eosinophilia in IL-6 overexpressing transgenic mice (Wang et al., 2000). Hence more studies are warranted for better understanding the role of IL-6 in airway diseases.

Despite remarkable progress in our understanding of IL-6 as a master player in the pathogenesis of lung diseases (Neveu et al., 2010) and a key modulator of the overall immune response, the mechanisms by which IL-6 interferes with lung function and potentiates airway disease pathogenesis is yet unclear. Moreover, due to the pleotropic nature of IL-6 (Kishimoto, 2006), it provides a variety of mechanisms by which this cytokine can contribute to specific aspects of complex respiratory diseases including asthma. Future studies required to address the clinical importance of IL-6 and ascertain its role in asthmatic inflammation. We therefore aim to explore the role of IL-6 in asthmatic inflammation using respiratory epithelial and airway smooth muscle cells.

1.8.1.1.2 IL-8

IL-8 is a critical proinflammatory cytokine implicated in asthma. One of the important asthma endotypes is neutrophilic (non-eosinophilic) asthma, which is mainly driven by the chemokine CXCL8 (IL-8) (Simpson et al., 2009). Neutrophils are implicated in the pathogenesis of many inflammatory lung diseases, including acute respiratory distress syndrome, chronic obstructive pulmonary disease and asthma. IL-8 is a potent neutrophil recruiting and activating factor (Pease and Sabroe, 2002), hence IL-8 detection from the patients with these diseases has led clinicians to believe that targeting IL-8 may be a potential therapeutic strategy for the management of these diseases. Thus, studies into the molecular pathways that upregulate this neutrophil chemoattractant will allow us to gain greater insight of the underlying pathogenic mechanisms

and suggest potential pharmacotherapeutic strategies for treating the neutrophilic asthma endotype in the future.

It is reported that IL-8 plays a primary role in the activation of both neutrophils and eosinophils in the airways of patients with asthma and COPD. It is also reported that IL-8 concentration in the sputum is closely associated with the extent of airflow obstruction in patients with COPD and hence it may serve as a marker in evaluating the severity of airway inflammation (Norzila et al., 2000). The causes of neutrophilic asthma are currently uncertain. Innate immunity dysregulation through TLR2 plays an important role (Simpson et al., 2007), as may T-helper17 (Th17) regulation (Newcomb and Peebles, 2013) and NACHT, LRR and PYD domains containing protein3 (NLRP3) inflammasome activation (Simpson et al., 2013). Activation of these cellular pathways has been reported to increase neutrophilic inflammation in the airways in an IL-8-driven manner; which is the key characteristic of the disease endotype. Airway structural cells (such as alveolar epithelium and ASM) serve as important contributors to IL-8 chemokine production and in this way they may orchestrate neutrophil chemoattraction in response to inflammatory mediators (Henness et al., 2006; Manetsch et al., 2012c; Zijlstra et al., 2012).

1.8.1.2 Chemokines

Chemokines are a large family of cytokines and are capable of attracting different cell types and thereby they mediate inflammatory tissue destruction in a wide variety of human diseases such as rheumatoid arthritis, myocardial infarction, adult respiratory distress syndrome and asthma (Baggiolini and Dahinden, 1994; Strieter et al., 1996; Lukacs, 2001). Chemokines serve as potent leukocyte chemoattractants, cellular activating factors and histamine-releasing factors; hence they are important in the pathogenesis of allergic inflammation. Several studies demonstrate that

chemokines play a vital role in orchestrating multiple aspects of asthmatic the response including injury and repair (Zimmermann et al., 2003).

The eotaxin subfamily of chemokines appears to be a central regulator of the asthmatic response and is relatively selective for eosinophils. Thymus, activation-regulated chemokines (TARCs) and macrophage-derived chemokines (MDCs) recruit Th2 cells, which subsequently induce asthma via secreting a number of cytokines (Zimmermann et al., 2003). Chemokines bind with a wide range of receptors and are promiscuous in the context of asthmatic inflammation (Oliveira and Lukacs, 2003). Hence, determining the exact role of individual chemokines and receptors is crucial to identifying the effective targets. Exploring the function of eosinophils and their chemokine receptors during inflammation may be an excellent approach to understanding the determinants of asthma severity and developing novel therapeutic targets.

1.8.1.3 Cysteinyl-leukotrienes

Cysteinyl leukotrienes are inflammatory molecules derived from mast cells during asthma attack and are mainly responsible for bronchoconstriction. It is reported that inhibition of this mediator is associated with the improvement in asthma symptoms and lung function (Busse, 1996). Leukotrienes are critical both in accelerating acute asthma attacks and in causing long term hypersensitivity in chronic asthma (Berger, 1999). Leukotrienes have two distinct families; the first group primarily acts on neutrophils dependent of inflammatory conditions, whereas the other group of leukotrienes, cysteinyl-leukotrienes, are mainly concerned with the bronchoconstriction in asthma induced by mast and eosinophil cells. Leukotrienes selectively bind with the receptors located on bronchial smooth muscle and other airway tissues (O'Byrne et al., 1997) and cause asthma symptoms.

1.8.1.4 Prostanoids

Prostanoids are a subclass of eicosanoids comprising of both prostaglandins and thromboxanes, which are derived from arachidonic acid. Several studies demonstrate the regulatory role of prostanoids in allergic inflammation and pathogenesis of allergic bronchial asthma. Some *in-vivo* studies showed that the disruption of the D prostanoid (DP) gene attenuated the allergen-induced airway eosinophilic inflammation (Tanaka and Nagai, 2001). Excessive prostanoid production and signaling contribute to increase susceptibility to infections and subsequently drive COPD exacerbations and inadequate alveolar repair (Zaslona and Peters-Golden, 2015).

1.8.1.5 Nitric oxide

Mounting evidence indicates that endogenous nitric oxide (NO) plays a significant role in the physiological regulation of airway functions and is implicated in airway diseases, including asthma (Barnes and Belvisi, 1993; Gaston et al., 1994). It is predominantly produced from airway epithelial cells by the action of inducible NO synthase. High concentrations of NO defend against microorganisms' at the same time it is toxic to airway epithelial cells and may contribute to epithelial shedding in asthma (Barnes and Liew, 1995). Hence there is increasing evidence that high concentrations of NO may have effects on the immune system and the inflammatory response. Recent studies demonstrate the association between exhaled nitric oxide levels (FeNO) and asthmatic inflammation. Hence measuring the fraction of exhaled NO can be a useful tool to monitor the response of anti-asthmatic drugs in asthma treatment (Green et al., 2002).

1.8.1.6 Immunoglobulin E (IgE)

IgE is a critical factor involved in allergic inflammation. It plays an important role in the pathogenesis of allergic diseases including asthma and the development of persistence inflammation (Boyce, 2003; Rosenwasser, 2011). IgE is predominantly found in mast cells and activated by the interaction with a specific antigen. Activated IgE initiates acute bronchospasm and releases pro-inflammatory cytokines to perpetuate airway inflammation (Boyce, 2003). It is reported that reduction of IgE using monoclonal antibodies is effective in asthma treatment, which further supports the importance of IgE in asthma (Busse and Lemanske, 2001).

1.8.2 Exogenous inflammatory stimuli

There are a number of clinically proven exogenous inflammatory stimuli such as TNFα, IFN-γ, S1P, LPS, TGFβ, IL-17, IL-1β, bacterial infection (Pam3CSK4) and viral infection, which are widely used in asthma research. These stimuli can either initiate or trigger acute or chronic inflammation in airway diseases and subsequently cause diseases pathogenesis. In this section we will discuss a few well studied exogenous inflammatory stimuli which are widely used in asthma research. Most importantly we also used them in this study.

1.8.2.1 TNFα

TNFα is a pleiotropic pro-inflammatory cytokine of the TNF superfamily. The human TNF gene (TNFA) maps to chromosome 6p21.3 and contains 4 exons. Among these four, the last exon codes for more than 80% of the secreted protein (Nedwin et al., 1985). The 3' UTR of TNFα has an AU-rich element (ARE). TNFα is 233-amino acids in length, containing transmembrane proteins arranged in stable homotrimers. Matrix metalloprotease, a TNFα

converting enzyme (TACE) mediates proteolytic cleavage and releases soluble homotrimeric cytokine (sTNF) from the membrane-integrated form. Both the membrane bound and the soluble forms of TNFα have distinct biological activities (Kriegler et al.; Tang et al., 1996). It is reported that TNFα has two commonly expressed but specific cell-surface receptors (Brockhaus et al., 1990), TNF receptor 1 (TNF-R1) and TNF receptor 2 (TNF-R2), which differ in their expression profiles, ligand affinity and downstream signalling pathway activation (Matera et al., 2010). TNF-R1 is constitutively expressed in most tissues and cell types, and is activated by either the soluble or the membrane-bound trimeric forms of TNFα, whereas TNF-R2 is largely found in the immune system cells and is preferably activated by the trans-membrane protein form of TNFα and thereby they differentially regulate signalling pathways, leading to physiological and also pathophysiological effects (Al-Lamki et al., 2001).

TNFα is a critical inflammatory cytokine produced by a number of cells including activated macrophages (mostly produced by these cells), immune cells like mast cells, lymphocytes, endothelial cells and also by structural cells (which is very important for this study) such as fibroblasts, epithelial cells and smooth muscle cells (Wajant et al., 2003; Brightling et al., 2008). Though TNFα is detectable in healthy individuals, elevated levels of TNFα, mRNA and protein were reported in asthmatic airways compared to non-asthmatics (Ying et al., 1991; Bradding et al., 1994). TNFα plays key role in the pathogenesis of asthma (hyperresponsiveness, airway remodelling) via activating various pro-inflammatory signalling pathways, which includes activation of nuclear factor kappa B (NFκB) and activator protein-1 (AP-1) signalling pathways as well as other transcription factors (Natoli et al., 1997). It is also reported that with the response of inflammatory stimuli such as lipopolysaccharide (LPS), IL-1 and other bacterial products huge amounts of TNFα which is released from skin and mast cells

(Wajant et al., 2003). This cytokine is involved in the regulation of a wide spectrum of biological processes including cell growth promotion and inhibition, angiogenesis, immunomodulation and inflammation, and thereby plays an important role in modulating a wide range of inflammatory conditions such as rheumatoid arthritis (Sarzi-Puttini et al., 2005), cardiovascular diseases (Sack, 2002), psoriasis (Kleyn and Griffiths, 2006) and cancer (Li et al., 2015). TNFα is implicated in several aspects of the airway pathophysiology in respiratory diseases (Berry et al., 2007; Brightling et al., 2008; Matera et al., 2010) and we also highlighted the inflammatory role of TNFα in asthmatic conditions here in this study. It is well studied and clearly demonstrated that TNFα can activate MAPKs signalling pathways (51, 92), and thereby upregulate several pro-inflammatory cytokines and chemokines including those which are implicated in asthmatic inflammation (Wajant et al., 2003). Interestingly TNFα can also upregulate anti-inflammatory proteins MKP-1 (Quante et al., 2008) in ASM cells. Further, it has also been shown that p38 MAPK is involved in the biosynthesis of TNFα (Kotlyarov et al., 1999). In support of the earlier research, in our present study we demonstrate that TNFα is a model of inflammatory mediator that secretes significant amount of pro-inflammatory cytokines in respiratory epithelial cells (A549, BEAS-2B and ASM cells) and confirmed its role in the pathogenesis of inflammatory lung diseases such as asthma.

1.8.2.2 Pam3CSK4

Pam3CSK4, a synthetic bacterial lipoprotein, has been widely used to mimic bacterial infections (gram-positive bacteria) to mediate inflammatory responses via TLR2 (Manukyan et al., 2005; Beckett et al., 2012; Manetsch et al., 2012c). Pam3CSK4 is a potent TLR2 agonist, which is capable of modulating both Th1 and Th2 responses (Patel et al., 2005). Mounting evidence

demonstrates that TLRs are the primary sensors of both innate and adaptive immune systems that recognize invading pathogens and their synthesized molecules to coordinate the inflammatory responses (Patel et al., 2005). It is also reported that TLRs recognized a number of microbial products, which includes LPS, peptidoglycans, lipotechoic acids and bacterial pathogens producing lipoproteins (BLPs) (Henderson et al., 1996; Aliprantis et al., 1999). Among all these microbial products BLPs play the key role in inducing pro-inflammatory cytokines (Kostyal et al., 1994). BLPs are bacterial cell wall components found both in gram-negative and gram-positive bacteria and mediate their immunomodulatory effects through their amino acid terminus (Manukyan et al., 2005). It is well studied and demonstrated that Pam3CSK4 induces the production of pro-inflammatory cytokines via activation of MAPK/AP-1 (Thaikoottathil and Chu, 2011) and NF-κB (Aliprantis et al., 1999) signalling pathways. However alternative mechanisms are also reported. We previously reported that Pam3CSK4 significantly increased TNFα-induced IL-6 and IL-8 mRNA expression and protein release and neutrophil chemotactic activity via phosphorylation of CREB protein at Ser^{133} and CRE-mediated transcription but not augmentation of NF-κB or MAPK signalling pathways or activation of TLR2 (Manetsch et al., 2012c). In addition, Pam3CSK4 can also modulate RSV infection in a manner independent of TLR2 activation and accelerate acute respiratory disease in infants, immunocompromised subjects and the elderly (Nguyen et al., 2010)

Synthetic lipopeptide is considered an important tool for studying innate immune recognition mechanisms as it is not only more easily obtained than the lipopeptide (directly obtained from organisms) but is also free of other contaminating bacterial components (Manukyan et al., 2005). Several studies demonstrate that TLRs interact with synthetic ligands or bacterial products to augment TLR2 function and stimulate pro-inflammatory cytokine secretion from human ASM

cells, including eotaxin and IL-6, IL-8 (Sukkar et al., 2006; Manetsch et al., 2012c) . In addition, it is reported that corticosteroid insensitivity is linked with certain cytokines and chemokines expression including IL-6 and IL-8 in respiratory cells (Bouazza et al., 2012; Chang et al., 2012). Hence in our study we used Pam3CSK4 to induce IL-6 and IL-8 expression in the context of corticosteroid insensitivity in respiratory epithelial cells to gain insight into the molecular mechanisms of this event as well as explore the possible therapeutic target to bypass corticosteroid insensitivity.

1.8.2.3 S1P

S1P is a bioactive sphingolipid metabolite with pleiotropic functions (Spiegel and Milstien, 2003). S1P is increasingly recognized as playing an important role in asthma and airway inflammation (Oskeritzian et al., 2007). A growing body of evidence supports that S1P can regulate airway smooth muscle functions associated with asthmatic inflammation and remodelling, including cytokine secretion (Ammit et al., 2001). A number of stimuli such as cytokines, antigen and receptor tyrosine kinase and G protein-coupled receptors (GPCR) agonists can induce S1P expression in multiple cell types (Pyne and Pyne, 2000). S1P is involved in many cellular processes, acting both as an extracellular mediator via a family of plasma membrane GPCRs, and as an intracellular second messenger for growth factors, by altering the activity of specific intracellular target proteins (Pyne and Pyne, 2000). It is reported that S1P can regulate cell growth, proliferation, differentiation, and chemotaxis of various cell types. Further, the involvement of S1P in certain disease states such as atherosclerosis (Wang et al., 1999) and certain cancers (Hong et al., 1999) is also well recognized. Elevated levels of S1P were detected in bronchoalveolar lavage (BAL) fluid of asthmatics after challenge with antigen (Ammit et al.,

2001), which is correlated with the eosinophil numbers in asthmatic subjects. Most importantly, S1P is recognized as a key regulator of ASM cell growth, contraction and synthetic functions. Further, it is evident that a number of signalling pathways are activated by S1P and thereby it regulates physiological and pathophysiological conditions.

Elevated levels of S1P were also found in the airways of asthmatics and can increase IL-8 secretion from human alveolar epithelial cells (A549) to regulate neutrophil–epithelial interactions *in vitro* (Milara et al., 2009). That S1P induces secretion of an important chemokine (IL-8) responsible for neutrophilia in airway inflammation is relatively unexplored. Therefore, in this study we aim to investigate whether S1P induces IL-8 gene expression and secretion to enhance neutrophil chemotaxis *in vitro,* as well as examine the molecular mechanisms responsible for repression by the corticosteroid, especially dexamethasone. We believe that our understanding of signaling mechanisms underlying S1P induced IL-8 gene regulation will create an avenue for the development of anti-inflammatory therapeutic strategies for better asthma management.

1.8.3 MAPKs and asthmatic inflammation

MAPKs are protein kinases that are evolutionary conserved eukaryotic signal transducing enzymes. Activation of MAPKs signalling pathways in airway structural cells produce a number of pro-inflammatory molecules, which elicit complex inflammatory conditions and cause structural changes that ultimately contribute to the hallmark of asthma such as T cell activation, eosinophil and mast cell infiltration, bronchial hyperresponsiveness and airway remodelling (Pelaia et al., 2005). MAPKs regulate multiple signalling pathways; hence they affect several aspects of normal airway functions as well as contribute to asthma pathophysiology (shown in

Table 1.3). The MAPKs superfamily comprises of several subgroups such as p38 MAPK, c-Jun N-terminal kinase (JNK 1, JNK 2 and JNK) and extracellular signal-regulated kinase (ERK, also known as p44/p42 or ERK1/ERK2) (Johnson and Lapadat, 2002). The MAPKs subfamilies share a common activation mechanism; they are activated via phosphorylation of three sequentially activated kinases. Importantly, like their substrates (other protein kinases and transcription factors), MAPKs functions are also regulated by phosphorylation. MAPKs activation requires dual phosphorylation at threonine (Thr) and tyrosine (Tyr) residues of the Thr-X- Tyr, where X is glutamic acid in the case of ERKs, proline in JNKs, and glycine in p38 MAPK (Widmann et al., 1999; Pearson et al., 2001; Turjanski et al., 2007). After activation MAPKs migrate to the nucleus, where they phosphorylate various transcription factors via phosphorylation at their specific sites and thereby control gene expression (Karin, 1998; Widmann et al., 1999).

Table 1.3 MAPKs activation and their impact in asthmatic inflammation (Johnson and Lapadat, 2002; Subhashini et al., 2016).

MAPKs	Activated airway cells (clinical outcomes)
p38 MAPKs	-**Eosinophils** (chemotaxis, degranulation) -**Mast cells** (cell migration, production of IL-8) - **Neutrophils** (cell recruitment) -**Monocytes & macrophages** (differentiation, chemotaxis, release TNFα and MIP-2) -**T cells** (release IL-5 & IL-13) -**Epithelial cells** (apoptosis, release IL-8 and RANTES) -**Fibroblasts** (release IL-6) -**Smooth muscles** (cytokines production)
ERK MAPKs	-**Eosinophils** (chemotaxis) -**Mast cells** (production of IL-8) -**T cells** (Th2 differentiation) -**Epithelial cells** (release IL-8 and RANTES) -**Endothelial cells** (angiogenesis) -**Smooth muscles** (cytokine production, cell proliferation)
JNK MAPks	-**Epithelial cells** (release IL-8 and RANTES) -**Fibroblasts** (myofibroblasts differentiation -**Smooth muscles** (cell proliferation)

Compiling evidence from published data support the importance of MAPKs family members in regulating the expression of cytokines, including two important cytokines implicated in asthmatic inflammation, IL-6 and IL-8 (Amrani et al., 2001; Henness et al., 2006; Quante et al., 2008; Moutzouris et al., 2010). Our recent studies also clearly demonstrate the importance of

the MAPK superfamily signalling molecules in asthmatic inflammation using airway structural cells (A549, BEAS-2B and ASM) (Henness et al., 2006; Quante et al., 2008). Hence as part of our ongoing projects in this current study we used the same molecular approach to reveal this unexplored avenue to successfully combat asthmatic inflammation.

1.8.3.1 p38 MAPKs

The four isoforms of p38 MAPK subfamily are p38α, p38β, p38γ, and p38δ. Among these isoform p38α is well studied and shown to be expressed from most of the cells, whereas the others are expressed in a more tissue specific manner (Cuadrado and Nebreda, 2010). It is reported that distinct genes are responsible for p38 isoforms expression. p38 MAPKs are localized in the nucleus or cytosol or in both compartments and can be activated by several pro-inflammatory stimuli; hormones, ligands for GPCRs and cellular stress (such as osmotic shock and heat shock). The p38 MAPKs regulate the expression of many cytokines (including those which are implicated in asthma and autoimmunity), transcription factors, and cell surface receptors (Roux and Blenis, 2004; Keshet and Seger, 2010). In addition, they also play an important role in the functional response of immune and inflammatory cells by controlling chemotaxis, cell migration and differentiation, apoptosis, and cytokine production (Ono and Han, 2000), which suggest that p38 activity is critical for normal immune and inflammatory responses. Further, numerous studies demonstrate that p38 MAPKs regulate cell migration, proliferation, apoptosis and several inflammatory events in airway structural cells via increasing secretion of pro-inflammatory signalling molecules (Moutzouris et al., 2010; Munoz et al., 2010), which confirm their involvement in airway diseases pathogenesis.

Activation of p38 MAPKs lead to phosphorylation of a number of substrates including transcription factors (such as ATF2/6, Chop, Max, MEF2C), nuclear histones (H3), heat shock proteins (hsp27), various kinases like MAPKAP-K2 (mitogen-activated protein kinase-activated protein kinase 2), MSK1 (mitogen and stress-activated kinase 1), MNK1/2 (MAPK-interacting kinase 1/2) and PRAK (p38-regulated/activated protein kinase). Mediator generating enzymes such as the inducible isoform of COX-2 are also an important targets of p38 (Lasa et al., 2000). Most importantly, p38 MAPKs control the dynamic relation between phosphorylated (inactive) and unphosphorylated (active) TTP, a critical anti-inflammatory protein.

1.8.3.2 c-Jun N-terminal kinase (JNK) MAPKs

The JNK MAPK subgroup is encoded by three genes; JNK1, JNK2 and JNK3 and has ten isomers. JNK MAPKs are also known as stress-activated protein kinases due to their activation in response to inhibition of protein synthesis (Yan et al., 1994). Like p38 MAPK, JNK MAPKs are also responsive to stress stimuli, such as ultraviolet irradiation, cytokines, pro-inflammatory cytokines, heat shock, and osmotic shock (Pelaia et al., 2005; Munoz et al., 2010). JNK1 and JNK2 are ubiquitously expressed, whereas the JNK3 protein is found mainly in brain, heart and testis (Bode and Dong, 2007). Upon activation, JNKs translocate to the nucleus and phosphorylate a number of substrates including several transcription factors such as the c-Jun component of AP-1, Elk-1 and ATF2 and thereby they play important role in stress responses, apoptosis and inflammation (Cowan and Storey, 2003; Pelaia et al., 2005). It is reported that JNK is regulated by PP2A and plays an important role in IL-8 production (Kobayashi et al., 2011; Kobayashi et al., 2012).

1.8.3.3 ERK MAPKs

ERK MAPKs have several isoforms, among which the isoforms ERK1 and ERK2 are two well-studied types, which are widely expressed and are involved in the regulation of meiosis, mitosis, and postmitotic functions in differentiated cells (Johnson and Lapadat, 2002). ERK MAPKs are also known as p42/p44 MAP kinases, which are activated by growth factors, mitogens or GPCRs, cytokines, virus infection transforming agents, and carcinogens (Sugden and Clerk, 1997). Upon activation, ERK MAPKs phosphorylate diverse substrates, either in the cytoplasm or in the nucleus depending on the prior stimulus. ERK MAPKs have a wide variety of substrates including transcription factors (such as cMyc, Elk1, STATs, Ets1, Sap1a, Tal), growth factors such as EGF and pro-inflammatory enzymes like cytosolic phospholipase A2 (Widmann et al., 1999; Cowan and Storey, 2003). The ERK subgroup of MAPKs plays an important role in diverse physiological and pathological processes such as proliferation, differentiation and transformation, survival and apoptosis, stress response, inflammation, cell growth and also contributes to airway inflammation. Further activation of ERK MAPKs induce cyclin D1 expression in airway monocytes and regulate ASM progression (Ramakrishnan et al., 1998), and thereby they increase ASM mass in asthmatic patients and contributes to bronchial remodelling and excessive airway narrowing.

1.9 Endogenous anti-inflammatory proteins

Inflammation is an immune response which is beneficial under normal physiological conditions. Inflammation plays important role in host defence mechanism via eliminating harmful foreign organisms or materials (Van Hove et al., 2008). However, persistent inflammation is harmful to the host and often accelerates the pathogenesis of a number of

diseases including asthma due to overexpression of multiple inflammatory proteins (Barnes, 2008). There are a number of endogenous anti-inflammatory proteins including MKP-1, TTP (Prabhala and Ammit, 2015), and PP2A (Frasca et al., 2010), which play crucial roles in alleviating this inflammation. However, a growing body of evidence clearly demonstrates that these anti-inflammatory proteins become inactive during acute inflammatory conditions; mostly via phosphorylation. Hence understanding their regulation process and underlying molecular mechanisms may provide a therapeutic avenue for an alternative anti-inflammatory strategy with reduced side effects to fight inflammatory diseases. In this study, we mainly focused on some key endogenous anti-inflammatory proteins such as PP2A, TTP and MKP-1 with the hope of combatting asthmatic inflammation via enhancing the power of these anti-inflammatory proteins.

1.9.1 Protein phosphatase 2A (PP2A)

PP2A comprises a family of serine/threonine phosphatases, which have broad substrate specificity and diverse cellular functions (Janssens and Rebollo, 2012). Typically, PP2A is a tri-molecular complex consisting of a catalytic C subunit (PP2A-C) together with a core scaffold structural A subunit (PP2A-A). These interact with a third regulatory B subunit (PP2A-B) to form PP2A (Janssens et al., 2008; Shi, 2009), as shown in Figure 1.6. The structural subunit is required for correct function whereas the catalytic subunit controls phosphatase activity. The regulatory subunit confers substrate specificity via reversible methylation and phosphorylation of the C-terminal tail of the catalytic subunit (Janssens et al., 2008; Shi, 2009).

PP2A is a key eukaryotic phosphatase, regulating a wide variety of cellular functions such as transcription, DNA replication, RNA splicing, metabolism, translation, cell cycle progression,

cell transformation, cell senescence and apoptosis, morphogenesis, development, and neurotransmission (Chen et al., 2009). Moreover, PP2A is a master controller of multiple inflammatory signalling pathways (Lechward et al., 2001; Van Hoof and Goris, 2003). It is an exciting target for anti-cancer therapy (Janssens et al., 2005; Perrotti and Neviani, 2008) and has more recently emerged as a druggable target in respiratory disease (Kobayashi et al., 2011; Collison et al., 2013a; Kobayashi et al., 2013). Importantly, PP2A is also involved in restoring corticosteroid insensitivity (Kobayashi et al., 2013).

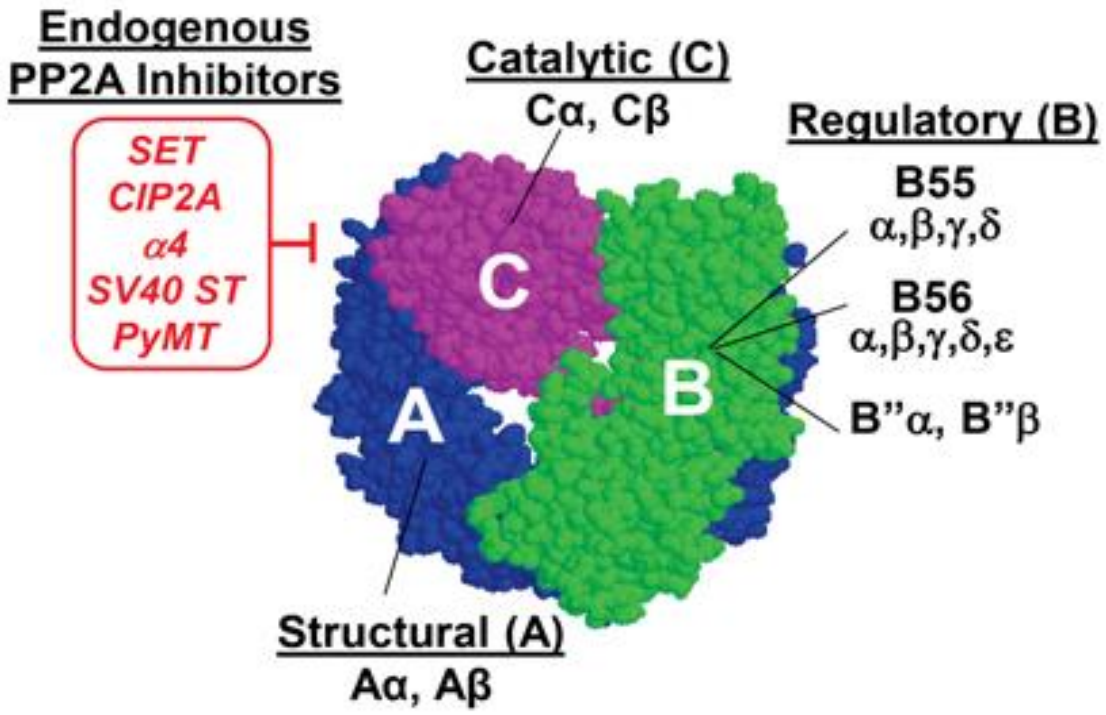


Figure 1.6 Structure and regulation of PP2A (Cho and Xu, 2007)

Despite the exciting potential of PP2A in asthma, research on PP2A has primarily focused on its role in cancer (Janssens et al., 2005; Perrotti and Neviani, 2008), leaving investigations into the

anti-inflammatory function of PP2A in airway disease relatively unexplored. To date, there are only a few studies that have investigated PP2A in airway structural cells in the context of asthmatic inflammation and corticosteroid insensitivity. As it is evident that the catalytic subunit of PP2A plays the key role in cellular functions, in our study we mainly focused on PP2A-C.

1.9.1.1 Regulation of PP2A

PP2A is a multimeric holoenzyme, which is highly regulated at multiple levels (Virshup and Shenolikar, 2009); including transcriptional and post-transcriptional regulation, post-translational modifications and ubiquitin-mediated proteasomal degradation. In addition, PP2A enzymatic activity is regulated by endogenous inhibitory proteins (red: Figure 1.6) and by the nature and identity of the PP2A subunits. The most important regulatory mechanisms modulating cellular functions are phosphorylation and dephosphorylation. More than one third of total cellular protein is regulated by phosphorylation and dephosphorylation (Olsen et al., 2006). In the following section we will briefly discuss the transcriptional and post translational modification of PP2A.

Transcriptional regulation of PP2A: PP2A comprises of a 65 kDa scaffolding protein (known as A or PR65 subunit) and a 36 kDa catalytic subunit or C subunit. Molecular cloning revealed that in mammalian cells, the catalytic subunit has two isoforms (PP2A-Cα and PP2A-Cβ), which share high (97%) sequence similarity and are encoded by the PPP2CA and PPP2CB genes, respectively. These isoforms are localized to human chromosome 5q23±q31 for α and to 8p12±p11.2 for β (Jones et al., 1993). Both isoforms are ubiquitously expressed, but abundantly found in brain and heart. Importantly, PP2A-Cα isoform is more abundant than PP2A-Cβ (Khew-Goodall and Hemmings, 1988). Further, expression from the PP2A-Cα gene promoter is

7±10-fold stronger than from the PP2A-Cβ gene promoter, which may explain the difference in protein levels between the isoforms. The PP2A-C subunit has only two isoforms; however the number of possible combinatorial associations with different A and B regulatory subunits is very large, which provides many possibilities for regulation on PP2A-C. There are two A, two C, four B, at least eight B′, four B″ and two B‴ isoforms, which can be generated a total of about 75 different dimeric and trimeric PP2A holoenzymes.

Structural subunit A is closely associated with catalytic subunit C and forms a scaffold to which regulatory subunit B can bind firmly. Like the catalytic subunit, the structural subunit in mammalia also comprises of two distinct isoforms (α and β), which are ubiquitously expressed and share 86% sequence identity (Hemmings et al., 1990).

Molecular cloning revealed that regulatory subunit B is encoded by four genes in mammalia such as PR55α, PR55β, PR*55γ* and PR55δ. Importantly, these genes are expressed in a tissue specific manner (Healy et al., 1991; Mayer et al., 1991). PR55α and PR55β are widely distributed whereas PR55γ and PR55δ are mainly found in brain (Zolnierowicz et al., 1994). Regulatory subunits comprise five degenerate WD-40 repeats, which are conserved as at least 40 amino acid sequences (Neer et al., 1994).

Post-translational modifications of PP2A: Post-translational modification of PP2A-C includes phosphorylation and methylation, which are thought to be important regulatory devices. Despite remarkable progress in the research of post translational modulation of PP2A-C, many aspects are not clear yet. Moreover there are conflicts in regard to the effect of PP2A-C phosphorylation and methylation on its catalytic activity. Hence post-translational modification of PP2A-C warrant more research for better understanding this event.

Phosphorylation: Wide ranging factors are responsible for the phosphorylation of PP2A-C, such as tyrosine kinases pp60v-src, pp56lck, epidermal growth factor and insulin receptors. Phosphorylation occurs on Tyr^{307} of the conserved C-terminal of PP2A-C and subsequently causes inactivation of the enzyme. The presence of a phosphatase inhibitor such as OA enhances the phosphorylation of PP2A-C, which suggests that under normal conditions PP2A can undergo autophosphorylation to quickly re-activate itself. It is reported that inflammatory stimuli such as IL-1β or TNFα can cause transient tyrosine phosphorylation and inactivation of PP2A-C. Concomitant transient inactivation of PP2A-C can cause acceleration of signals transmission through kinase cascades and hence PP2A-C plays a pivotal role to modulate signal transduction cascades. Regulatory subunit B is also regulated by phosphorylation, especially the B′ family. Studies reveal that the substrate specificity of regulatory subunits alter due to phosphorylation of PR61δ by PKA (Usui et al., 1998).

Methylation: Methylation plays an important role in post translational regulation of PP2A. Methylation occurs on the carboxyl group of the C-terminal residue Leu^{309}. *In vivo* methylation is a reversible process due to the presence of a specific methylesterase. PP2A-C sequences contain a $T^{304}PDYFL^{309}$ motif at their C-terminus. In addition this motif also contains Tyr^{307} and a recognition site (a specific carboxyl methyltransferase) for carboxymethylation (Janssens and Goris, 2001). Studies revealed that OA prevents transferase access to its target site via inhibition of methylation of PP2A (Floer and Stock, 1994; Li and Damuni, 1994). Importantly, methylation of PP2A-C depends on cell type, for example cAMP moderately stimulates PP2A-C methylation in *Xenipus oocyte* extracts; however in pancreatic cells it has no influence (Kowluru et al., 1996) and most interestingly it also varies during the cell cycle.

PP2A activation: A number of small molecules have been reported to activate PP2A, as shown in Table 1.4 (Perrotti and Neviani, 2013). The best known of these is the sphingosine analog, FTY720, which is FDA approved and widely used as an immunomodulating drug for treating multiple sclerosis (MS) (Kappos et al., 2006). However FTY720 has other targets such as PP1. Most notably in the context of respiratory disease, FTY720 can induce S1P signalling. In a previous study we reported that S1P is elevated in BAL fluid of asthmatic subjects (Ammit et al., 2001) and drives development of a pro-inflammatory phenotype, including IL-6 and IL-8 expression *in vitro* (Che et al., 2014). Hence there is a compelling need to develop PP2A activators that are devoid of S1P agonism. Recent study has developed a chiral analog of FTY720 called AAL(S) that does not bind S1P receptors, (Don et al., 2007), and is as effective as FTY720 at activating PP2A complexes *in vitro* and significantly represses airways disease in mouse model *in vivo* (Collison et al., 2013a).

PP2A inhibition: There are a number of natural occurring small molecules that potently inhibit the catalytic activity of certain PPP-family ser/thr protein phosphatises (such as PP1, PP2B, PP5, and PP2A), shown in Table 1.3 (Smallie et al., 2015). Among these small molecules OA is widely used to inhibit PP2A phosphatase activity (Bialojan and Takai, 1988; Mao et al., 2005; Cornell et al., 2009; Kranias et al., 2010), however OA is a non-selective pharmacological inhibitor of PP2A (Swingle et al., 2007; Cornell et al., 2009). In our study we also used OA as a pharmacological inhibitor of PP2A. However, as OA is a non-selective inhibitor of PP2A, to demonstrate that OA-mediated effects are specific to PP2A, we used precise tools like siRNA against PP2A.

Table 1.4 Pharmacological activators and inhibitors of PP2A (McConnell and Wadzinski, 2009)

PP2A activator molecules	PP2A inhibitor molecules
• FTY720 (Fingolimod) or FTY720P • Sphingosine analog such as AAL(S) • Forskolin and 1,9-dideoxy forskolin • SET Binding Peptides such as COG112, COG/OP449 and apoE • Other PP2A activating drugs -promethylating agents like the Chloroethyl Nitrosourea (CENU) -α-tocopheryl succinate (α-TOS) -Vorinostat and sorafenib -Carnosic acid -Methylprednisolone -Ceramide -Exogenous TGFβ	○ Okadaic acid ○ Calyculin A ○ Dragmacidins ○ Microcystins ○ Nodularins ○ Tautomycin ○ Tautomycetin, ○ Cytostatins ○ Phospholine ○ Leustroducsins ○ Phoslactomycins ○ Fostriecin ○ Cantharidin

1.9.1.2 Relationship between PP2A and TTP

Several studies demonstrate that PP2A has a dynamic relationship with TTP (Figure 1.7). Recently we reported that PP2A inhibition in respiratory epithelial cells (A549 and BEAS-2B) has a significant impact on the critical anti-inflammatory protein, TTP (Rahman et al., 2015). TTP is an mRNA destabilizing protein that targets numerous cytokines (Brooks and Blackshear, 2013), including those involved in asthmatic inflammation. Its expression and function are p38 MAPK-dependent (Mahtani et al., 2001; Sun et al., 2007). There is a dynamic equilibrium between unphosphorylated and phosphorylated forms of TTP, shown in Figure 1.7. The

unphosphorylated form of TTP is active and capable of decaying cytokine mRNA. However, this form of TTP is unstable and undergoes proteosomal degradation, whereas phosphorylated TTP is stable but unable to target cytokines mRNA for decay. Importantly, TTP is a direct target of PP2A and PP2A phosphatase activity is responsible for dephosphorylation of TTP at two key serines (Ser^{52} and Ser^{178}) (Brook et al., 2006). In this way, PP2A shifts this balance of TTP towards unphosphorylated TTP via dephosphorylation (Brook et al., 2006; Sun et al., 2007).

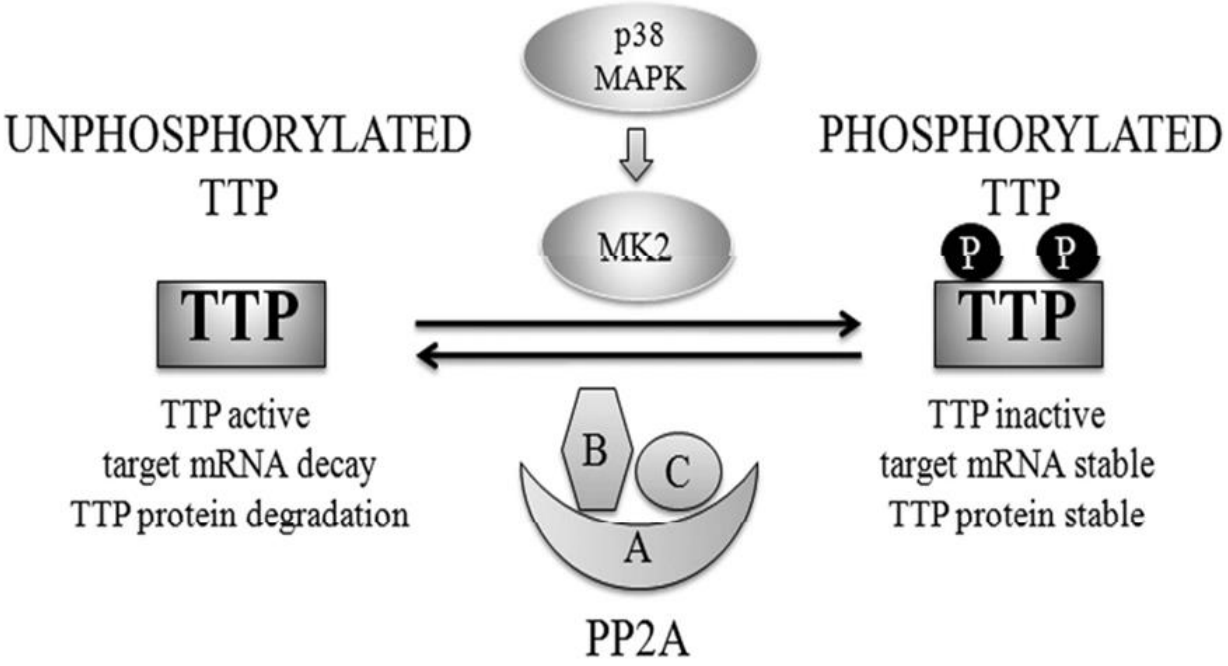


Figure 1.7 Relation between PP2A and TTP

1.8.1.3 Relationship between PP2A, TTP and MAPKs

As mentioned previously, TTP is a critical anti-inflammatory protein and is controlled by MAPKs mediated phosphorylation processes. The unphosphorylated form of TTP is active and capable of decaying cytokines mRNA (Brooks and Blackshear, 2013), which are implicated in

asthmatic inflammation. On the other hand, PP2A dephosphorylates a number of kinases that drive inflammatory cell signaling (Shanley et al., 2001; Junttila et al., 2008). Notably, PP2A can dephosphorylate members of the MAPK superfamily, including p38 MAPK (Junttila et al., 2008). By regulating MAPKs, PP2A exerts significant control over cytokine regulatory networks; although the molecular mechanisms responsible remain relatively unexplored in airway inflammation.

As shown in Figure 1.8, p38 MAPK shifts the TTP balance towards the phosphorylated state (inactive but stable) via phosphorylation of two key serines such as Ser^{52} and Ser^{178}. Interestingly, PP2A shifts this balance towards the unphosphrylated state (active but unstable) via dephosphorylating the same serine sites. Therefore it is evident that MAPKs (especially p38, ERK and JNK mediated) signalling pathways play critical roles in the relationship between PP2A and TTP. Recently we reported that inhibition of basal PP2A activity by OA releases restraint on MAPKs and thereby increase MAPK-mediated pro-asthmatic cytokines, including IL-6 and IL-8 (Rahman et al., 2015).

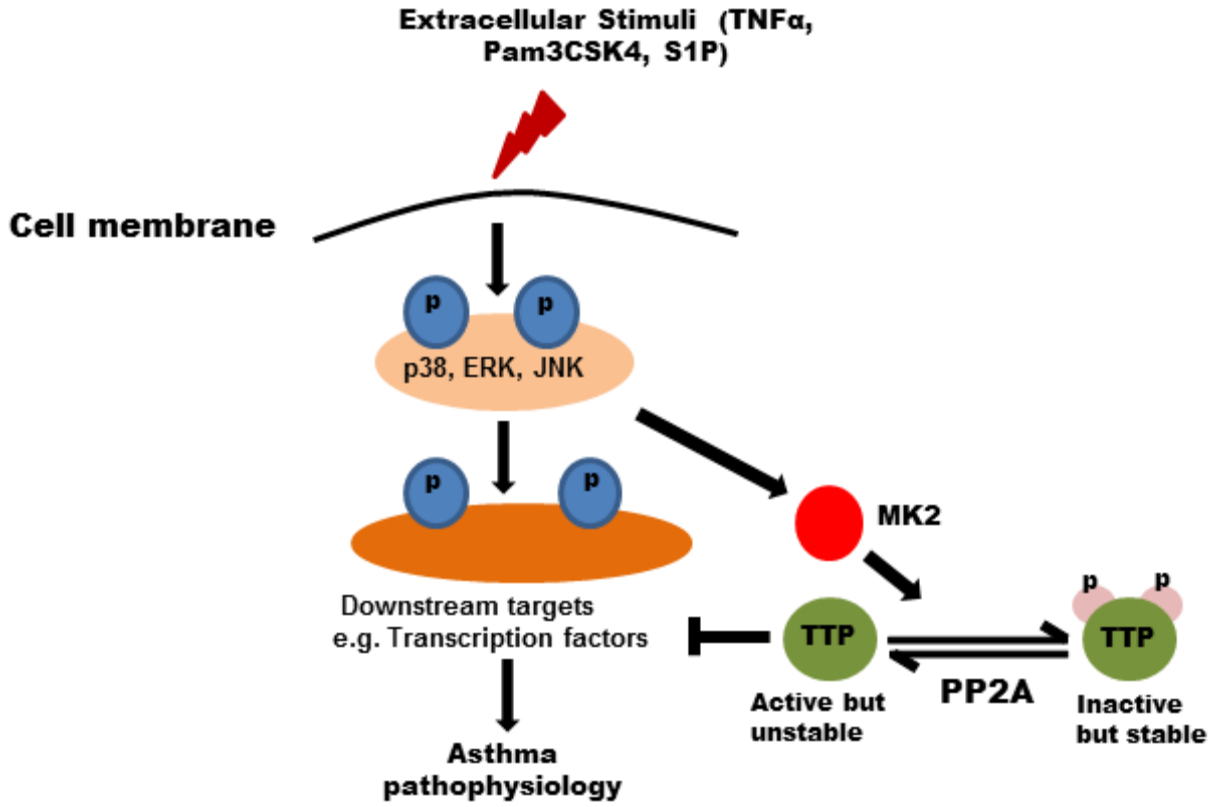


Figure 1.8 Interrelations between PP2A, TTP and MAPKs

1.9.2 TTP

TTP is a critical endogenous anti-inflammatory protein. TTP is also known as zinc finger protein 36 homolog (ZFP36), because it is encoded by the *ZFP36* gene in humans, mice and rats (DuBois et al., 1990). Zinc ion serves to stabilize the integration of the protein itself but not involved in targets binding. The "finger" refers to the secondary structures consisting of α-helix and β-sheet, which are held together by the Zn ion. Zinc finger containing domains typically serve as interactors, binding DNA, RNA, proteins or small molecules (Laity et al., 2001). TTP is a destabilizing RNA binding protein that binds to AU-rich elements within the 3'-untranslated region and increases mRNA decay of inflammatory cytokines such as IL-6, IL-8, IL-2, GM-CSF,

GROα, TNFα and many more. Importantly all these cytokines are implicated in asthmatic inflammation and drive airway remodeling. TTP is largely controlled by phosphorylation. Several studies demonstrate that various inflammatory stimuli mediated MAPKs (especially p38) signaling rapidly and robustly activate TTP gene expression (Tchen et al., 2004; Brook et al., 2006) and at the same time p38 MAPK also activates downstream kinase MK2. Upon activation MK2 phosphorylates TTP protein on two key serine residues (Ser^{52} and Ser^{178}) (Hitti et al., 2006) and thereby prevents the recruitment of the deadenylase complex (Marchese et al., 2010) and stops the initiation of mRNA decay. Therefore, the anti-inflammatory function of the destabilising RNA binding protein TTP is dictated by the phosphorylation status of TTP at Ser^{52} and Ser^{178}.

Taylor *et al* clearly demonstrate the role of TTP as a critical anti-inflammatory protein using knock-in mice (Taylor et al., 1996). TTP deficient mice appear normal at birth, but develop a profound inflammatory phenotype with severe arthritis, cachexia and autoimmunity, resulting from increased mRNA stability of the cytokine TNFα, and excessive levels of circulating TNFα protein (Carrick et al., 2004). Several studies demonstrate that in addition to TNFα, TTP also targets many other clinically relevant cytokines, which are implicated in chronic inflammatory disease (Lai et al., 2006; Tudor et al., 2009). 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. Moreover TTP has dynamic relation with PP2A as described in relevant section. Hence in this study we aim to explore the molecular mechanisms of TTP anti-inflammatory functions in the context of asthmatic inflammation and eventually find out the strategy to enhance its anti-inflammatory function during asthmatic inflammation.

1.9.3 MAPK phosphatase 1 (MKP-1)

Cellular functions are greatly affected by the strength and duration of MAPKs activation. Therefore, controlling functional outcomes of MAPKs is very important, and can be achieved by balancing the MAPK activation by diverse environmental and chemical stimuli and the negative feedback mechanism mediated by protein phosphatases such as MAPK phosphatases (MKPs) (Owens and Keyse, 2007). MKPs are classified into three major groups depending on the preference of the relative MKPs to dephosphorylate tyrosine, serine/threonine, or both the tyrosine and threonine residues in the activation loop "Thr-X- Tyr-motif" of these signalling enzymes. As these phosphatases dephosphorylate both the threonine and the tyrosine residues, they are also called dual-specificity phosphatases (DUSPs). To date there have been at least 10 DUSP genes identified in the human and other mammalian genomes (Theodosiou and Ashworth, 2002; Jeffrey et al., 2007). Though MKPs vary in substrate specificity, subcellular localization tissue distribution and also in their response to various stimuli, all of them contain highly conserved typical structural elements (Lang et al., 2006). MKPs comprise a subfamily of 10 catalytically active proteins with a common structure that consists of an N-terminal non-catalytic domain and a C-terminal catalytic domain. The kinase interaction motif (KIM), situated in the N-terminal half, is very important for the quality of the interaction between MKPs and MAPKs and, thereby, plays a crucial role in the regulation of the enzymatic specificity (Farooq and Zhou, 2004; Kondoh and Nishida, 2007).

MKP-1 is a 367 amino acid protein expressed by an immediate early gene located in the nuclear compartment. As it is located in the nuclear compartment, upon activation with diverse stimuli it can induce transcription instantly. MKPs share a number of stimuli with MAPKs (Keyse, 2000; Wong et al., 2005; Kondoh and Nishida, 2007); hence MKP-acts on MAPKs via a negative

feedback mechanism to control the strength and duration of MAPKs-mediated signalling. Diverse stimuli and pro- inflammatory mediators cause activation of MAPKs; thus they play a significant regulatory role in inflammatory and innate immune responses (Abraham and Clark, 2006), and subsequently in inflammatory airway diseases, such as asthma. MKP-1 is a promising target to attenuate the inflammatory responses mediated by MAPK activation. The inhibitory effect of MKP-1 on p38 MAPK, JNK and ERK (p38>JNK>>ERK (Franklin and Kraft, 1997)) activation as well as the modulation of pro-inflammatory processes indicate the importance of MKP-1 in these cellular pathways.

As MKP-1 regulates pro-remodelling signal transduction via negative feedback mechanisms, exploring the underlying molecular mechanisms of MKP-1 in the context of asthmatic inflammation and airway remodelling may be an alternative and novel anti-inflammatory strategy. Recent studies demonstrate that exogenous stimuli especially ROS and infection (both bacterial and viral) cause MKP-1 inactive via oxidation and thereby develop resistance or corticosteroid insensitivity in asthmatic patients (Aeberli et al., 2006; Cao et al., 2008; Wancket et al., 2012). In addition, MAPKs mediated pro-inflammatory signaling pathways has a profound impact on asthma pathogenesis and MKP-1 inhibits these MAPKs mediated pro-remodeling functions; however the underlying molecular mechanisms are still unclear.

In this study we aim to investigate the molecular mechanisms that mimic bacterial infection (Pam3CSK4) induced corticosteroid insensitivity taking MKP-1 oxidation into consideration. At the same time we will also check whether PP2A activators can bypass corticosteroid sensitivity, which is hindered by Pam3CSK4 in asthmatic inflammation. We expect that our work may reveal the mechanistic basis of corticosteroid insensitivity and explore novel alternative anti-inflammatory strategies for treating and preventing airway inflammation.

1.10 Corticosteroid insensitivity

Corticosteroids are the mainstay of anti-inflammatory therapy in respiratory disease. Although corticosteroids (glucocorticoids) have proven clinical efficacy in the treatment of chronic inflammatory diseases, such as asthma, some patients show poor response either due to an inherent difference in the glucocorticoid sensitivity as part of the disease process or due to exogenous stressors such as cigarette smoke and other oxidative insults. Hence increasing knowledge in relation to the underlying molecular mechanisms of corticosteroid insensitivity in respiratory diseases is crucial to achieve future therapeutic targets.

Based on research to date we know that relative responsiveness to corticosteroids depends on exogenous (such as cigarette smoking, obesity and even social stress) or endogenous (genomic difference) factors (Hew and Chung, 2010; Marwick and Chung, 2010; Keenan et al., 2012). Most importantly these factors may interfere with the components of the corticosteroids' anti-inflammatory pathways and modify their functions, as shown in Figure 1.9. That's why more research is warranted to wholly understand the mechanistic basis of corticosteroids' insensitivity and to identify the effective therapeutic target to restore their responsiveness or develop an alternative anti-inflammatory strategy to bypass corticosteroid insensitivity.

Viral and bacterial infections are the most common modulators of respiratory inflammation that eventually lead to asthma exacerbation via interfering with our innate immune system. Importantly, this infection associated asthma exacerbation is controlled by various pattern recognition receptors including TLRs. To date TLR2 is well studied and it is evident that TLR2 plays key role in innate immunity (Takeda and Akira, 2005) and evokes inflammatory responses in recognition of bacterial and viral components (Mogensen, 2009).

Pam3CSK4, a synthetic bacterial lipoprotein, has been widely used to mimic bacterial infections (gram-positive bacteria) to mediate inflammatory responses via TLR2 (Beckett et al., 2012; Manetsch et al., 2012c). In our study, we modeled bacterial exacerbation *in vitro* using the synthetic bacterial lipoprotein Pam3CSK4 to mimic bacterial infection and TNFα to simulate inflammation and explore their role in corticosteroid (particularly dexamethasone) insensitivity. Compiling evidence from recent studies show that both Pam3CSK4 and TNFα increase IL-6, IL-8 and exotoxin secretion via TLR2 pathway (Tliba and Panettieri, 2009). Hence in our study we measured the relative secretion of IL-6 and IL-8 using the same inflammatory stimuli.

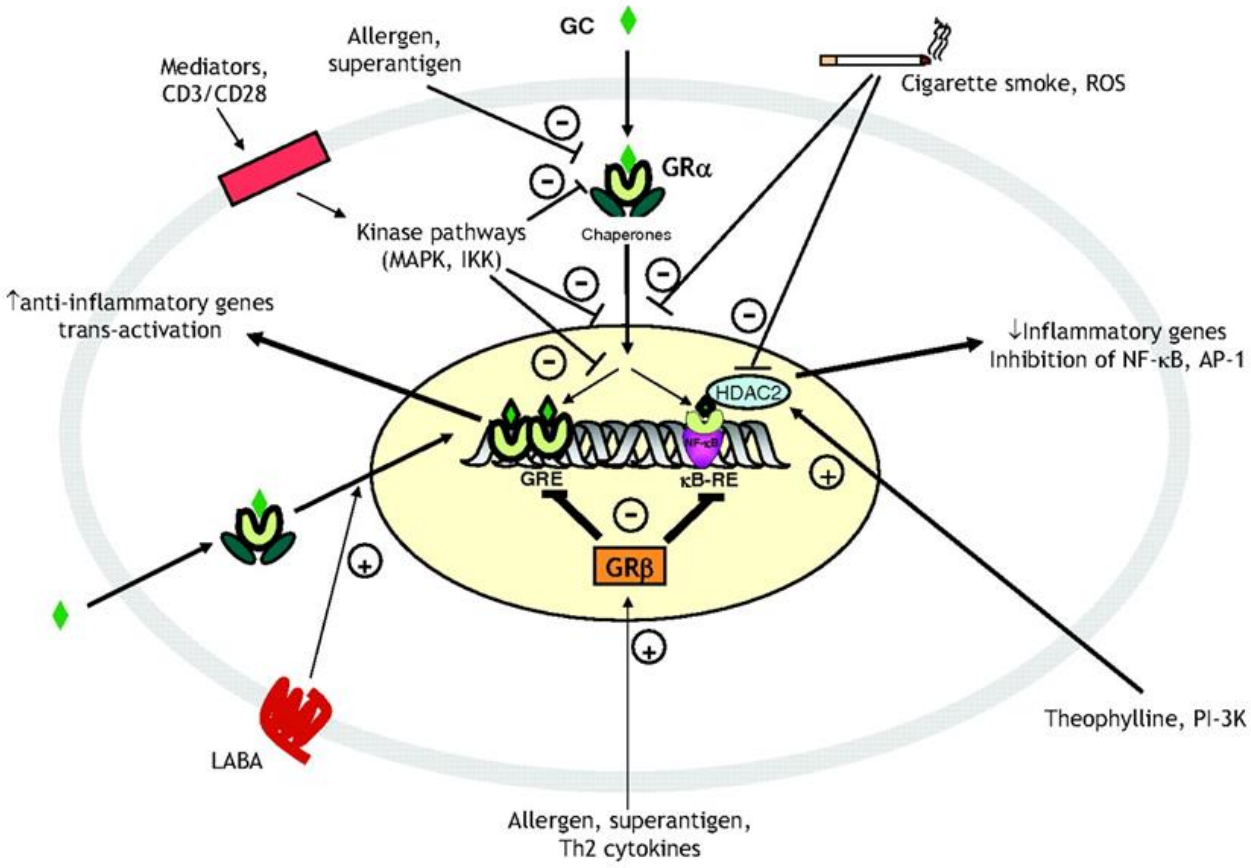


Figure 1.9 Mechanisms of glucocorticosteroid (GC) insensitivity (Petsky et al., 2012)

Pharmacological approaches to restore corticosteroid sensitivity have revealed that there are three principle mechanisms involved in this phenomenon. These are: glucocorticoid receptor (GR) post-translational modification, GR-mediated recruitment of transcriptional co-repressors and post-transcriptional regulation (Ammit, 2013). Recent evidence demonstrates that post-translational modifications of GR have a functional role in glucocorticoid responsiveness. GR isoforms have a number of key sites for post-translational modification (including sumoylation, ubiquitylation, acetylation and phosphorylation) that can affect their translocation, transcriptional activity and degradation and thus eventually their function (Galliher-Beckley and Cidlowski, 2009).

To date serine (Ser) phosphorylation is well studied in the context of GR function in respiratory disease, which eventually identified two critical phosphorylation sites (Ser^{211} and Ser^{226}) for GR transcriptional activity. Most importantly, studies have also revealed that these sites can be regulated via dephosphorylation by Ser/threonine protein phosphatases 2A and 5 (PP2A and PP5), which have emerged as exciting mechanisms responsible for glucocorticoid insensitivity.

Another promising approach to bypass corticosteroid sensitivity is activation of MKP-1. Several studies demonstrate that exogenous stimuli, especially ROS and infection (both bacterial and viral), cause MKP-1 inactivation via oxidation and thereby develop resistance or corticosteroid insensitivity in asthmatics (Aeberli et al., 2006; Cao et al., 2008; Wancket et al., 2012). Hence, MKP-1 is also an exciting target in corticosteroid insensitivity.

In our present study, we therefore aim to unveil the fascinating role of endogenous PP2A and its activators (FTY720 and AAL(S)) to pinpoint their role in glucocorticoid insensitivity as a source of future alternative anti-inflammatory strategy in asthma and COPD. We also aim to explore the

role of a critical endogenous anti-inflammatory protein (MKP-1) in corticosteroid insensitivity to unveil the therapeutic avenue for alternative anti-inflammatory strategies.

1.11 Targeting PP2A in corticosteroid insensitivity

PP2A dephosphorylates a number of kinases that drive inflammatory cell signalling (Shanley et al., 2001; Junttila et al., 2008). Thus its inhibition allows unrestrained action of a number of downstream effectors. MAPKs family members (p38 MAPK, ERK and JNK) are important regulators of cytokine expression and are known to drive expression of two important cytokines implicated in asthmatic inflammation, IL-6 and IL-8 (Amrani et al., 2001; Henness et al., 2006; Quante et al., 2008; Moutzouris et al., 2010). By regulating MAPKs, PP2A exerts significant control over cytokine regulatory networks. Few recent studies support the role of PP2A in respiratory disease and demonstrate the promise of PP2A as a druggable target. It is reported that activating PP2A represses the hallmark of allergic and rhinovirus-induced airway disease in *in vivo* models (Collison et al., 2013a). Kobayashi *et al* (Kobayashi et al., 2011) has shown that defects in PP2A cause corticosteroid insensitivity in severe asthma and activation of PP2A can repress expression of pro-inflammatory chemokines, the neutrophil chemoattractant IL-8 in particular (Kobayashi et al., 2012). Intriguingly, PP2A activity was increased in the lungs of people with emphysema and in response to cigarette smoke exposure in mice (Wallace et al., 2012). This was considered evidence of a homeostatic mechanism that serves to restrain or resolve oxidative lung injury. This was supported by a study showing that intra-tracheal transfection of PP2A prevented smoke-induced lung inflammation *in vivo* and knock-down of PP2A exacerbated smoke-mediated responses *in vitro* (Wallace et al., 2012). These studies serve to underscore the key role played by PP2A in the control of inflammation in respiratory disease,

and in corticosteroid responsiveness. Thus, modulating PP2A has the potential to treat asthmatic inflammation and bypass corticosteroid sensitivity. We examined this in this study and demonstrated that enhancing the power of endogenous PP2A activity can repress MAPKs mediated inflammatory cytokines and increase active TTP level; demonstrating an alternative anti-inflammatory strategy in corticosteroid sensitivity in airway structural cells.

1.12 Targeting MKP-1 in corticosteroid insensitivity

MKP-1 is an important anti-inflammatory protein that plays a vital role in limiting the strength and duration of MAPK signalling pathways (Manetsch et al., 2012a). A growing body of evidence demonstrates that glucorticoids-induced MKP-1 is partially responsible for anti-inflammatory functions in clinically relevant airway cell types (Kang et al., 2008; Kaur et al., 2008; Quante et al., 2008; Newton et al., 2010). Though several studies have shown that long-acting β2-agonists alone and in combination with corticosteroid can increase both MKP-1 mRNA and protein and limit inflammatory gene expression in asthma (Kaur et al., 2008; Newton et al., 2010), MKP-1 function is dysregulated in asthma. In addition it is reported that ROS and infection can cause dephosphorylation/inactivation of one important anti-inflammatory protein, MKP-1, which is associated with corticosteroid insensitivity (Sakai et al., 2004; Wancket et al., 2012; Pinart et al., 2014). Therefore, in this study we aim to explore whether Pam3CSK4 is able to induce MKP-1 oxidation and subsequent corticosteroid insensitivity in respiratory structural cells.

1.13 Aim of this study

Asthma is a clinically and socioeconomically significant chronic airway disease driven by inflammation. Inflammation plays multifaceted roles in asthma pathogenesis and exacerbation. Therefore anti-inflammatory strategies are still considered the most effective approach to combat asthmatic inflammation as well as asthma management. Corticosteroids are the mainstay of anti-inflammatory therapy in respiratory disease and although they have proven clinical efficacy in asthma, many asthmatic inflammatory conditions (e.g. infection, exacerbation) are not responsive to them. Moreover, typical asthma management insists on long term use of corticosteroids, which can often lead to severe side effects. Therefore, alternative anti-inflammatory strategies are urgently needed and enhancing the function of endogenous phosphatases, especially PP2A, offers great promise.

The overall aim of this study is to explore the underlying molecular mechanisms and reveal the potential of endogenous PP2A to combat asthmatic inflammation using airway structural cells. It is hoped that our present study will offer better understanding about the endogenous PP2A in the context of its relationship with TTP and MAPKs and a therapeutic avenue of alternative anti- inflammatory strategy. We addressed our aim in the following projects:

(1) Sphingosine 1-Phosphate Induces Neutrophil Chemoattractant IL-8: Repression by Steroids

In searching for alternative anti-inflammatory strategies, we initially aimed to confirm the role of one important imflammatory mediator, bioactive sphingolipid S1P, in asthmatic inflammation. Several studies demonstrate an increase in the amount of S1P in the airways of asthmatics. It is also reported that S1P can regulate airway smooth muscle functions associated with asthmatic

inflammation and remodeling, including cytokine secretion. To date, however, whether or not S1P induces secretion of an important chemokine responsible for neutrophilia in airway inflammation IL-8 remained unexplored. In this project we explored this and found that S1P may contribute to the development of IL-8-driven neutrophilia in airway inflammation.

(II) Basal protein phosphatase 2A activity restrains cytokine expression: role for MAPKs and tristetraprolin

Next, we proposed to reveal the potential of basal endogenous phosphatase, PP2A as a potential target of an alternative anti-inflammatory strategy. PP2A is a master controller of multiple inflammatory signalling pathways driven by MAPKs. It is a target in asthma; however the molecular mechanism by which PP2A controls airway inflammation is yet unclear. A growing body of evidence support that PP2A has important role in respiratory disease and demonstrate the promise of PP2A as a druggable target. Despite the exciting potential of PP2A in asthma treatment, research into PP2A has primarily focused on its role in cancer (Janssens et al., 2005; Perrotti and Neviani, 2008), leaving investigations into the anti-inflammatory function of PP2A in airway disease relatively unexplored. We addressed this issue in this study.

(III) Activating Protein Phosphatase 2A (PP2A) Enhances Tristetraprolin (TTP) Anti-Inflammatory Function in A549 Lung Epithelial Cells

In aim (II) we described the anti-inflammatory role of basal PP2A and highlighted its potential link with the critical anti-inflammatory protein TTP. As our next target we will therefore explore the interrelationship between PP2A and TTP. TTP function induces mRNA decay of cytokines frequently overexpressed in inflammation; including cytokines that have been implicated in

asthma. TTP is controlled by phosphorylation and in the unphosphorylated state is capable of inducing mRNA decay of cytokines. It is evident that PP2A has a dynamic relation with TTP. However the underlying molecular mechanisms and interrelationship between the anti-inflammatory function of PP2A and TTP dephosphorylation remains unclear. We have addressed this in this project.

(IV) TLR2 ligation induces corticosteroid insensitivity in A549 lung epithelial cells: anti-inflammatory impact of PP2A activators

Our studies successfully demonstrate that PP2A activators offer a great promise as alternative anti-inflammatory strategies in asthmatic inflammation. Though corticosteroids have proven efficacy in asthmatic inflammation, bacterial and viral infection mediated asthma exacerbation is insensitive to corticosteroids. Hence the aim was to check whether alternative anti-inflammatory molecules/PP2A activators known (FTY720) and novel (Theophylline) are effective when corticosteroid insensitivity exists. In this section we also aimed to explore the underlying molecular mechanisms of mimic bacterial infection, Pam3CSK4-induced corticosteroid insensitivity.

Chapter 2
General Materials and Methods

Chapter 2
General Materials and Methods

2.1 Materials

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

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Solutions

Cell culture/feeding media for the culture of primary ASM cells is composed of Dulbecco's Modified Eagle's Medium (DMEM) with phenol red, enriched with 200 mM L-glutamine, 1 M HEPES (*4-(2-hydroxyethyl)-1- piperazineethanesulfonic acid*), antibiotic-antimycotic liquid (containing 10,000 units/mL 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 consists of 0.1 % (v/v) BSA (30% w/v solution, sterile) in DMEM.

Whereas, airway epithelial cells, A549 (originated from cancerous lung) and BEAS2B (originated from normal lung) were cultured in Ham's 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%.

2.2.1.2 Culture of primary human ASM cells

Human bronchi from the large bronchial airways were obtained from patients undergoing surgical resection for carcinoma or lung transplant donors in accordance with procedures approved by the Central Sydney Area Health Service and the Human Ethics Committee of the University of Sydney. Primary ASM cells were dissected, purified and cultured as previously described by Johnson *et al.* (Johnson et al., 1995). Specific to our settings, after dissection of the bronchi from the surrounding tissue and removing of the epithelium layer individual ASM tissue bundles were plucked out and placed into 25cm^2 tissue culture flask (Sarstedt, Nümbrecht Germany) with DMEM containing phenol red and supplemented with 2 mM L-Glutamine, 20 mM HEPES, 10% heat inactivated FBS and 2% Antibiotic-Antimycotic mix (200 U/L Penicillin G, 0.5 µg/mL Amphotericin B and 200 µg/mL Streptomycin sulphate). The flasks were then placed in a 5% CO_2 humidified incubator at 37°C. Cells grew to confluence over 14-21 days and were passaged when confluent.

2.2.1.3 Passaging ASM cells

Before trypsinizing the cells (2 min at 37°C) the media was aspirated and the cells were washed two times with sterile PBS (Ca^{2+}/Mg^{2+}-free) to remove all traces of FBS. Cell passaging was completed using a trypsin solution (0.5 g/l porcine trypsin and 0.2 g/l EDTA in Hank's balanced salt solution with phenol red). The cell attachment was checked microscopically (Olympus CKX31, Olympus, Center Valley, PA) and if necessary the detachment was facilitated by agitation. After detachment, feeding media was added to inhibit further tryptic activity. Cell numbers were assessed on a haemocytometer using trypan blue solution. Cells were plated at a density of 1x10^6 cells/T175 flask (Sarstedt, Nümbrecht, Germany) placed at 5% CO_2 in

air at 37°C, media change every week and passage into further T175 flasks (split 1:3) every 2 weeks, depending on cell need. For all experiments in this thesis, smooth muscle cells were used between passage 4 and 8. Primary ASM cells tipically use maximum passage number 8 to 10.

2.2.1.4 Passaging A549 and BEAS2B cells

Before trypsinizing the cells (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. Cell passaging was completed using a trypsin solution (0.5 g/l porcine trypsin and 0.2 g/l EDTA in Hank's balanced salt solution with phenol red). The cell attachment was checked microscopically (Olympus CKX31, Olympus, Center Valley, PA) and if necessary the detachment was facilitated by agitation. After detachment, feeding media was added to inhibit further tryptic activity. Cells were splited 1/3 two times per week (e.g. Monday and Thursday or Tuesday and Friday) and placed at 5% CO_2 in air at 37°C.

2.2.1.5 Plating and starvation 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 commonly grown for one week. Feeding media was changed approximately 72 hours 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 starving media was added for 48 h.

Table 2.1 Guidelines for plating of ASM cells for experiments

TYPE OF PLATE	PLATING DENSITY	MEDIA PER WELL
6-well	96,000 cells/well	2 mL
100 mm	785,000 cells/well	10 mL

2.2.1.6 Plating and starvation A549 and BEAS2B cells

Cells were plated at a density of 250,000/ml so that when 2 mL is added to 6 well dishes 500,000 cells/well is achieved according to the guidelines in Table 2.2. Commonly cells were allowed to settle for 4-6 h and growth arrested before performing the experiments. The feeding media was aspirated, the cells were washed twice with sterile PBS, and then starving media was added for 14-16 h.

Table 2.2 Guidelines for plating of A549 and BEAS2B cells for experiments

TYPE OF PLATE	PLATING DENSITY	MEDIA PER WELL
6-well	500,000 cells/well	2 mL
100 mm	2500,000 cells/well	10 mL

2.2.1.7 ASM cell characterization

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

2.2.2 Western blotting

2.2.2.1 Solutions

5x Cell lysis/sample buffer is composed of TrisHCl 250 mM, DTT 500 mM, SDS 10% (w/v), bromophenol blue 0.5% (w/v), glycerol 50%, pH 6.8 and was used to collect cell lysates. The separating gel (8% if not otherwise specified) consisted of a 30% acrylamide- 0.8% bisacrylamide- solution, TRIS (pH 8.8).

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

2.2.2.2 Sample preparation and immunoblotting

According to cell needs and experimental plans, ASM cells were plated in petridishes, 6 well plates (BD Bioscience, Franklin Lakes, NJ) and cultured until they reached confluence. After growth arresting for 48 hours, cells were treated according to the experimental plans as indicated in the respective Chapters. Cells were then washed two times with ice-cold PBS, 1x sample buffer was added and after scraping, the lysates were collected in eppendorf tubes and boiled

for 5 min. To fractionize the samples by size and to detect specific proteins of interest, a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed. The SDS-PAGE was run at 200 V constant using the Power Pac 200 (Bio-Rad, Hercules, CA) until the bromophenol blue in the sample buffer completely reached the end of the gel. Proteins were then transferred onto a nitrocellulose membrane (Pall Corporation, Port Washington, NY) by electrotransfer (100 V constant on the Power Pac 200, 1 hour). To check the uniformity and the overall effectiveness of the protein the membranes were stained with Ponceau. After blocking the membranes for 1 hour at room temperature with blocking buffer on the rocking platform mixer (Ratek Instruments, Boronia, VIC, Australia) and subsequent washing with TBS-T the membranes were incubated with the primary antibody specific to the target protein (see Table 2.2). After 3 other wash steps, primary antibodies were detected with goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (see Table 2.2) and visualized by enhanced chemiluminescence (PerkinElmer, Wellesley, MA). The blots were exposed to a medical film (Fujifilm, Tokyo, Japan) and then developed with an x-ray processor (SRX-101A, Konica, Tokyo, Japan).

Table 2.3 Primary and secondary antibodies used for Immunoblotting

	Buffer	**Incubation**	**Company**	**C/N**	**Dilution**
Primary antibody					
TTP	Blocking buffer	Over night	Andy Clark	Sak21	1:1000
Phospho-p38	Primary antibody dilution buffer	Over night	Cell signaling	9211	1:1000
Total p38	Primary antibody dilution buffer	Over night	Cell signaling	9212	1:1000
Phospho-ERK	Primary antibody dilution buffer	Over night	Cell signaling	9101	1:1000
Total ERK	Primary antibody dilution buffer	Over night	Cell signaling	9102	1:1000
Phospho-JNK	Primary antibody dilution buffer	Over night	Cell signaling	9251	1:1000
Total JNK	Blocking buffer	Over night	Cell signaling	9258	1:1000
MKP-1	Blocking buffer	Over night	SantaCruz Biotechnology	G3013	1:50
I2PP2A	Blocking buffer	Over night	SantaCruz Biotechnology	Sc-25564	1:1000
PP2A-C	Blocking buffer	1 h RT	Millipore	05-421	1µg/10 ml
GRα	Blocking buffer	Over night	Abcam	Ab53692	1:1000
α-Tubulin	Blocking buffer	30 min RT	Santacruz Biotechnology	sc-32293	1:10000
Lamin B	Blocking buffer	1 h RT	Cell signaling	2031	1:10000
Secondary antibody					
Anti-mouse IgG	Blocking buffer	1 h RT	Cell signaling	7076	1:10000
Anti-rabbit IgG	Blocking buffer	1 h RT	Cell signaling	7074	1:10000

2.2.3 Nuclear and Cytoplasmic protein extraction

2.2.3.1 Cell culture and sample collection

To prepare highly enriched fractions of cytoplasmic and nuclear proteins from cultured cells for subsequent Western blot studies, cytoplasmic and nuclear protein extraction was performed using the NE-PER nuclear and cytosolic extraction reagents from Pierce (Thermo Fischer Scientific Rockford, IL).

A549 cells were cultured in 100 mm petri dishes according to the standard protocol described earlier and then growth-arrested 14-16 hours prior to the planned experiment. After aspirating the feeding media the cells were washed two times with PBS and Trypsin-EDTA was added to the petri dishes to detach and harvest the cells. After incubation for approximately 2 min at 37°C, the cell attachment was checked microscopically and if necessary the detachment was facilitated by agitation. Feeding media was added to inactivate the trypsin and the cell suspensions were transferred to a 1.5 mL microcentrifuge tube. After centrifugation for 5 min at 1100 rpm the cell pellet was washed with PBS and another centrifugation step (5 min at 1100 rpm) was performed.

2.2.3.2 Cytoplasmic and nuclear protein extraction

To avoid dilution of the CER I (Cytoplasmic Extraction Reagent I) lysis buffer, and therefore a decrease in cell lysis efficiency, all PBS had to be removed. Protease inhibitor cocktail (provided with the kit) had to be added to CER I and NER (Nuclear Extraction Reagent) before use to maintain integrity and function of the extracted proteins.

For the extraction, ice-cold CER I had to be added to the cell pellet. After vortexing the pellet

on the highest setting for 15 sec, the cell suspension had to be incubated on ice for 10 min. In a second step CER II (Cytoplasmic Extraction Reagent II) was added to the tube and after vortexing on the highest setting for 5 sec, the tubes were incubated on ice for 1 min. Following vortexing the tubes were centrifuged for 5 min at maximum speed (4°C, 13,000 rpm). The supernatants (cytoplasmic fraction) were transferred to pre-chilled tubes and placed on ice until use or stored at -80°C.

To get the nuclear fraction the remaining the cell pellet (insoluble fraction that contains the nuclei) was washed with PBS and centrifuged for 5 min at maximum speed (4°C, 13,000 rpm). After removing all PBS ice-cold NER was added to the tubes. Following vortexing on the highest settings for 15 sec the tubes were placed on ice for 10 min and for a total of 40 min the vortexing was continued for 15 sec every 10 min. After subsequent centrifugation at maximum speed (4°C, 13,000 rpm) for 10 min the supernatants containing the nuclear extracts were transferred to pre-chilled tubes and placed on ice until use or stored at -80°C.

2.2.3.3 Western blot preparation

Before analysing the samples using Western blotting, equal amounts of cell sample buffer had to be added to the cytosolic and nuclear fraction and the samples had to be boiled for 5 min. Western blotting was performed as described in Chapter 2.2.2 but to check equal loading the membranes were probed with Lamin A/C for the nuclear and α-tubulin (see Table 2.2) for the cytosolic fraction. α-tubulin should not give a signal for nuclear protein samples to ensure the nuclear fraction is not contaminated with the cytosolic fraction.

2.2.4 RT-PCR

2.2.4.1 RNA sample collection and RNA extraction

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

µL of RNA loading buffer) were run on a 1% agarose/TAE gel in 1x TAE (Tris-acetate-EDTA) at 100 V for about 30 min. The RNA integrity was assessed using the denaturing agarose gel electrophoresis method followed by ethidium bromide staining. Assessing the gels on the transilluminator, both 18S and 28S rRNA appeared as sharp, distinct bands after electrophoresis of total eukaryotic RNA and therefore the RNA was considered to be intact.

2.2.4.2 Reverse transcription and polymerase chain reaction

After this control step reverse transcription was performed to prepare single- stranded DNA from the RNA samples using the Fermentas RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Thermo Scientific, Burlington, Canada). To avoid any contamination and to ensure the preparation of a high- quality complementary DNA (cDNA) all tubes used were autoclaved and only filter tips were used for the preparation of the samples. All the following reactions were prepared on ice. The water/primer master mix, containing Random Hexamer Primer and bottled water (Livingstone International, Rosebery, NSW, Australia), was prepared and aliquoted to the appropriate number of autoclaved, thin-walled 200 µl PCR tubes. Then the extracted RNA samples were added to each PCR tube. The tubes were placed into the PCR machine (MJ Research, PTC-200 Thermal Cycler, GMI inc, Ramsey, Mn) and heated at 70°C for 5 min for denaturation and to provide the right annealing conditions for the primers. After that step the tubes were placed on ice and reaction buffer, RNase inhibitor and dNTPs were added to the samples and the tubes were heated at 25°C for 5 min in the PCR machine. After adding the reverse transcriptase (RevertAid™ M-MuLV Reverse Transcriptase, Fermentas, Thermo Scientific, Burlington, Canada) to each tube the rest of the program was run to obtain the cDNA. The conditions were the following: 25°C for 10 min then 42°C for 60 min, 70°C for 10 min,

and then the samples were cooled to 4°C and the cDNA was collected. Subsequent mRNA expression of the target genes was quantified by real-time RT-PCR using an ABI Prism 7500 real-time PCR machine (Applied Biosystems, Foster City, CA) and the appropriate primer set (Assays on Demand; Applied Biosystems, Foster City, CA). The samples were multiplexed with a eukaryotic 18S rRNA endogenous control probe (Applied Biosystems, Foster City, CA) and subjected to the following cycle parameters: 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.

2.2.5 Enzyme-linked immunosorbent assay (ELISA)

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

To perform an ELISA a 96-well ELISA plate (NUNC Maxisorp Immuno Plate F96; Noble Park North, VIC, Australia) was coated with a capture antibody diluted in coating buffer (0.1 M sodium carbonate, pH 9.5) specific for the target protein. For all recommended antibody dilutions in, the manufacturers' lot-specific Instruction/Analysis Certificate was consulted. The plate was sealed and incubated overnight at 4°C. The wells were washed 3 times with wash buffer (PBS with 0.05% Tween-20) to wash off possible unbound capture antibody. Subsequently the plate was blocked with assay diluent (PBS with 10% FBS, pH 7.0) and incubated for 1 h at RT to block all unbound sites and therefore to prevent false positive results. After 3 other wash steps the standards and cell supernatant dilutions (prepared in 0.1% BSA in DMEM) were added as set out in the experimental protocol, the

plate was sealed and incubated for 2 h at RT. During this incubation the protein of interest binds to the capture antibody. Following 5 other wash steps a biotinylated detection antibody together with a streptavidin-horseradish peroxidase conjugates (SAv- HRP) in assay diluent was added, in 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 streptavidin-horse radish peroxydase (HRP), bound to the detection antibody, converts the added substrate in to a coloured product and the colour is proportional to the amount of bound protein. To stop this process an acidic stop solution was added to the wells and the colour was measured using a spectrometer at 450 nm wave length (Microplate Reader, Model 680, Bio-rad, Hercules, CA).

2.2.6 Transient transfection

A549 cells (5×10^5 cells/well) were transfected with 1 µg of pEGFP HA-PP2A-C (PP2A-C WT) or pEGFP-TTP-S52A/S178A (TTP knock-in) or pEGFP-TTP (TTP WT) or empty vector control, using Lipofectamine 2000 (Invitrogen). After transfection, cells were cultured for 24 h in media without antibiotics, and then growth-arrested for 16 h in Ham's F-12K supplemented with 0.1% BSA, supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), before cells were assayed.

2.2.7 siRNA transfection

A549 cells (5×10^5 cells/well) were transfected with siRNA against PP2A-C or TTP, or scrambled control, by reverse transfection with RNAiMAX according to the manufacturer's protocols (Invitrogen, NY, USA). Specifically, for each well of 6-well plates, 800 ng of ON-Target plus Control Non-targeting siRNA (scrambled control) or ON-target plus SMART pool Human PPP2CA siRNA (aka PP2A-C, both from Dharmacon, Thermo-Fisher Scientific, Waltham, MA) or ON-target plus SMART pool Human TTP siRNA (aka Zfp36: both from Dharmacon, Thermo-Fisher Scientific, Waltham, MA) were diluted in 500 μL of Opti-MEM Reduced Serum Media (Invitrogen). This was followed by the addition of 5 μL of RNAiMAX reagent (Invitrogen) into each well and incubation at room temperature for 20 min. After transfection, cells were cultured for 24 h in media without antibiotics, and then growth-arrested for 16 h in Ham's F-12K supplemented with 0.1% BSA, supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), before stimulation with TNFα (4 ng/ml).

2.2.8 PP2A activity assay

PP2A activity was determined using the PP2A immunoprecipitation phosphatase assay kit (Merck Millipore, Darmstadt, Germany) according to the manufacturer's instructions. In brief, whole-cell lysates were prepared in 20 mM imidazole-HCl, 2 mM EDTA, 2 mM EGTA pH 7.0, protease inhibitor cocktail (10 μl/ml) and 1 mM PMSF. PP2A-C was immunoprecipitated from 250 μg of cell lysate by 4 μg of anti-PP2A-C mAb (Upstate, clone 1D6) and protein-A agarose slurry for 2 h at 4 °C. Lysates were washed in Tris-buffered saline followed by serine-threonine phosphatase assay buffer (50 mM Tris HCl, 100 μM CaCl2, pH 7.0). Threonine phosphopeptide (K-R-pT-I-R-R) was added to a final concentration of 750 μM. and incubated for 15 min at 30

°C. Samples were centrifuged briefly, 25 µl of supernatant was transferred to a half-volume 96-well plate, and 100 µl Malachite Green detection solution was added for 10 min at room temperature. Amount of phosphate cleaved by PP2A was then determined by absorbance at 655 nm on a microplate reader (Model 680, Bio-Rad Laboratories).

2.2.9 Neutrophil chemotaxis

Neutrophils were purified from anti-coagulated venous blood of a healthy volunteer by positive selection using magnetic-activated cell sorting (MACS) as previously described (Baines et al., 2009). Written informed consent from the volunteer was obtained for use of this sample in research in accordance with procedures approved by the Human Research Ethics Committee of the University of Sydney. Briefly, erythrocytes were removed by dextran sedimentation followed by ficoll centrifugation to separate mononuclear cells from granulocytes. Remaining erythrocytes were lysed by osmotic shock and the pellet which contains eosinophils/neutrophils was mixed with anti-CD16 conjugated immunomagnetic beads (Miltenyi Biotec, Sunnyvale, CA) and incubated for 30 min at 4 $^{\circ}$C. The cells were then separated using MACS where the eluent was discarded. Neutrophils bound to the micro-beads were taken out of the magnetic field, flushed and prepared at a cell density of 2 x 10^6/ml in DMEM + 0.1% BSA to be used in chemotaxis assays. Neutrophil chemotaxis towards conditioned media from treated ASM cells was quantified microscopically using a 96-well microchemotaxis chamber (Neuroprobe, Gaithersburg, MD) as previously published (Henness et al., 2006).

2.2.10 NF-κB activity assays

NF-κB activity assays were performed with the NF-κB reporter vector, pNF-κB-Luc, according to our previously published methods (Henness et al., 2006; Moutzouris et al., 2010).

The NF-κB reporter vector, pNF-κB-Luc, was purchased from Clontech (BD Biosciences). Transient transfection of ASM cells was performed using Lipofectamine 2000 (Invitrogen). In brief, ASM cells were plated onto six-well plates at a density of ~2.5 × 10^5 cells/well for 24 h and then transfected with 2.4 μg of pNF-κB-Luc as well as 1.4 μg of pSV-β-galactosidase control vector (Promega) to normalize transfection efficiencies. After transfection, cells were cultured for 8 h and then growth-arrested for 16 h in DMEM supplemented with 0.1% BSA, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Invitrogen). Cells were then treated with vehicle or TNF-α (10 ng/ml), in the absence or presence of S1P (1 μM), for 6 h. Cells were then harvested, and luciferase and β-galactosidase activities were assessed according to manufacturer's instructions (Promega). Data represent normalized luciferase activity, relative to vehicle-treated cells (expressed as fold difference).

2.2.11 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
S1P-induces neutrophil chemoattractant IL-8: repression by steroids

This work has been published in the article entitled "Sphingosine 1-phosphate induces neutrophil chemoattractant IL-8: repression by steroids. **Md. Mostafizur Rahman**, Hatem Alkhouri, Francesca Tang, Wenchi Che, Qi Ge and Alaina J. Ammit·. PLoS One. 2014 Mar, 19;9(3):e92466.

Chapter 3
S1P-induces neutrophil chemoattractant IL-8: repression by steroids

3.1 Introduction

Asthma can be considered a heterogeneous syndrome made up of a number of disease phenotypes characterized by their underlying pathophysiology into "asthma endotypes" (Anderson, 2008; Lötvall et al., 2011). Defining asthma in this way will enable the design of tailored therapeutic strategies that specifically target the fundamental mechanisms responsible for the disease endotypes. One important asthma endotype is non-eosinophilic (neutrophilic) asthma. Neutrophilic asthma is driven by the chemokine CXCL8 (Simpson et al., 2009); thus studies into the molecular pathways that upregulate this neutrophil chemoattractant will allow us to gain greater insight into the underlying pathogenic mechanisms and suggest potential pharmacotherapeutic strategies for treating the neutrophilic asthma endotype in the future.

The causes of neutrophilic asthma are currently uncertain. Innate immunity dysregulation through TLR2 plays an important role (Simpson et al., 2007), as may Th17 regulation (Newcomb and Peebles Jr) and NLRP3 inflammasome activation (Simpson et al., 2013). Activation of these cellular pathways has been reported to increase neutrophilic inflammation in the airways in an IL-8-driven manner; key characteristics of the disease endotype. Airway structural cells (such as alveolar epithelium and ASM) serve as important contributors to IL-8 chemokine production and in this way may orchestrate neutrophil chemoattraction in response to inflammatory mediators (Henness et al., 2006; Manetsch et al., 2012c; Zijlstra et al., 2012).

In this study we focus on primary ASM cells in order to examine whether IL-8 is produced in response to stimulation with S1P. S1P is a bioactive sphingolipid found elevated in the airways of asthmatics (Ammit et al., 2001) and can increase IL-8 secretion from human alveolar epithelial cells (A549) to regulate neutrophil–epithelial interactions *in vitro* (Milara et al., 2009). Herein we are the first to show that S1P induces IL-8 secretion from primary ASM cells and demonstrate the molecular mechanisms responsible. We show that the anti-inflammatory corticosteroid dexamethasone inhibits S1P-induced IL-8 driven neutrophil chemotaxis and show that the molecular pathways responsible for corticosteroid-mediated repression converge on MSK1. These studies may provide further insight into the pathogenic mechanisms responsible for the neutrophilic asthma endotype.

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. Written informed consent from the donor was obtained for use of this sample in research. Primary ASM cells were dissected, purified and cultured as previously described by Johnson *et al.* (Johnson et al., 1995). A minimum of three different ASM primary cell lines were used for each experiment.

3.2.2 Chemicals

S1P (Biomol) was purchased through Enzo Life Sciences (Exeter, UK) and tumour necrosis factor α (TNFα) purchased from R&D Systems (Minneapolis, MN). Unless otherwise specified, all chemicals were from Sigma-Aldrich (St. Louis, MO).

3.2.3 ELISAs

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

3.2.4 Real-time RT-PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen Australia, Doncaster, VIC, Australia) and reverse transcription performed by using the RevertAid First strand cDNA Synthesis Kit (Fermentas Life Sciences, Hanover, MD) as per the manufacturer's protocol. MKP-1 mRNA levels were measured using real-time RT-PCR on an ABI Prism 7500 (Applied Biosystems, Foster City, CA) with the IL-8 (Hs00174103_m1) TaqMan® Gene Expression Assay 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.

3.2.5 NF-κB activity assays

NF-κB activity assays were performed with the NF-κB reporter vector, pNF-κB-Luc, according to our previously published methods (Henness et al., 2006; Moutzouris et al., 2010). For detailed description, see Chapter 2.2.10.

3.2.6 siRNA knock-down

Primary ASM cells were transiently transfected using nucleofection with 1 μg MSK1-specific SMART pool siRNA as described previously (Che et al., 2014) and cell supernatants removed for IL-8 protein measurement by ELISA and lysates utilized for MSK1 Western blotting (Cell Signaling Technology, Danvers, MA) .

3.2.7 Neutrophil chemotaxis

Neutrophils were purified from anti-coagulated venous blood of a healthy volunteer by positive selection using magnetic-activated cell sorting (MACS) as previously described (Baines et al., 2009). Written informed consent from the volunteer was obtained for use of this sample in research in accordance with procedures approved by the Human Research Ethics Committee of the University of Sydney. Briefly, erythrocytes were removed by dextran sedimentation followed by ficoll centrifugation to separate mononuclear cells from granulocytes. Remaining erythrocytes were lysed by osmotic shock and the pellet which contains eosinophils/neutrophils was mixed with anti-CD16 conjugated immunomagnetic beads (Miltenyi Biotec, Sunnyvale, CA) and incubated for 30 min at 4 $^{\circ}$C. The cells were then separated using MACS where the eluent was discarded. Neutrophils bound to the micro-beads were taken out of the magnetic field, flushed and prepared at a cell density of 2 x 10^6/ml in DMEM + 0.1% BSA to be used in chemotaxis

assays. Neutrophil chemotaxis towards conditioned media from treated ASM cells was quantified microscopically using a 96-well microchemotaxis chamber (Neuroprobe, Gaithersburg, MD) as previously published (Henness et al., 2006).

3.2.8 Statistical analysis

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

3.3 Results

3.3.1 The bioactive sphingolipid S1P induces secretion of IL-8 from ASM cells, but this can be repressed by the corticosteroid dexamethasone in a concentration-dependent manner

In our previous studies we have shown that the bioactive sphingolipid S1P is increased in the airways of asthmatics (Ammit et al., 2001) and as airway neutrophilia has shown to be linked to asthmatic inflammation we were interested to explore whether S1P increases secretion of the neutrophilic chemoattractant IL-8 from ASM cells. To address this, primary ASM cells were stimulated with 1 μM S1P for 24 h and the resultant IL-8 secretion measured by ELISA. As shown in Figure 3.1A, S1P significantly increases secretion of IL-8 ($P<0.05$). Importantly, S1P-increased IL-8 secretion from ASM cells can be repressed by the corticosteroid dexamethasone in a concentration-dependent manner (Figure 3.1B: $P<0.05$).

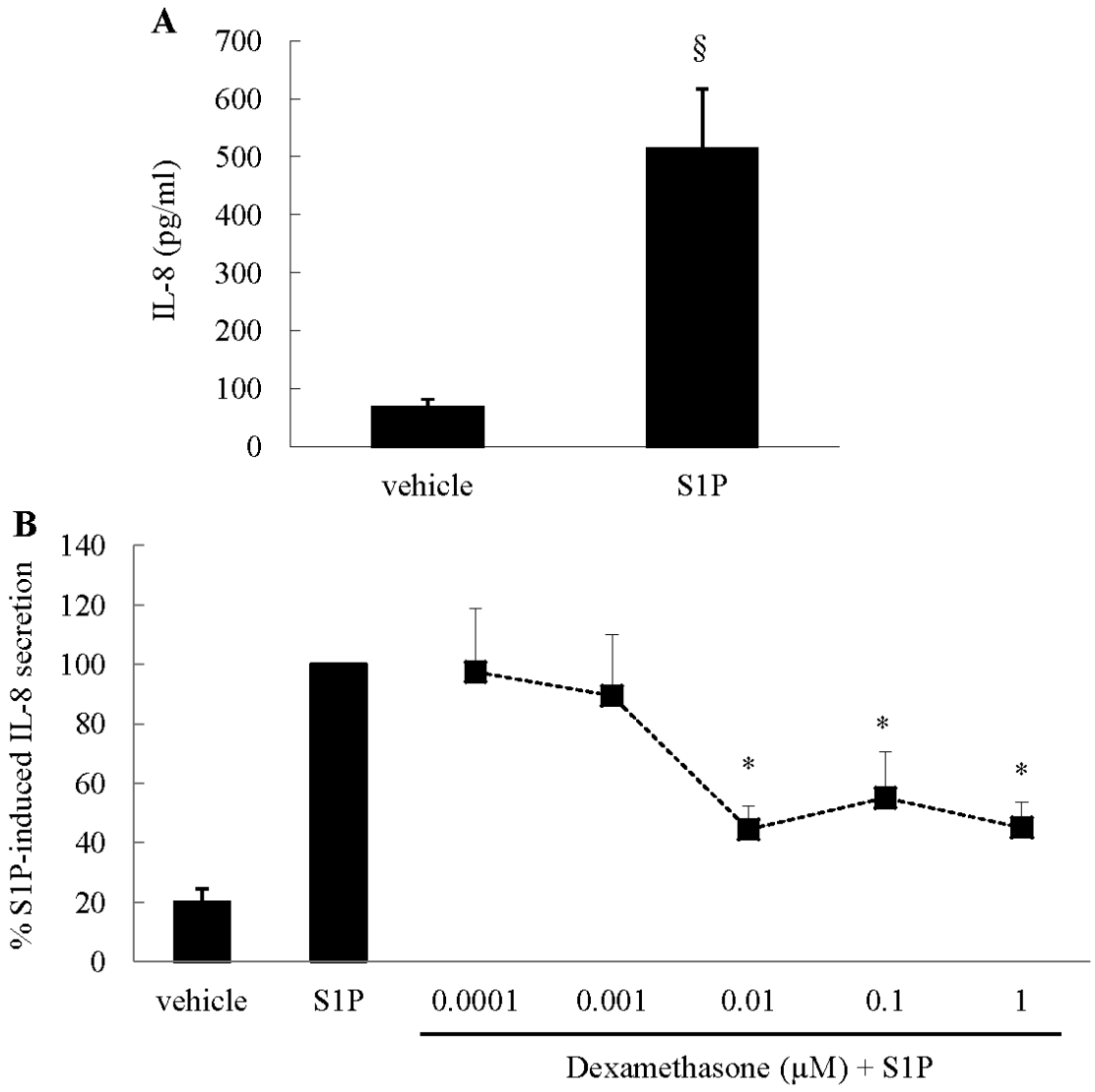


Figure 3.1 The bioactive sphingolipid S1P induces secretion of IL-8 from ASM cells, but this can be repressed by the corticosteroid dexamethasone in a concentration-dependent manner. (A) Growth-arrested **primary** ASM cells were treated with vehicle or S1P (1 μM) for 24 h and IL-8 secretion measured by ELISA. Statistical analysis was performed using the Student's unpaired *t* test, where § denotes a significant effect of S1P ($P<0.05$). (B) To demonstrate corticosteroid-mediated repression of S1P-induced IL-8 secretion we then pre-treated growth-arrested **primary** ASM cells for 1 h with vehicle or dexamethasone (0.0001-1 μM), before stimulation for 24 h with S1P (1 μM). IL-8 protein was measured by ELISA and results expressed as % S1P-induced IL-8 secretion at 24 h. Statistical analysis was performed using one-way ANOVA then Fisher's post-hoc multiple comparison test, where * denotes significant repression by dexamethasone ($P<0.05$). Data are mean+SEM values from n=12 primary ASM cell lines.

3.3.2 Time course of S1P-induced IL-8 mRNA expression and protein secretion and its repression by dexamethasone

In order to explore the molecular mechanism of repression by dexamethasone we first performed an analysis of the temporal kinetics of S1P-induced IL-8 mRNA expression and subsequent protein secretion and its repression by dexamethasone. As shown in Figure 3.2A, S1P robustly increases IL-8 mRNA expression as early as 1 h after stimulation. This expression is sustained from 2-8 h, before receding to baseline levels by 24 h ($P<0.05$). This upregulation is repressed by dexamethasone pre-treatment with S1P-induced IL-8 mRNA expression being significantly inhibited by dexamethasone at 2 h ($P<0.05$). The pattern of protein secretion follows the same course with significant amounts of IL-8 being secreted from primary ASM cells by 8 h and accumulating further at 24 h (Figure 3.2B: $P<0.05$). Secretion of IL-8 was substantially and significantly repressed by the corticosteroid dexamethasone at 24 h (Figure 3.2B: $P<0.05$).

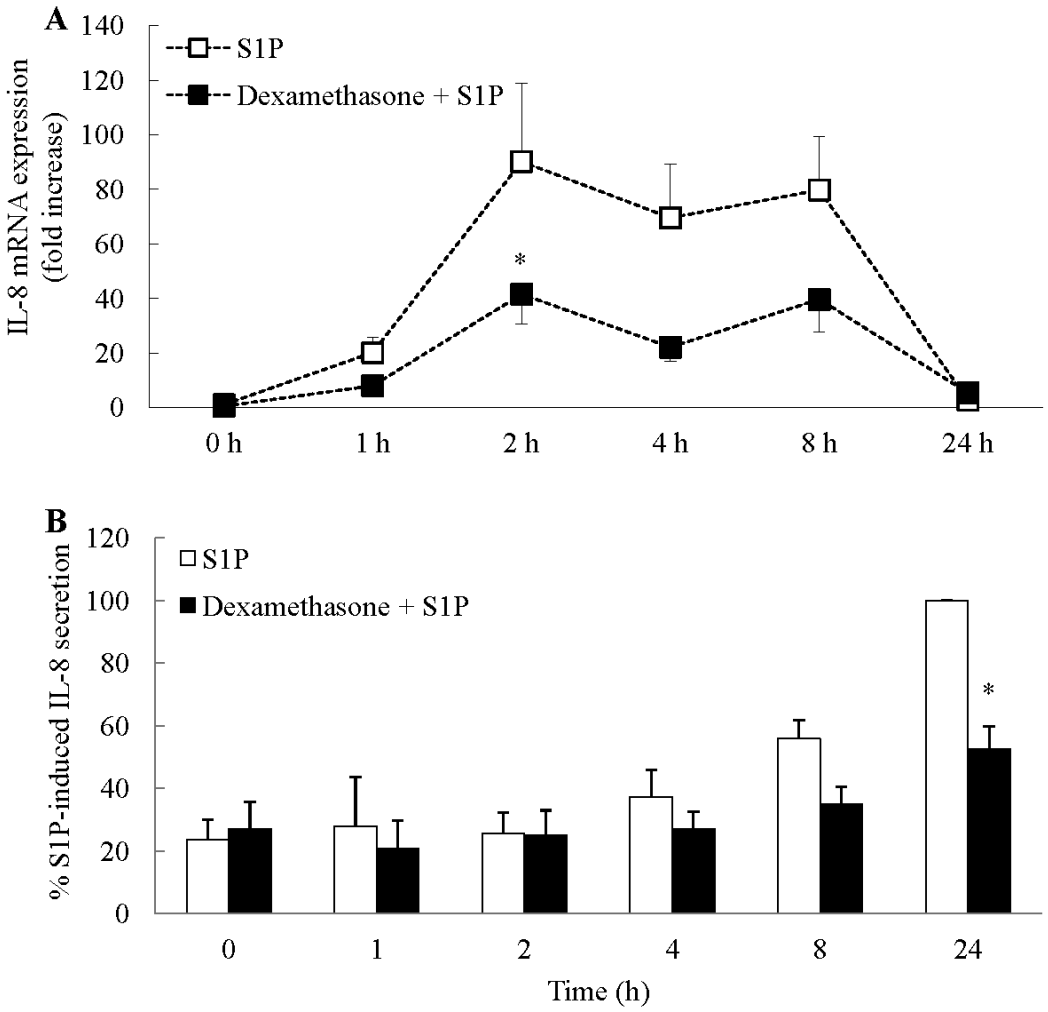


Figure 3.2 Time course of S1P-induced IL-8 mRNA expression and protein secretion and its repression by dexamethasone. Growth-arrested **primary** ASM cells were pre-treated for 1 h with vehicle or 100 nM dexamethasone, followed by treatment with vehicle or S1P (1 μM) for 0, 1, 2, 4, 8, and 24 h. (A) IL-8 mRNA expression was quantified by real-time RT-PCR and results expressed as fold increase compared to vehicle-treated cells at 0 h. (B) IL-8 protein secretion was measured by ELISA and results expressed as % S1P-induced IL-8 secretion at 24 h. Statistical analysis was performed using two-way ANOVA then Bonferroni's post-test where * indicates significant repression by dexamethasone, compared to vehicle-treated cells at the same time point (P<0.05). Data are mean+SEM values from n=7 primary ASM cell lines.

3.3.3 Dexamethasone totally inhibits S1P-induced IL-8 protein secretion, but only partially inhibits TNFα-induced IL-8 induced in the presence of S1P

As we had observed (in Figures 3.1 & 3.2) that dexamethasone represses S1P-induced IL-8 expression relatively effectively, S1P secretion alone cannot solely account for the more corticosteroid insensitive phenotype associated with neutrophilic asthma. As numerous inflammatory mediators can be found elevated in asthma at the same time, it is of interest to examine the relative corticosteroid sensitivity of IL-8 induced by another inflammatory mediator added in combination with S1P. TNFα is a key example (Broide et al., 1992). Therefore we stimulated cells with S1P + TNFα in order to determine if the model is more corticosteroid resistant under these experimental conditions. We first performed a side-by-side comparison of IL-8 secretion in response to S1P or TNFα, added separately or in combination. As shown in Figure 3.3A, and in confirmation of our earlier studies (Henness et al., 2006), TNFα potently and significantly increases IL-8 secretion. Notably, TNFα-induced IL-8 secretion is significantly potentiated in the presence of S1P (Figure 3.3A: $P<0.05$). We then compared the relative corticosteroid sensitivity of IL-8 induced by each stimulant, alone or in combination. As shown in Figure 3.3B, IL-8 induced by S1P can be completely repressed by dexamethasone. Dexamethasone concentrations as low as 0.01 μM can repress S1P-induced IL-8 secretion to unstimulated levels. In contrast, TNFα-induced IL-8 cannot be is relatively resistant to repression by dexamethasone (Figure 3.3C). Even after 1 μM dexamethasone pretreatment, TNFα-induced IL-8 secretion cannot be completely repressed (Figure 3.3C). Accordingly, when then the two pro-inflammatory mediators are added together, S1P in addition to the potent pro-inflammatory cytokine TNFα induces IL-8 expression that is relatively corticosteroid resistant (Figure 3.3D).

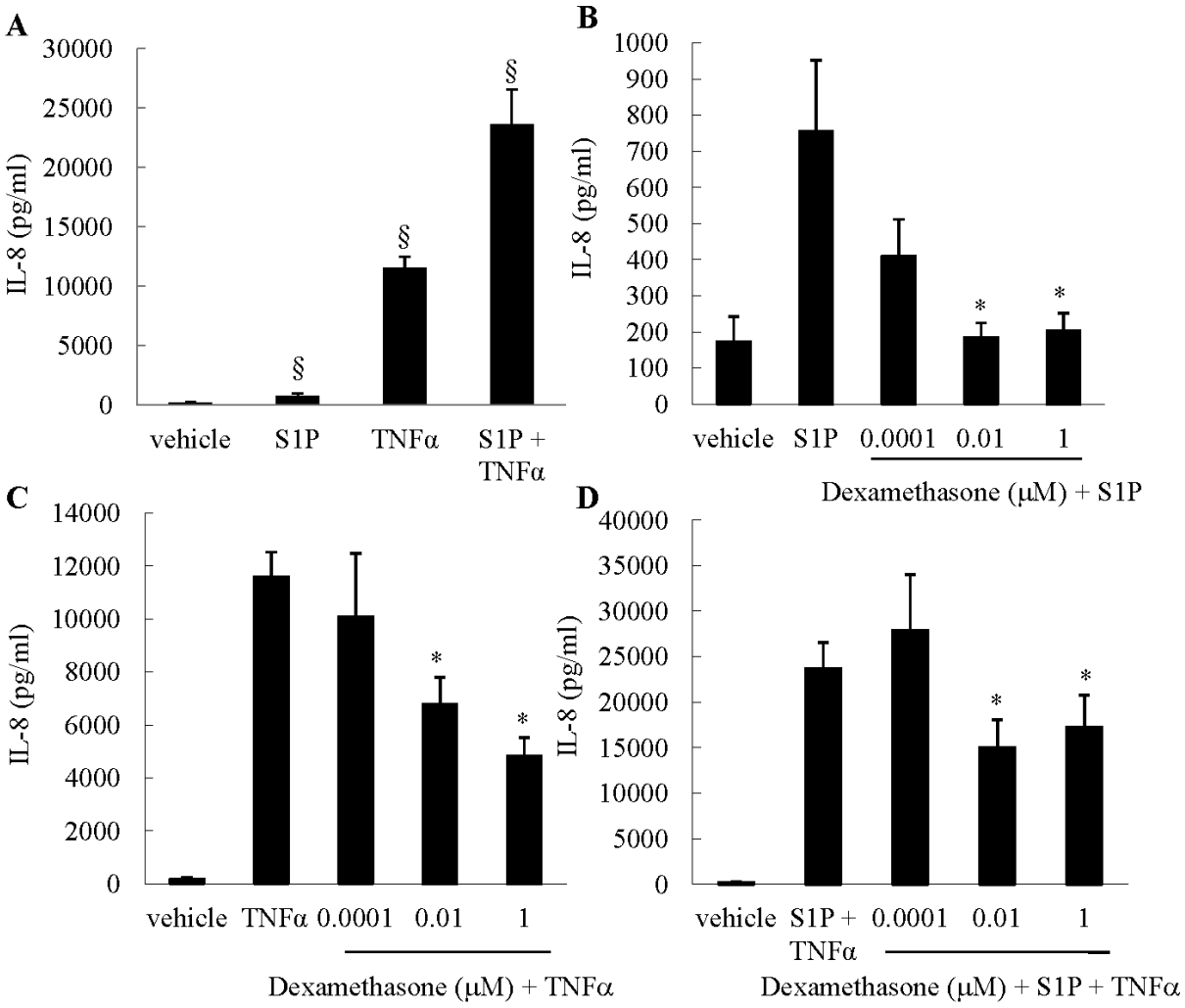


Figure 3.3 Dexamethasone totally inhibits S1P-induced IL-8 protein secretion, but only partially inhibits TNFα-induced IL-8 induced in the presence of S1P. (A) To compare IL-8 secretion, growth-arrested **primary** ASM cells were treated with vehicle, S1P (1 μM), TNFα (10 ng/ml), or S1P (1 μM) in combination with TNFα (10 ng/ml) (B-C). To examine the relative corticosteroid sensitivity, cells were pre-treated for 1 h with vehicle or dexamethasone (0.0001, 0.01, 1 μM) then: (B) stimulated with S1P (1 μM); (C) stimulated with TNFα (10 ng/ml); (D) stimulated with S1P (1 μM) + TNFα (10 ng/ml). After 24 h, secreted IL-8 was measured by ELISA. Statistical analysis was performed using one-way ANOVA then Fisher's post-hoc multiple comparison test, where § denotes a significant upregulation of IL-8, and * denotes a significant effect of dexamethasone on IL-8 secretion ($P < 0.05$). Data are mean+SEM values from n=7 primary ASM cell lines.

3.3.4 S1P does not activate NF-κB in ASM cells

In order to examine whether the difference in corticosteroid sensitivity between mediators is due to the involvement of NF-κB signaling pathway in mediating IL-8 expression we utilized an NF-κB reporter vector, pNF-κB-Luc. As shown in Figure 3.4, TNFα activates NF-κB; in confirmation of our earlier studies (Ammit et al., 2002). Importantly, S1P alone does not increase NF-κB-luciferase activity, nor increase TNFα-induced NF-κB activity. As NF-κB activity in ASM cells is relatively insensitive to repression by corticosteroids (Amrani et al., 1999; Catley et al., 2006; Quante et al., 2008), our *in vitro* results may reflect the *in vivo* situation where multiple inflammatory mediators orchestrate chemokine expression and is consistent with airway neutrophilia being corticosteroid resistant.

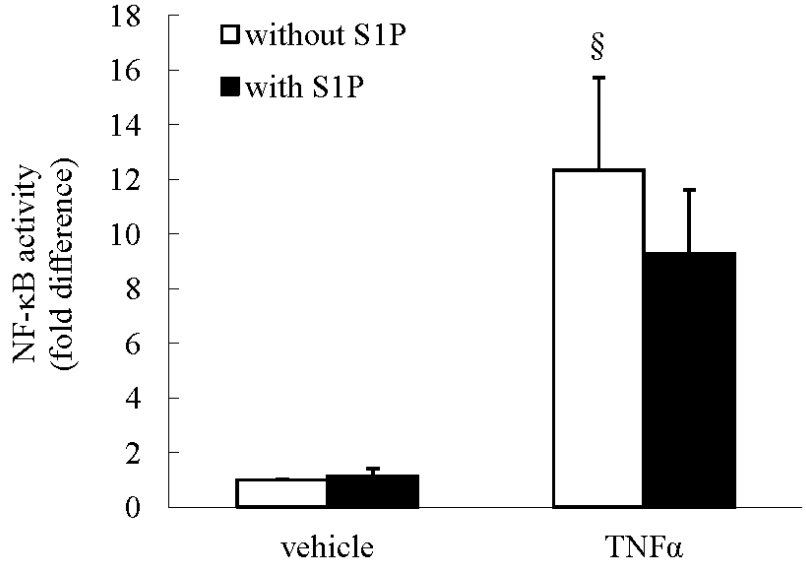


Figure 3.4 S1P does not activate NF-κB in ASM cells. Primary ASM cells transfected with a NF-κB reporter vector, pNF-κB-Luc, were growth-arrested, then treated with vehicle or TNFα (10 ng/ml), in the absence or presence of S1P (1 μM) for 6 h. Cells were then harvested and luciferase and β-galactosidase activities assessed. Data represent normalized luciferase activity, relative to vehicle-treated cells (expressed as fold difference). Statistical analysis was performed using the Student's unpaired *t* test (where § denotes significant effect of TNFα on NF-κB activity ($P<0.05$)). Data are mean+SEM values from n=6 primary ASM cell lines.

3.3.5 S1P-induced IL-8 protein secretion is repressed by inhibitors of the p38 MAPK- and ERK-mediated pathways

We had previously shown that S1P rapidly activates all members of the MAPK superfamily (Che et al., 2012) and had earlier implicated p38 MAPK and ERK pathways in IL-8 secretion in response to another stimulus – TNFα (Moutzouris et al., 2010). For these reasons we now explored the role of the p38 MAPK and ERK pathways in S1P-induced IL-8 secretion. We utilized well-established pharmacological inhibitors of p38 MAPK and ERK, i.e. 1 μM SB2035680 and 10 μM PD98059, respectively, and investigated their repressive effects on S1P-induced IL-8 protein secretion from primary ASM cells. We routinely utilize these inhibitors to repress p38 MAPK and ERK signalling in ASM cells *in vitro* (Henness et al., 2006; Quante et al., 2008; Moutzouris et al., 2010; Munoz and Ammit, 2010; Manetsch et al., 2012a; Che et al., 2013) as they specifically inhibit p38 MAPK and ERK phosphorylation in primary ASM cells at these concentration (Che et al., 2012). As shown in Figure 3.5, S1P-induced IL-8 protein secretion was significantly repressed by both SB203580 and PD98059 pre-treatment ($P<0.05$).

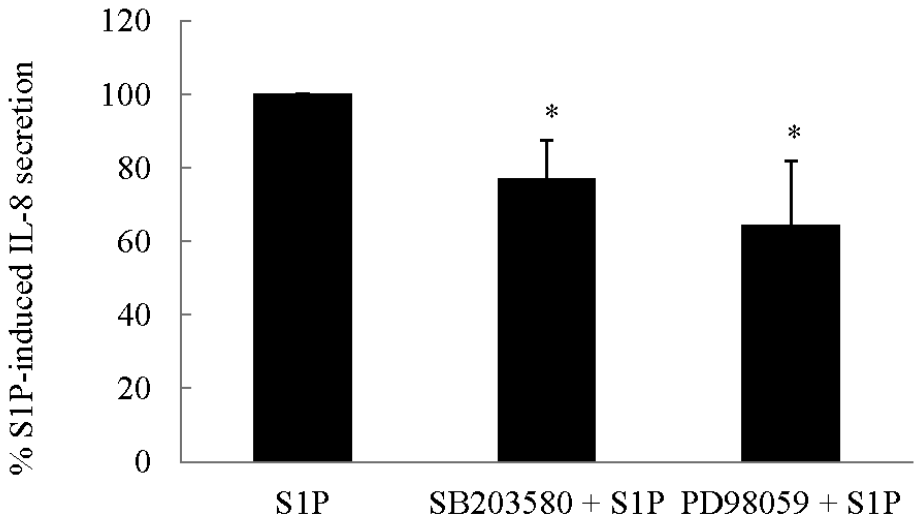


Figure 3.5 S1P-induced IL-8 protein secretion is repressed by inhibitors of the p38 MAPK- and ERK-mediated pathways. Growth-arrested primary ASM cells were pre-treated for 30 min with vehicle, 1 μM SB203580, or 10 μM PD98059 to inhibit p38 MAPK and ERK, respectively. Cells were then stimulated with 1 μM S1P for 24 h. IL-8 protein was measured by ELISA and results expressed as % S1P-induced IL-8 secretion at 24 h. Statistical analysis was performed using the Student's unpaired *t* test, where * denotes significant inhibition ($P<0.05$). Data are mean+SEM values from n=10 primary ASM cell lines.

3.3.6 IL-8 induced by S1P causes human neutrophil chemotaxis *in vitro* and this can be repressed by corticosteroids or by blocking the p38 MAPK- or ERK-mediated pathways

Taken together, our results thus far demonstrate that S1P induces IL-8 secretion from primary ASM cells and that this secretion can be repressed by the corticosteroid dexamethasone and pharmacological inhibitors of the mitogen- and stress-activated protein kinases; p38 MAPK and ERK. IL-8 is a key neutrophil chemoattractant cytokine and in Figure 3.6 we show that human neutrophils undergo significant chemotaxis towards conditioned media from cells stimulated with S1P. Importantly, this S1P-induced chemotaxis was significantly reduced when cells were pre-treated with dexamethasone, SB203580 or PD98059 prior to S1P stimulation (Figure 6: $P<0.05$). These data suggest that the bioactive sphingolipid S1P may play a role in IL-8-driven

neutrophilic inflammation and implicate molecular pathways that may be targeted to reduce expression of this chemokine.

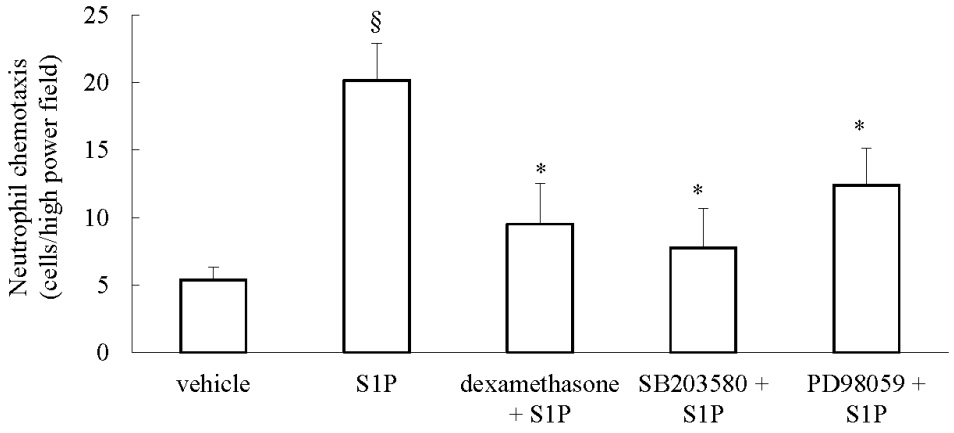


Figure 3.6 IL-8 induced by S1P causes human neutrophil chemotaxis in vitro and this can be repressed by corticosteroids or by blocking the p38 MAPK- or ERK-mediated pathways. Chemotaxis of human neutrophils toward conditioned media from growth-arrested primary ASM cells pre-treated with vehicle, dexamethasone (100 nM), SB203580 (1 μM) or PD98059 (10 μM), followed by treatment with vehicle or S1P (1 μM) for 24 h, was measured using microchemotaxis chambers. Results are expressed as cells per high-power (x200) field. Statistical analysis was performed using the Student's unpaired *t* test where § denotes a significant effect of S1P on neutrophil chemotaxis, while * denotes significant repression ($P<0.05$). Data are mean+SEM values using conditioned media from n=3 primary ASM cell lines.

3.3.7 S1P induces IL-8 secretion via a MSK1-dependent pathway and MSK1 knock-down by siRNA attenuates S1P-induced neutrophil chemotaxis

S1P-induced IL-8 is mediated via p38 MAPK and ERK-mediated pathway. One of the important downstream effectors of these mitogen- and stress-activated kinases is MSK1. We have recently demonstrated that S1P activation leads to MSK1 phosphorylation in primary ASM cells (Che et al., 2013). Thus, to address whether S1P-induced IL-8 secretion is mediated via a MSK1-dependent pathway we transiently transfected primary ASM cells using nucleofection with

scrambled control or MSK1 siRNA and assessed the impact of reduced MSK1 on IL-8 secretion induced by S1P. Figure 3.7A demonstrates successful knock-down of MSK1 with siRNA. As shown in Figure 3.7B, the degree of IL-8 secretion was significantly less in cells transfected with MSK1 siRNA as compared to scrambled control ($P<0.05$). Accordingly, by reducing S1P-induced IL-8 secretion MSK1 knock-down also reduces S1P-induced neutrophil chemotaxis. This is demonstrated in Figure 3.7C, where neutrophil chemotaxis in supernatants from cells nucleofected with MSK1 siRNA was 57.2±18.2% when compared to supernatants from scrambled control cells (designated as 100%)($P<0.05$).

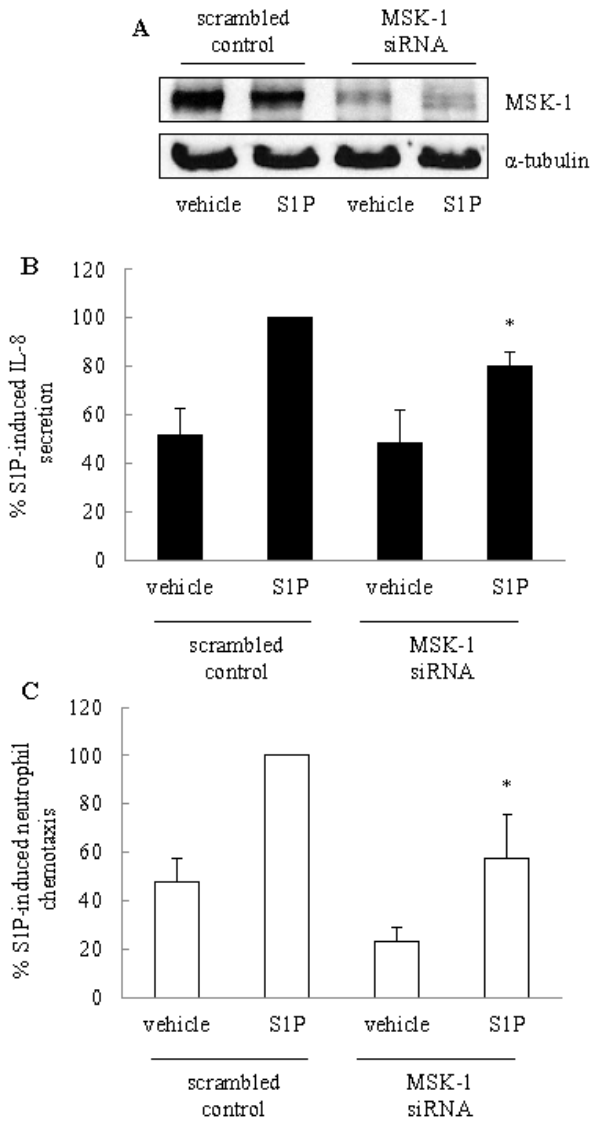


Figure 3.7 S1P induces IL-8 secretion via a MSK1 dependent pathway and MSK1 knock-down by siRNA attenuates S1P-induced neutrophil chemotaxis. Primary ASM cells were transiently transfected using nucleofection with scrambled control or MSK1 siRNA, growth-arrested, then treated for 24 h with vehicle or S1P (1 μM). (A) To confirm that MSK1 siRNA reduces protein levels of MSK1, cells were lysed and immunoblotted for MSK1 (90 kDa), using α-tubulin (55 kDa) as the loading control. To measure the effect of MSK1 knock-down on IL-8 inducibility and neutrophil chemotaxis, supernatants were removed and (B) IL-8 protein measured by ELISA and (C) neutrophil chemotaxis assessed using microchemotaxis chambers. Results are expressed as: (A) representative Western blots; (B) % S1P-induced IL-8 secretion in cells transfected with scrambled control; or (C) % S1P-induced neutrophil chemotaxis in the corresponding conditioned media (data are mean+SEM values from n=6 primary primary ASM cell lines). Statistical analysis was performed using the Student's unpaired *t* test where * indicates that knocking down MSK-1 significantly attenuates S1P-induced effects ($P<0.05$).

3.4 Discussion

S1P is found elevated in airways of asthmatics and in this study we explore its stimulatory effect on an important chemokine responsible for neutrophilia in airway inflammation – IL-8. We demonstrate that stimulation of primary ASM cells with S1P results in IL-8 gene expression and protein secretion. We determine the temporal kinetics of IL-8 upregulation and show that mRNA expression and protein secretion can be repressed by the corticosteroid dexamethasone. Additionally, we reveal that S1P-induced IL-8 secretion is p38 MAPK- and ERK-dependent and that these key phosphoproteins act on the downstream effector MSK1 to control secretion of the neutrophil chemoattractant cytokine IL-8. We demonstrate the functional relevance of our *in vitro* data by performing neutrophil chemotaxis assays. We show that S1P-induced effects can be significantly attenuated by pre-treatment with dexamethasone, pharmacological inhibition of p38 MAPK- or ERK-mediated pathways, or by knocking down MSK-1 with siRNA. Taken together these studies help us appreciate the molecular pathways responsible for IL-8 secretion from primary ASM cells in response to S1P and indicate ways in which its impact on IL-8-driven neutrophilia may be repressed.

S1P is increasingly recognized as playing an important role in asthma and airway inflammation. We were the first to show that the levels of this bioactive sphingolipid were increased in the broncho-alveolar lavage fluid of allergic asthmatics (Ammit et al., 2001) and since that time the important immunomodulatory role for S1P in asthma and airway remodelling has clearly emerged (reviewed in (Lai et al., 2011; Olivera and Rivera, 2011; Yang and Uhlig, 2011)). The source of S1P in allergic asthma is considered to be the mast cell (Jolly et al., 2002; Olivera and Rivera, 2011). This is of relevance to the current study, where we focus on the immunostimulatory effects of S1P on airway structural cells, because we have previously shown

that increased numbers of mast cells are close proximity to ASM in airways of sensitized individuals (Ammit et al., 1997); a finding confirmed in the airways of asthmatics (Brightling et al., 2002). These studies suggest that S1P released from mast cells may act upon the surrounding airway tissue to initiate, perpetuate or perhaps amplify airway responses, including cytokine secretion. In support, we have previously shown that S1P potently induces secretion of IL-6 from primary ASM (Ammit et al., 2001; Che et al., 2013) and herein show that expression of IL-8 is also stimulated by S1P. Our study is in accord with a recent publication that demonstrated that S1P also enhanced IL-8 secretion from alveolar epithelial cells (Milara et al., 2009). These studies suggest that the bioactive sphingolipid S1P can orchestrate cytokine production in airway inflammation and in the current study we extend our investigation to demonstrate that S1P can enhance neutrophil chemoattraction. This study is of clinical relevance given the contribution of airway neutrophilia to asthma pathophysiology.

Our recent papers in primary ASM cells have highlighted some of the key signalling pathways activated by S1P in primary ASM cells. S1P rapidly activates all members of the MAPK superfamily and also induces robust and sustained phosphorylation of the cAMP-dependent transcription factor CREB (Che et al., 2012; Che et al., 2013). Taken together with the knowledge that IL-8 secretion from ASM cells can be inhibited by SB203580 and PD98059 (Moutzouris et al., 2010), but is independent of the cAMP/CREB-mediated pathway (Manetsch et al., 2013), we were able to narrow our focus for investigation in this study and reveal for the first time that S1P-induced IL-8 secretion is mediated by p38 MAPK- and ERK-dependent pathways. This is important as we have recently shown that the downstream effector of these mitogen- and stress-activated protein kinases – MSK1 - plays an essential role in controlling histone H3 phosphorylation, enhanced chromatin relaxation and regulation of gene expression.

Thus, by stimulating p38 MAPK/ERK, S1P induces IL-8 expression in primary ASM cells in an MSK1-dependent pathway. This was confirmed by knocking-down MSK1 with siRNA and showing that S1P-induced IL-8 secretion was attenuated.

Our study also reveals that the corticosteroid dexamethasone can reduce S1P-induced IL-8 gene expression, and resultant protein secretion, in a time- and concentration-dependent manner. Our recent study (Che et al., 2013) explored the signalling pathways responsible for secretion of another cytokine released by ASM cells after S1P stimulation, namely IL-6, and revealed how corticosteroids act to exert their anti-inflammatory effects. We know now that corticosteroids mediate their anti-inflammatory actions, in part, via upregulation of the anti-inflammatory protein-MKP-1 (Quante et al., 2008; Che et al., 2013). Importantly, inhibition of the MSK1/histone H3 pathways is one of the key ways in which corticosteroids mediate their repressive effects in ASM cells. This was shown in our recent study (Che et al., 2013) where corticosteroid-induced MKP-1 attenuated S1P-induced IL-6 secretion by dephosphorylating p38 MAPK and ERK-mediated activation of MSK1 and histone H3 phosphorylation. We propose that S1P-induced IL-8 is controlled in a similar MSK1-dependent manner. We illustrate the clinical relevance of targeting MSK1 by demonstrating that knocking-down MSK1 significantly represses IL-8-driven neutrophil chemotaxis induced by S1P. Thus MSK1 inhibition may represent a feasible therapeutic option for controlling S1P-mediated pro-inflammatory actions in the future.

The role of neutrophils in airway inflammation has recently come to the fore and therefore in this study we examined whether the asthma-related sphingolipid S1P increased secretion of the neutrophil chemoattractant IL-8 from primary ASM cells. Demonstration of S1P-induced neutrophil chemotaxis is clinically relevant given the important role played by IL-8-driven

airway neutrophilia. Interestingly, the degree of reduction in neutrophil chemotaxis achieved with the MAPK pharmacological inhibitors is greater than the attenuation of IL-8 secretion. This may indicate that other MAPK-dependent chemokines may also be upregulated by S1P. Although IL-8 is the classical chemotaxin for neutrophils, other chemokines such as GROα (CXCL1) and regulated on activation normal T cell expressed and secreted (CCL5: also known as RANTES) can also contribute to airway neutrophilia (Kaur and Singh, 2013). These chemokines can be secreted by ASM cells (John et al., 1997; Ammit et al., 2002; Henness et al., 2006; Issa et al., 2006) and their expression is regulated by p38 MAPK/ERK- dependent pathways (Amrani et al., 2001; Henness et al., 2006; Issa et al., 2006). We are the first to reveal the signalling pathways activated by S1P in primary ASM cells (Che et al., 2012; Che et al., 2013), but based on our demonstration that S1P induces IL-8 to enhance neutrophil chemotaxis in a p38 MAPK/ERK-manner we predict that other chemokines regulated by these signalling molecules may repressed by pharmacological inhibition in a similar manner. This study may have implications for revealing the pathogenic mechanisms responsible for neutrophilic asthma endotype, although to date, whether S1P levels are increased in BAL fluids from patients characterised with neutrophilic asthma is an open question.

Together, these studies indicate that inflammatory mediators such as S1P may contribute to the development of IL-8-driven neutrophilia in airway inflammation and suggests that further studies into the causal role played by S1P in the neutrophilic asthma endotype are warranted. Most importantly, these studied clearly demonstrate the role of MAPKs in asthmatic inflammation and the role of one important corticosteroid, dexamethasone in repressing neutrophilic asthma.

Chapter 4
Basal Protein phosphatase2A activity restrains cytokine expression: role for MAPKs and TTP

This work has been published in the article entitled "Basal protein phosphatase 2A activity restrains cytokine expression: role for MAPKs and tristetraprolin. **Md. Mostafizur Rahman**, Nowshin N. Rumzhum, Jonathan C. Morris, Andrew R. Clark, Nicole M. Verrills and Alaina J. Ammit. *Sci Rep.* 2015 May 18;5:10063. doi: 10.1038/srep10063

Chapter 4
Basal PP2A activity restrains cytokine expression: role for MAPKs and TTP

4.1 Introduction

PP2A is the key controller of a number of inflammatory signalling pathways mediated by MAPKs. Moreover, it controls a critical anti-inflammatory protein, TTP via dephosphorylation. Here in this study we therefore aim to explore the role basal PP2A in the expression of MAPKs and regulation of TTP.

PP2A is a ubiquitously expressed serine/threonine phosphatase that exists as a tri-molecular complex of a catalytic subunit (C), a structural subunit (A), and a variable regulatory subunit (B) of which there are at least 3 different families (B55, B56, B") each with several isoforms (Cho and Xu, 2007). PP2A has generated much excitement as a target for anti-cancer therapy (reviewed in (Perrotti and Neviani, 2013)) and has more recently emerged as a druggable target in respiratory disease (Collison et al., 2013a; Hatchwell et al.). But in order to accelerate the development of PP2A activators as a future pharmacotherapeutic strategy in respiratory medicine, it is essential that we gain an advanced understanding of the regulation and function of PP2A in cellular models of asthmatic inflammation *in vitro*.

PP2A dephosphorylates a number of kinases that drive inflammatory cell signaling (Shanley et al., 2001; Junttila et al., 2008). Notably, PP2A can dephosphorylate members of the MAPK superfamily, including p38 MAPK (Junttila et al., 2008). By regulating MAPKs, PP2A exerts significant control over cytokine regulatory networks; although the molecular mechanisms responsible remain relatively unexplored in airway inflammation.

We address this herein by utilizing the human alveolar epithelial cell line (A549); a transformed cancer cell line widely used to examine cytokine expression in the context of asthmatic inflammation (Cornell et al., 2009; King et al., 2009b; Papi et al., 2013). Confirmatory experiments were also performed with the human bronchial epithelial cell line (BEAS-2B) (Kaur et al., 2008; Cornell et al., 2009; Collison et al., 2013a). PP2A is a ubiquitously expressed phosphatase and our study shows that under unstimulated conditions there is a high level of PP2A enzymatic activity. This basal PP2A activity serves to restrain downstream effectors regulated by MAPKs. Inhibition of PP2A releases restraint and thereby increases MAPK-mediated pro-inflammatory cytokines, including IL-6 and IL-8, as well as disables the anti-inflammatory function of TTP, a destabilizing RNA binding protein regulated at multiple levels by p38 MAPK.

4.2 Materials and Methods

4.2.1 Chemicals

Okadaic acid (OA) was purchased from Enzo Life Sciences (Farmingdale, NY). FTY720 was purchased from Cayman Chemical Company (Ann Arbor, MI) and AAL(s) was synthesized (Don et al., 2007; Collison et al., 2013a; Hatchwell et al., 2014). TNFα is from R&D Systems (Minneapolis, MN). Unless otherwise specified, all chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO).

4.2.2 Cell culture

The human alveolar epithelial cancer cell line (A549) and human bronchial epithelial normal cell line (BEAS-2B) were cultured in Ham's F-12K (Kaighn's) Medium (Invitrogen, Carlsbad, CA)

supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), and fetal calf serum (10%), in accordance with culture conditions reported by Cornell *et al.* (Cornell et al., 2009). All experiments were performed after an overnight serum-starvation period (14-16 h) in Ham's F-12K supplemented with sterile BSA (0.1%). A minimum of three experimental replicates performed on separate days were used for each experiment.

4.2.3 PP2A activity assay

PP2A activity was determined using the PP2A immunoprecipitation phosphatase assay kit (Merck Millipore, Darmstadt, Germany) according to the manufacturer's instructions. For detailed description, see Chapter 2.2.8.

4.2.4 Western blotting

Western blotting was performed using rabbit monoclonal or polyclonal antibodies against phosphorylated (Thr^{180}/Tyr^{182}) and total p38 MAPK, phosphorylated (Thr^{202}/Tyr^{204}) and total ERK, phosphorylated (Thr^{183}/Tyr^{185}) and total JNK (all from Cell Signalling Technology, Danvers, MA). PP2A-C was detected with a mouse monoclonal antibody (IgG2bκ, clone 1D6: Merck Millipore, Darmstadt, Germany). TTP was measured by Western blotting using rabbit antisera against TTP (Sak21). Detection of α-tubulin was used as the loading control (mouse monoclonal IgG_1, DM1A: Santa Cruz Biotechnology, Santa Cruz, CA). Primary antibodies were detected with goat anti-rabbit and anti-mouse HRP-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA) and visualized by enhanced chemiluminescence (PerkinElmer, Wellesley, MA).

4.2.5 Real-time RT-PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen Australia, Doncaster, VIC, Australia) and reverse transcription performed by using the RevertAid First strand cDNA Synthesis Kit (Fermentas Life Sciences, Hanover, MD) according to the manufacturer's protocol. IL-6, IL-8 and TTP mRNA levels were measured using real-time RT-PCR on an ABI Prism 7500 (Applied Biosystems, Foster City, CA) with IL-6 (Hs00174131_m1), IL-8 (Hs00174103_m1) and TTP (Zfp36, Hs00185658_m1) 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.

4.2.6 ELISAs

IL-6 and IL-8 ELISAs were performed according to the manufacturer's instructions (BD Biosciences Pharmingen, San Diego, CA). For detailed description, see Chapter 2.2.5.

4.2.7 Transient transfection

A549 cells (5×10^5 cells/well) were transfected with 1 µg of pEGFP HA-PP2A-C, or empty vector control, using Lipofectamine 2000 (Invitrogen). After transfection, cells were cultured for 24 h in media without antibiotics, and then growth-arrested for 16 h in Ham's F-12K supplemented with 0.1% BSA, supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), before cells were assayed.

4.2.8 siRNA transfection

A549 cells (5×10^5 cells/well) were transfected with siRNA against PP2A-C, or scrambled control, by reverse transfection with RNAiMAX according to the manufacturer's protocols (Invitrogen, NY, USA). Specifically, for each well of 6-well plates, 800 ng of ON-Target plus Control Non-targeting siRNA (scrambled control) or ON-target plus SMART pool Human PPP2CA siRNA (aka PP2A-C: both from Dharmacon, Thermo-Fisher Scientific, Waltham, MA) was diluted in 500 μL of Opti-MEM Reduced Serum Media (Invitrogen). This was followed by the addition of 5 μL of RNAiMAX reagent (Invitrogen) into each well and incubation at room temperature for 20 min. After transfection, cells were cultured for 24 h in media without antibiotics, and then growth-arrested for 16 h in Ham's F-12K supplemented with 0.1% BSA, supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), before stimulation with TNFα (4 ng/ml).

4.2.9 Statistical analysis

Statistical analysis was performed using either the Student's unpaired *t* test, one-way or two-way ANOVA followed by Bonferroni's post-test. *P* values <0.05 were sufficient to reject the null hypothesis for all analyses. Data are mean±SEM of n≥3 independent replicates.

4.3 Results

4.3.1 Temporal regulation of basal PP2A enzymatic activity by OA

OA is a non-selective pharmacological inhibitor of PP2A (Swingle et al., 2007; Cornell et al., 2009) and widely used to potently inhibit PP2A phosphatase activity (Bialojan and Takai, 1988; Mao et al., 2005; Cornell et al., 2009; Kranias et al., 2010). To examine the temporal regulation

of basal PP2A enzymatic activity by OA, A549 cells were treated with 1 µM OA for 15 min or 45 min, then washed and left for a further 1 h before measuring PP2A enzymatic activity. PP2A is a ubiquitously expressed phosphatase and, as shown in Figure 4.1, the basal PP2A enzymatic activity in A549 cells is 863.7±98.9 pmol free phosphate. This activity can be significantly repressed by 45 min treatment with OA, while treatment for a shorter time period (i.e. 15 min) was without effect. These data indicate in part, the temporal regulation of basal PP2A activity by OA. Results from cells treated for both time points will be included throughout the study to demonstrate the link between repression of basal PP2A activity and effects on functional outcomes such as cell signalling and cytokine expression.

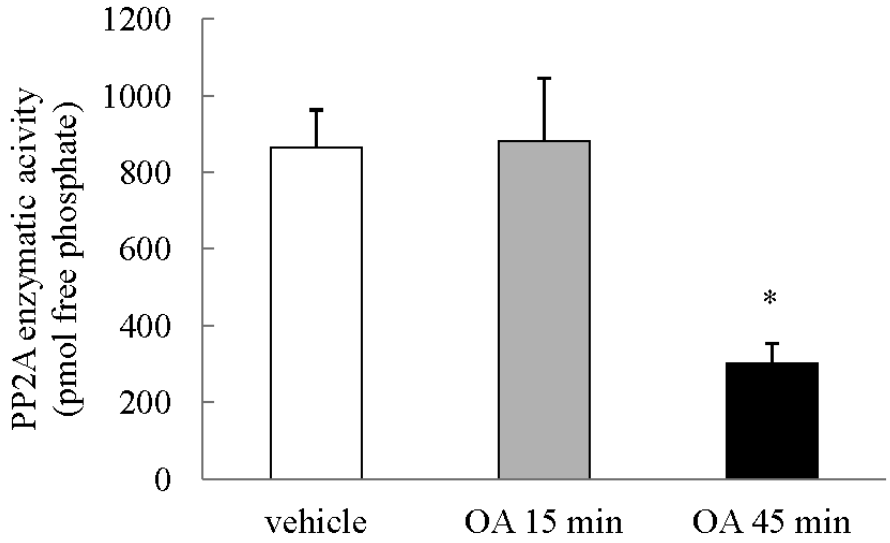


Figure 4.1 Temporal regulation of basal PP2A enzymatic activity by OA. PP2A enzymatic activity was measured in A549 cells treated for 15 min or 45 min with 1 µM OA, compared to vehicle-treated cells. Cells were washed and then PP2A enzymatic activity (measured as pmol free phosphate) detected at 1 h. Statistical analysis was performed using the Student's unpaired *t* test where * denotes a significant decrease in PP2A activity compared to vehicle (1 µM DMSO)-treated cells ($P<0.05$). Data are mean+SEM values from n=3 independent experiments.

4.3.2 Inhibition of basal PP2A phosphatase activity allows unrestrained action of MAPK phosphoproteins

PP2A dephosphorylates a number of kinases that drive inflammatory cell signaling; thus its inhibition allows unrestrained action of a number of downstream effectors. MAPKs family members (p38 MAPK, ERK and JNK) are important regulators of cytokine expression and are known to drive expression of two important cytokines implicated in asthmatic inflammation, IL-6 and IL-8 (Amrani et al., 2001; Henness et al., 2006; Quante et al., 2008; Moutzouris et al., 2010; Kobayashi et al., 2011). Accordingly, we examined the effect of OA on p38 MAPK, ERK and JNK phosphorylation by Western blotting. As shown in Figure 4.2A, treating cells for 45 min with OA robustly increased p38 MAPK phosphorylation at 0.5 and 1 h. ERK phosphorylation was enhanced at 30 min, and to a lesser extent at 1 h (Figure 4.2B). JNK is reported as an important inflammatory gene regulated by PP2A. It is also called early inflammatory gene (Shanley et al., 2001), in support of this statement we also found that inhibition of basal PP2A by OA enhanced early activation /phosphorylation of JNK at 30 min under these conditions (Figure 4.2C). Cells treated for the shorter time period of 15 min with OA did not show enhanced activity of MAPK phosphoproteins.

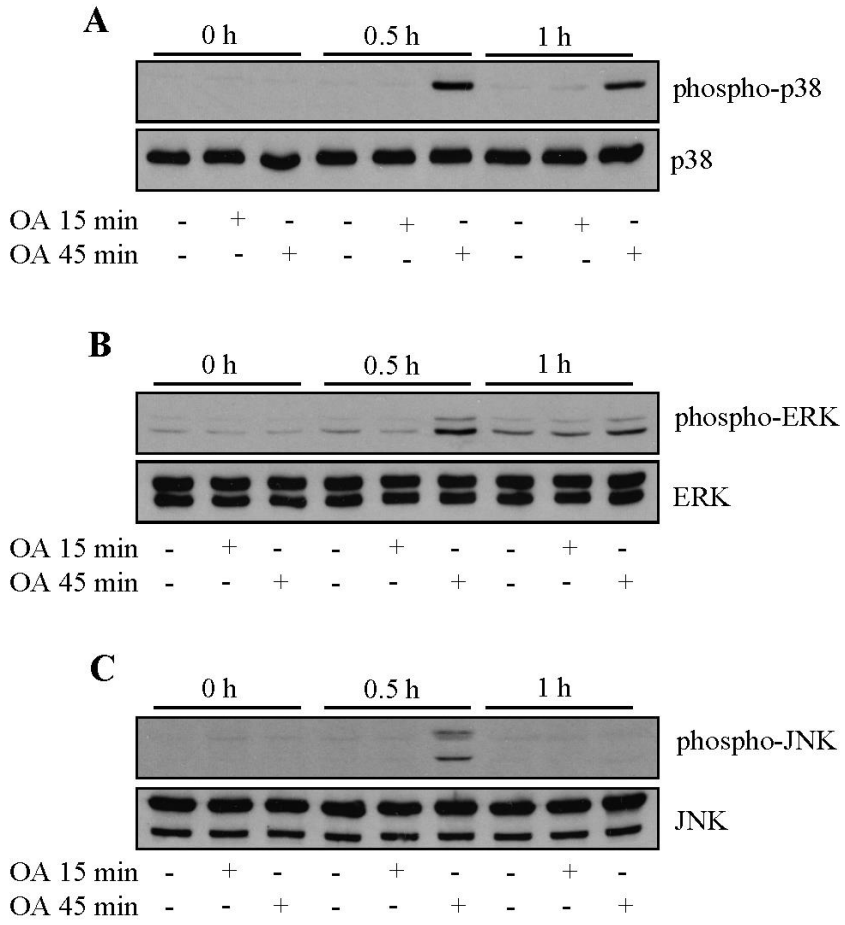


Figure 4.2 Inhibition of basal PP2A phosphatase activity allows unrestrained action of MAPK phosphoproteins. A549 cells were treated for 15 min or 45 min with 1 µM OA, compared to vehicle (1µM DMSO). Cells were washed and then lysates prepared at 0, 0.5 h, and 1 h, to compare temporal kinetics of (A) p38 MAPK (43 kDa), (B) ERK (42, 44 kDa) and (C) JNK (46, 54 kDa) phosphorylation by Western blotting (representative results (from n=5 independent experiments) are shown as cropped blots and full-length blots are presented in Supplementary Figure 1A (p38 MAPK), 1B (ERK) and 1C (JNK)).

4.3.3 Treating A549 cells with OA for 45 min, but not 15 min, significantly increases IL-6 mRNA expression and protein secretion

We then examined whether the inhibition of basal PP2A with OA has an effect on IL-6 mRNA expression and protein secretion. As shown in Figure 4.3B, 45 min treatment with OA induced significant upregulation of IL-6 mRNA expression in a temporally distinct manner, with the peak

of expression observed at 1 h (Figure 4.3B: $P<0.05$). This resulted in significant increase in IL-6 secretion observed at 8 and 24 h (Figure 4.3D: $P<0.05$). IL-6 production is p38 MAPK-mediated (Amrani et al., 2001; Quante et al., 2008), thus, taken together with our earlier results, these data indicate that significant repression of basal PP2A activity by 45 min treatment with OA allows p38 MAPK activation and corresponding increases in p38 MAPK-mediated cytokines such as IL-6. This PP2A-dependency of these results is confirmed by the lack of IL-6 mRNA expression and protein secretion in cells where basal PP2A activity was unaffected (see negative results in cells treated for only 15 min with OA (Figure 4.3A and 4.3C)).

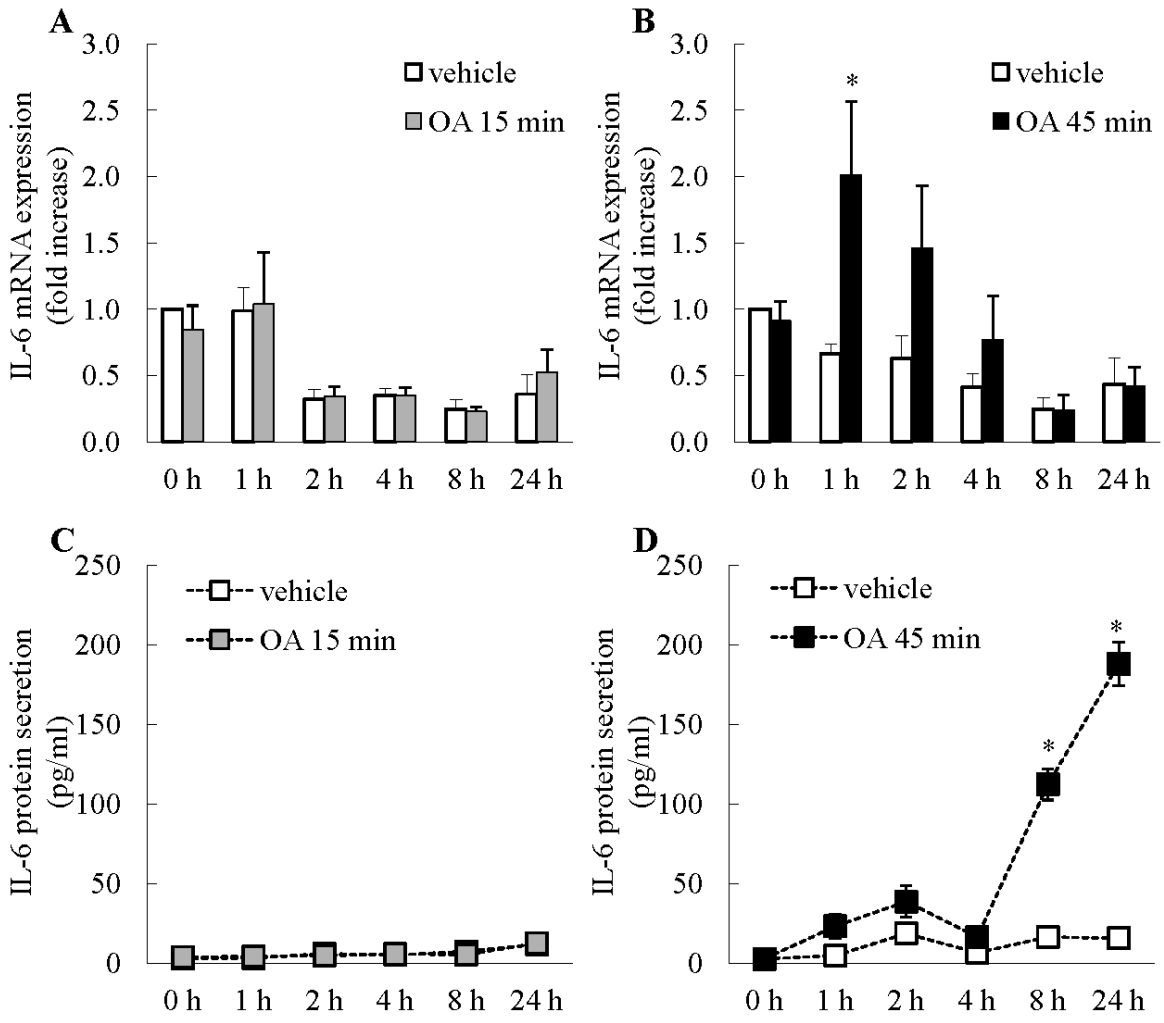


Figure 4.3 Treating A549 cells with OA for 45 min, but not 15 min, significantly increases IL-6 mRNA expression and protein secretion. A549 cells were treated for (A, C) 15 min or (B, D) 45 min with 1 µM OA, compared to vehicle (1µM DMSO). Cells were washed and then (A, B) IL-6 mRNA expression (results expressed as fold increase compared to vehicle-treated cells at 0 h) and (C, D) IL-6 protein secretion measured at 0, 1, 2, 4, 8, and 24 h. Statistical analysis was performed using two-way ANOVA then Bonferroni's post-test (where * denotes a significant effect compared to vehicle-treated cells (P<0.05)). Data are mean±SEM values from n=4 independent experiments.

4.3.4 Treating A549 cells with OA for 45 min, but not 15 min, significantly increases IL-8 mRNA expression and protein secretion

We then examined the effect of OA on IL-8 mRNA expression and protein secretion. Figure 4.4A reveals that 15 min treatment with OA has no effect on IL-8 mRNA expression but 45 min treatment significantly increased IL-8 mRNA at 1 h and 2 h time point (Figure 4.4B: $P<0.05$). Similarly, treatment with OA for 15 min did not increase IL-8 secretion above that achieved in cells treated with vehicle alone (Figure 4.4C), while significant increases in IL-8 secretion were observed at 4, 8, and 24 h after 45 min treatment with OA, compared to vehicle-treated cells (Figure 4.4D).

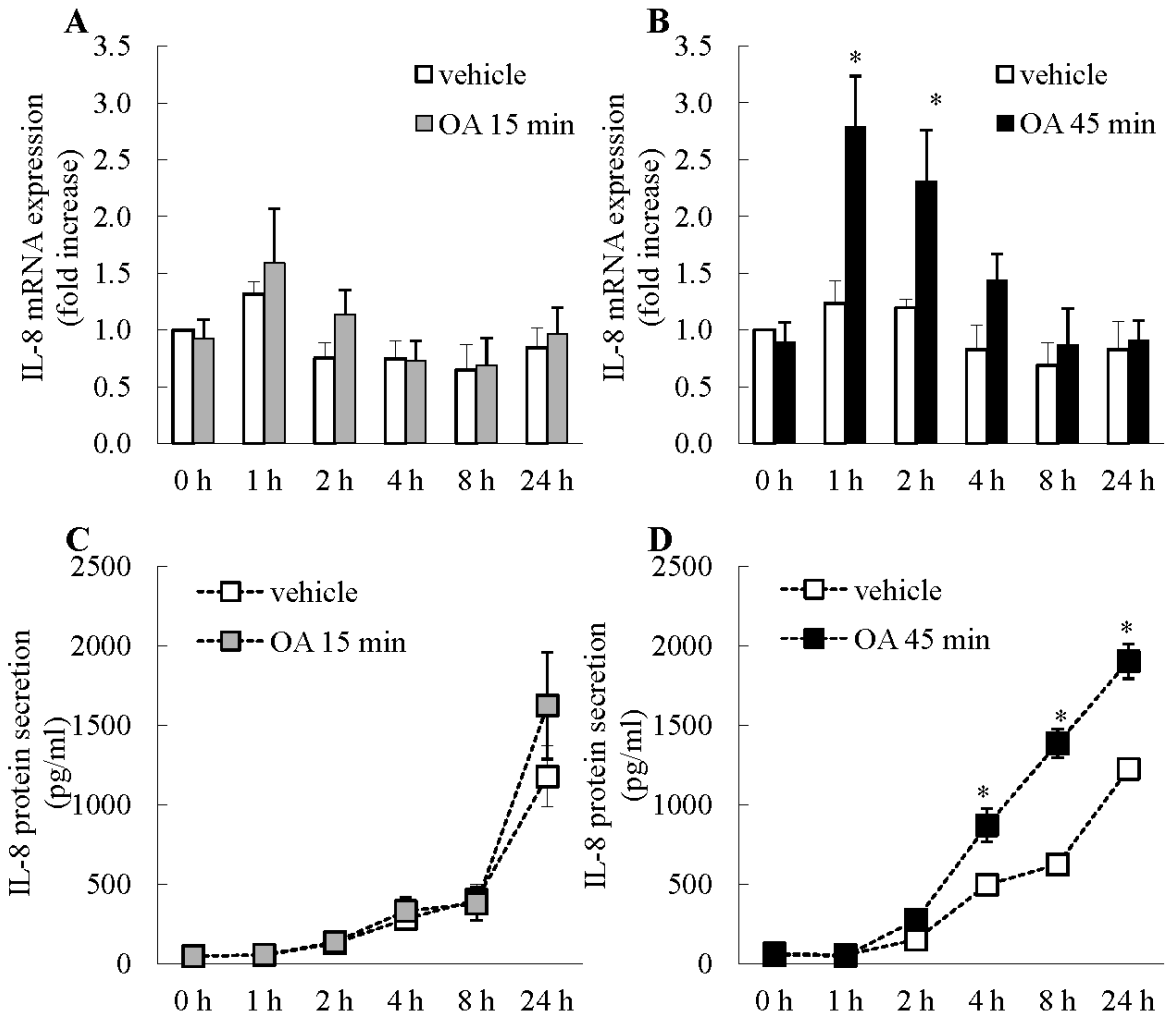
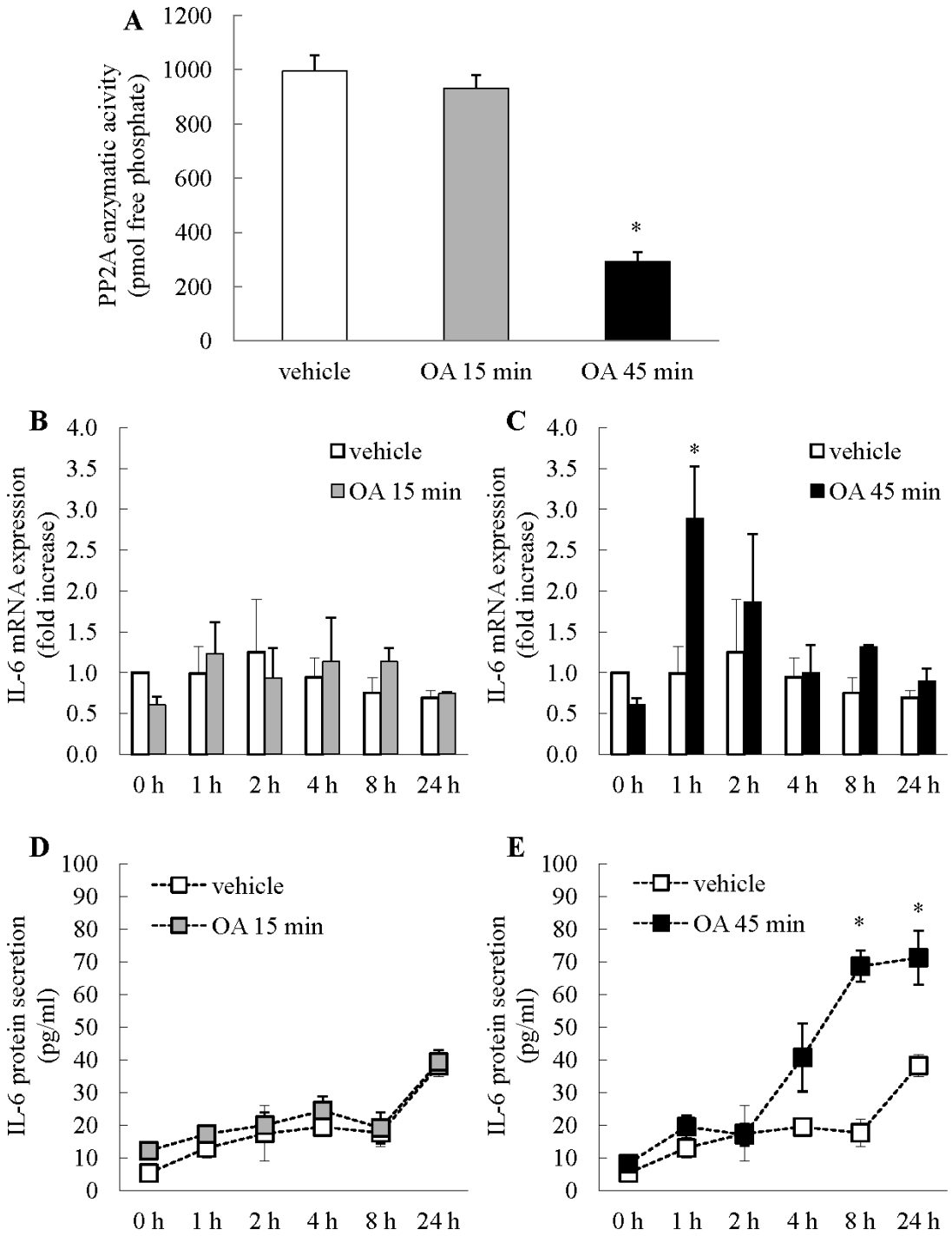


Figure 4.4 Treating A549 cells with OA for 45 min, but not 15 min, significantly increases IL-8 mRNA expression and protein secretion. A549 cells were treated for (A, C) 15 min or (B, D) 45 min with 1 µM OA, compared to vehicle (1µM DMSO). Cells were washed and then (A, B) IL-8 mRNA expression (results expressed as fold increase compared to vehicle-treated cells at 0 h) and (C, D) IL-8 protein secretion measured at 0, 1, 2, 4, 8, and 24 h. Statistical analysis was performed using two-way ANOVA then Bonferroni's post-test (where * denotes a significant effect compared to vehicle-treated cells (P<0.05)). Data are mean±SEM values from n=4 independent experiments.

4.3.5 Treating human bronchial epithelial cells (BEAS-2B) with OA for 45 min, but not 15 min, decreases PP2A enzymatic activity and significantly increases IL-6 and IL-8 mRNA expression and protein secretion

In order to confirm these findings in a second immortalized cell line, we utilized the human epithelial cell line BEAS-2B as they are commonly used in *in vitro* studies examining airway inflammation with relevance to asthma (Kaur et al., 2008; Cornell et al., 2009; King et al., 2009a; Holden et al., 2010; Collison et al., 2013a; Joshi et al., 2014). We conducted a series of confirmatory experiments (Figure 4.5) to demonstrate that treating BEAS-2B cells with OA for 45 min, but not 15 min, decreases PP2A enzymatic activity (Figure 4.5A) and significantly increases IL-6 mRNA expression (Figure 4.5C) and IL-6 secretion (Figure 4.5E) ($P<0.05$). IL-8 mRNA expression and protein secretion was also affected by 45 min treatment with OA (Figures 4.5G and 4.5I, respectively: $P<0.05$). These results confirm observations observed in A549 cells (Figures 4.3 and 4.4).


A
PP2A enzymatic acivity (pmol free phosphate)
1200
1000
800
600
400
200
0
*
vehicle
OA 15 min
OA 45 min
B
IL-6 mRNA expression (fold increase)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
vehicle
OA 15 min
0 h
1 h
2 h
4 h
8 h
24 h
C
IL-6 mRNA expression (fold increase)
*
vehicle
OA 45 min
D
IL-6 protein secretion (pg/ml)
100
90
80
70
60
50
40
30
20
10
0
vehicle
OA 15 min
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IL-6 protein secretion (pg/ml)
vehicle
OA 45 min
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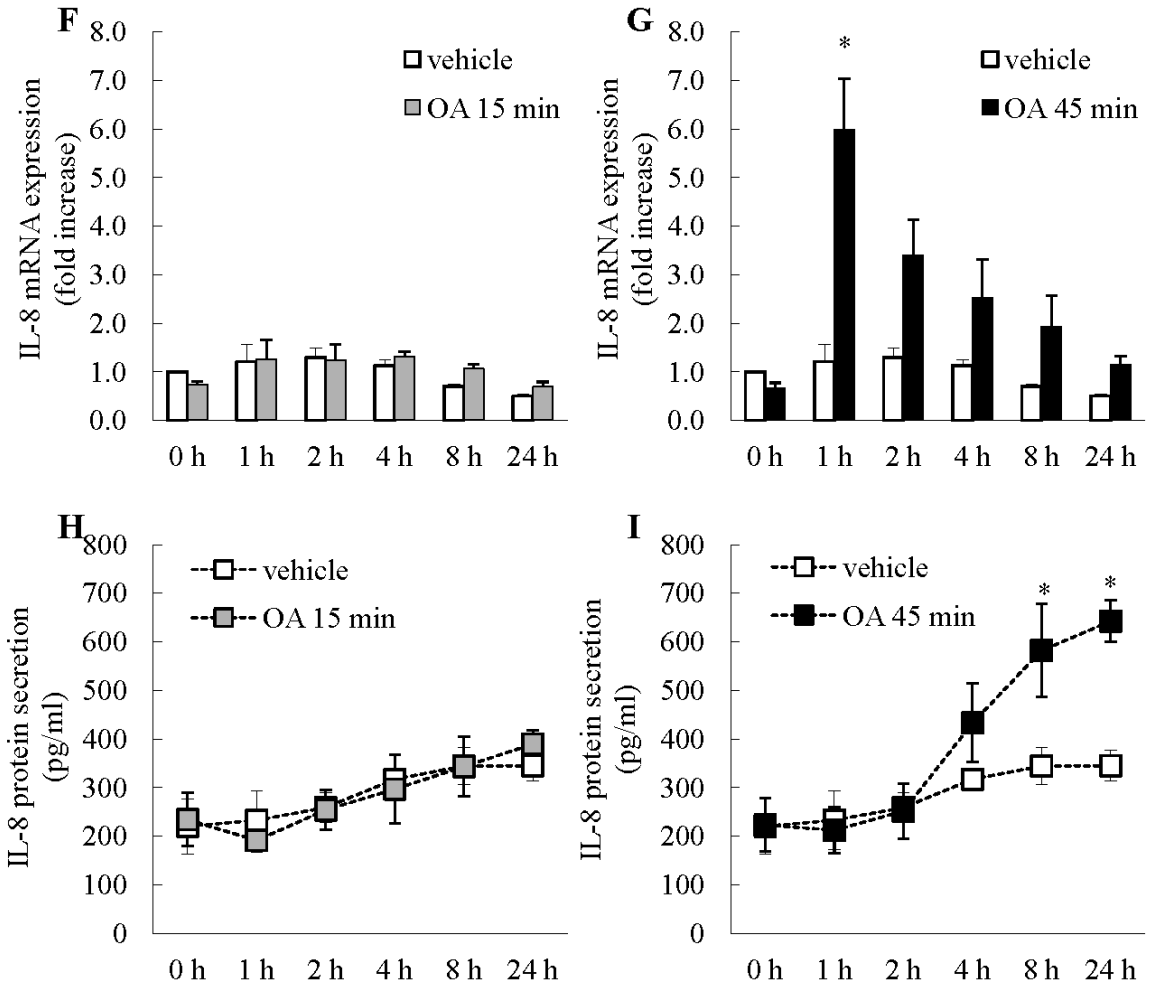


Figure 4.5 Treating human bronchial epithelial cells (BEAS-2B) with OA for 45 min, but not 15 min, decreases PP2A enzymatic activity and significantly increases IL-6 and IL-8 mRNA expression and protein secretion. (A) PP2A enzymatic activity was measured in BEAS-2B cells treated for 15 min or 45 min with 1 µM OA, compared to vehicle (1µM DMSO)-treated cells. Cells were washed and then PP2A enzymatic activity (measured as pmol free phosphate) detected at 1 h. Statistical analysis was performed using the Student's unpaired *t* test where * denotes a significant decrease in PP2A activity compared to vehicle-treated cells ($P<0.05$). BEAS-2B cells were treated for (B, D, F, H) 15 min or (C, E, G, I) 45 min with 1 µM OA, compared to vehicle. Cells were washed and then (B, C) IL-6 and (F, G) IL-8 mRNA expression (results expressed as fold increase compared to vehicle-treated cells at 0 h) and (D, E) IL-6 and (H, I) IL-8 protein secretion measured at 0, 1, 2, 4, 8, and 24 h. Statistical analysis was performed using two-way ANOVA then Bonferroni's post-test (where * denotes a significant effect compared to vehicle-treated cells ($P<0.05$)). Data are mean±SEM values from n=3 independent experiments.

4.3.6 Treating A549 cells with OA for 45 min, but not 15 min, significantly increases TTP mRNA expression and upregulation of TTP protein that is hyperphosphorylated

TTP is an important anti-inflammatory protein that is a direct target of PP2A (Brook et al., 2006) and can be regulated at multiple levels by p38 MAPK. TTP is an immediate early response gene whose expression is p38 MAPK-dependent (Mahtani et al., 2001) and once expressed its protein stability is regulated post-translationally by p38 MAPK-mediated phosphorylation of two key serines (Mahtani et al., 2001; Brook et al., 2006; King et al., 2009b). Importantly, this latter step also controls TTP function as an RNA destabilizing protein (phosphorylated – OFF; unphosphorylated – ON). Because PP2A controls TTP, we were interested to examine the impact of repression of PP2A activity on this TTP expression and function.

Firstly, we examined TTP mRNA expression, and as shown in Figure 4.6A, 15 min treatment with OA had no effect on the time course of TTP mRNA expression. In contrast, 45 min treatment with OA significantly increased TTP mRNA expression at 1 h (Figure 4.6B: $P<0.05$). Secondly, we measured TTP protein expression and phosphorylation with the rabbit antisera Sak21 (Mahtani et al., 2001; King et al., 2009b). As shown in Figure 4.6C, treating cells with OA for 45 min but not for 15 min, increases TTP protein level. Notably, we observe that the higher molecular weight immunoreactive bands for TTP predominate, especially at 1 h. This is consistent with earlier reports that inhibition of PP2A results in an equilibrium shift towards phosphorylated TTP, which is stable and builds up as a hyperphosphorylated (inactive) TTP (Brook et al., 2006; Sun et al., 2007).

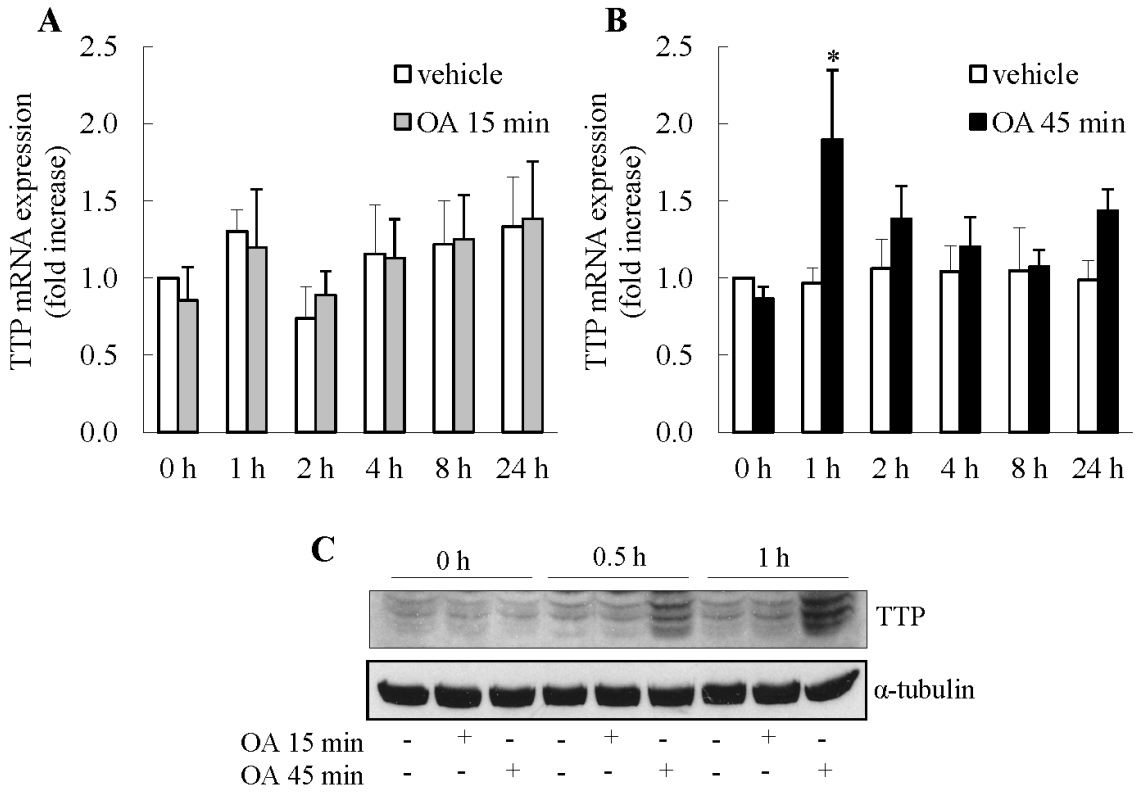


Figure 4.6 Treating A549 cells with OA for 45 min, but not 15 min, significantly increases TTP mRNA expression and upregulation of TTP protein that is hyperphosphorylated. A549 cells were treated for 15 min or 45 min with 1 μM OA, compared to vehicle (1μM DMSO). Cells were washed and at the indicated times (A, B) TTP mRNA expression (results expressed as fold increase compared to vehicle-treated cells at 0 h) and (C) TTP (43 to 49 kDa) protein upregulation was measured by Western blotting with α-tubulin as the loading control (representative results are shown as cropped blots and full-length blots are presented in Supplementary Figure 2). Please note that bands of immunoreactivity for TTP at higher molecular weight indicate phosphorylated forms of TTP. Statistical analysis was performed using two-way ANOVA then Bonferroni's post-test (where * denotes a significant effect compared to vehicle-treated cells (P<0.05)). Data are mean+SEM values from n=4 independent experiments.

4.3.7 The PP2A activator FTY720 overcomes OA-mediated inhibition of basal PP2A phosphatase activity and significantly represses IL-6 and IL-8 mRNA expression and cytokine secretion

Collectively, our data thus far implicates PP2A as a key regulator of cytokine expression via a MAPK/TTP-regulated network. That is, when PP2A is inhibited, cytokine secretion ensues. To demonstrate this further, we used a PP2A activator FTY720 (Roberts et al., 2010)) to overcome the effect of the PP2A inhibitor (OA). A549 cells were pre-treated with FTY720 before treatment for 45 min with OA, or vehicle. Firstly, we quantitated PP2A enzymatic activity and found that in FTY720-treated cells, OA is unable to inhibit PP2A activity to the same extent (Figure 4.7A: $P<0.05$). Secondly, we measured IL-6 and IL-8 mRNA expression at 1 h and protein secretion at 24 h under these conditions. Cells pre-treated with FTY720 had less significantly IL-6 (Figure 4.7B) and IL-8 (Figure 4.7C) mRNA expression than OA-treated controls. IL-6 and IL-8 protein secretion results also followed this pattern, as shown in Figure 4.7D and 4.7E, respectively.

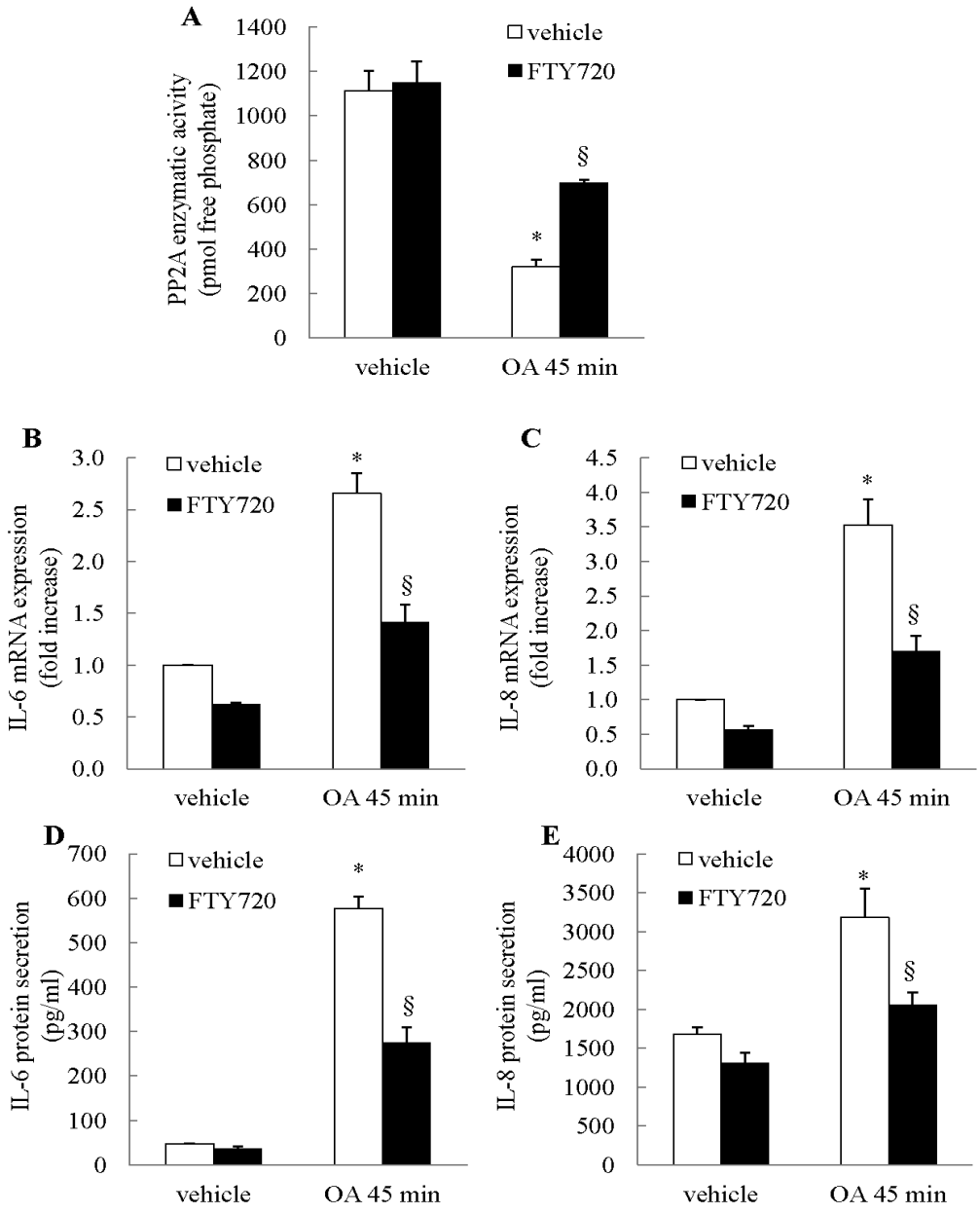


Figure 4.7 The PP2A activator FTY720 overcomes OA-mediated inhibition of basal PP2A phosphatase activity and significantly represses IL-6 and IL-8 mRNA expression and cytokine secretion. A549 cells were treated for 6 h with 2.5 µM FTY720 prior to 45 min with 1 µM OA, compared to vehicle (1µM DMSO). Cells were washed and then (A) PP2A activity measured at 1 h, (B, C) IL-6 and IL-8 mRNA expression measured at 1 h (results expressed as fold increase compared to vehicle-treated cells) and (D, E) IL-6 and IL-8 protein secretion measured at 24 h. Statistical analysis was performed using one-way ANOVA then Bonferroni's post-test (where * denotes a significant effect of OA or § FTY720) (P<0.05)). Data are mean+SEM values from n=3 independent experiments.

4.3.8 The PP2A activator devoid of sphingosine 1- phosphate agonism - AAL(S) - overcomes OA-mediated inhibition of basal PP2A phosphatase activity and significantly represses IL-6 and IL-8 mRNA expression and cytokine secretion

Although FTY720 is a known activator of PP2A, it also has other targets. Most notably in the context of asthma, FTY720 can induce S1P signalling and we have previously shown that S1P is elevated in allergic asthma (Ammit et al., 2001), drives development of the pro-asthmatic phenotype, and can induce IL-6 and IL-8 expression (Ammit et al., 2001; Rahman et al., 2014) . Although we did not observe upregulation of cytokine production with FTY720 in A549 cells, we still performed further experimentation with the FTY720 derivative AAL(S) (Don et al., 2007; Collison et al., 2013a; Hatchwell et al., 2014) because AAL(S) is devoid of S1P agonism. The data shown in Figure 4.8 serve to confirm that these effects are specific to PP2A by demonstrating that PP2A activation with AAL(S) can overcome OA-mediated inhibition of basal PP2A phosphatase activity and significantly repress IL-6 and IL-8 mRNA expression and cytokine secretion.

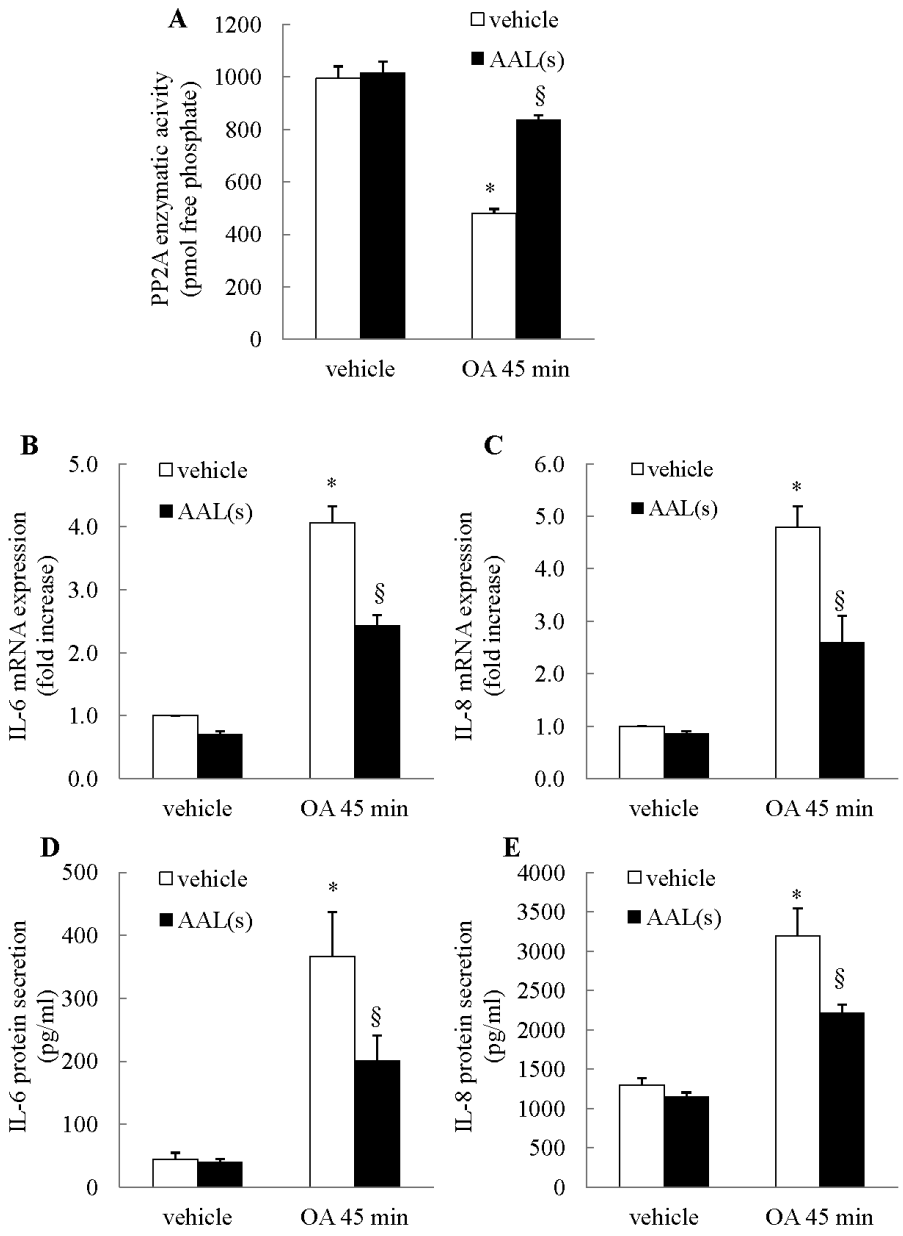


Figure 4.8 The PP2A activator devoid of sphingosine 1- phosphate agonism - AAL(S) - overcomes OA-mediated inhibition of basal PP2A phosphatase activity and significantly represses IL-6 and IL-8 mRNA expression and cytokine secretion. A549 cells were treated for 6 h with 2.5 µM AAL(S) prior to 45 min with 1 µM OA, compared to vehicle (1µM DMSO). Cells were washed and then (A) PP2A activity measured at 1 h, (B, C) IL-6 and IL-8 mRNA expression measured at 1 h (results expressed as fold increase compared to vehicle-treated cells) and (D, E) IL-6 and IL-8 protein secretion measured at 24 h. Statistical analysis was performed using one-way ANOVA then Bonferroni's post-test (where * denotes a significant effect of OA or § AAL(s) (P<0.05)). Data are mean+SEM values from n=3 independent experiments.

4.3.9 Overexpression of the catalytic subunit of PP2A (PP2A-C) overcomes OA-mediated inhibition of basal PP2A phosphatase activity and significantly represses IL-6 and IL-8 mRNA expression and cytokine secretion

As further substantiation of the role of PP2A in the control of cytokine secretion we overexpressed PP2A-C and measured the impact on IL-6 and IL-8 expression induced by OA. We first confirmed expression of PP2A-C in cells transfected with empty vector or PP2A-C (Figure 4.9A) and then examined the impact of OA treatment (45 min) on PP2A phosphatase activity and cytokine expression. As shown in Figure 4.9B, in cells where PP2A-C is overexpressed, OA is unable to inhibit PP2A activity to the same extent as controls. Further, cells transfected with PP2A-C plasmid significantly repressed OA-induced IL-6 and IL-8 mRNA expression (Figure 4.9C and 4.9D) and protein secretion (Figure 4.9E and 4.9F), respectively.

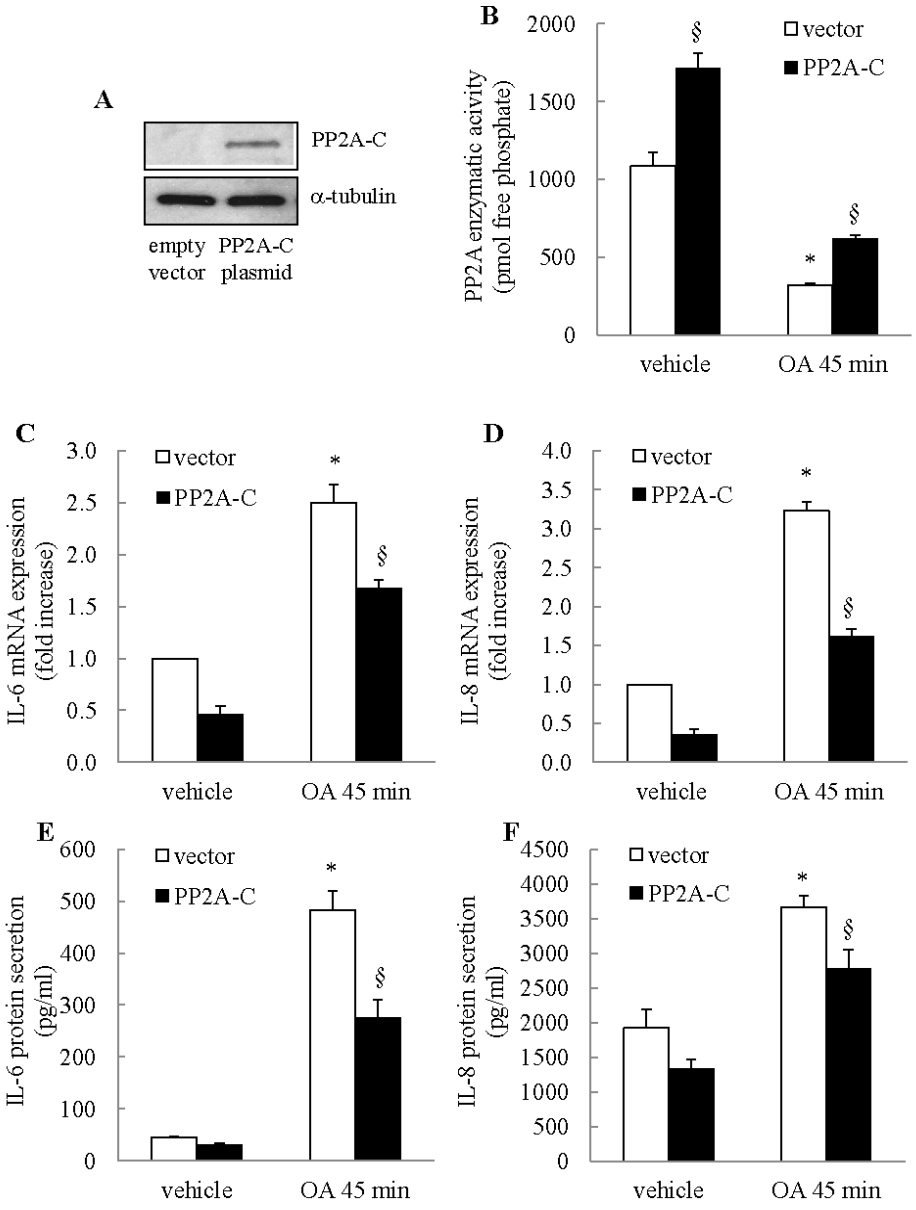


Figure 4.9 Overexpression of the catalytic subunit of PP2A (PP2A-C) overcomes OA-mediated inhibition of basal PP2A phosphatase activity and significantly represses IL-6 and IL-8 mRNA expression and cytokine secretion. A549 cells were transfected with empty vector or plasmid expressing PP2A-C, prior to 45 min with 1 µM OA, compared to vehicle (1µM DMSO). Cells were washed and then (A) PP2A-C (36 kDa) overexpression confirmed by Western blotting (representative results are shown as cropped blots and full-length blots are presented in Supplementary Figure 3), (B) PP2A activity measured at 1 h, (C, D) IL-6 and IL-8 mRNA expression measured at 1 h (results expressed as fold increase compared to vehicle-treated cells) and (E, F) IL-6 and IL-8 protein secretion measured at 24 h. Statistical analysis was performed using one-way ANOVA then Bonferroni's post-test (where * denotes a significant effect of OA or § PP2A-C) (P<0.05)). Data are mean+SEM values from n=3 independent experiments.

4.3.10 OA inhibits TNFα-induced PP2A phosphatase activity and increases TNFα-induced IL-6 and IL-8 mRNA expression and protein secretion

Our study thus far shows the important role played by basal PP2A enzymatic activity in restraining cytokine expression in unstimulated cells. To mimic the inflammatory milieu in asthma we now examined the impact of OA (for 45 min) on IL-6 and IL-8 expression stimulated by TNFα – a pro-inflammatory cytokine implicated in asthma. As shown in Figure 4.10A, TNFα increases PP2A activity and this can be significantly repressed by OA. We then examined the time course of TNFα-induced IL-6 (Figure 4.10B) and IL-8 (Figure 4.10C) mRNA expression and found that OA treatment significantly increased TNFα-induced cytokine expression at a number of time-points and that this resulted in significant increase in TNFα-induced IL-6 and IL-8 secretion at 24 h (Figures 4.10D and 4.10E, respectively: $P<0.05$).

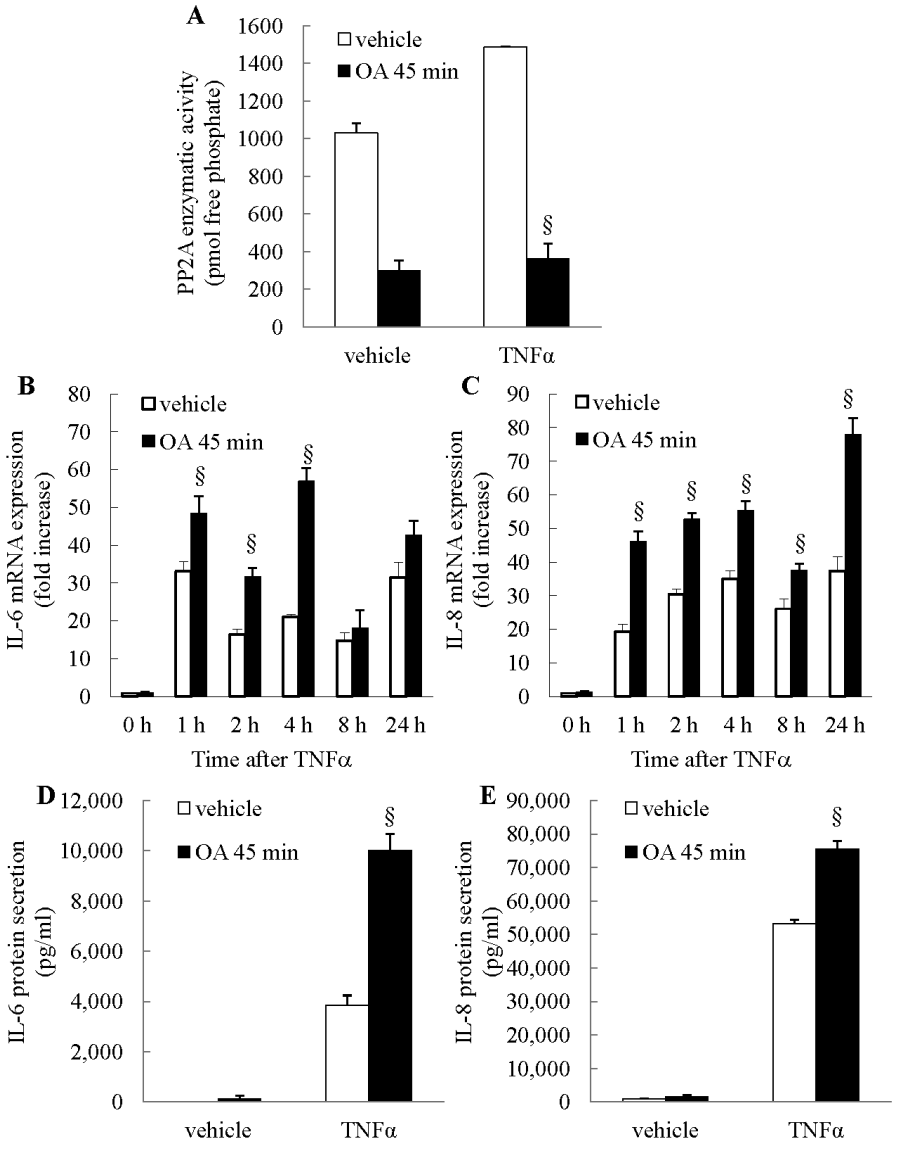


Figure 4.10 OA inhibits TNFα-induced PP2A phosphatase activity and increases TNFα-induced IL-6 and IL-8 mRNA expression and protein secretion. (A) PP2A enzymatic activity was measured in A549 cells treated for 45 min with vehicle (1µM DMSO) or 1 µM OA. Cells were washed before stimulation with TNFα (4 ng/ml) and then PP2A enzymatic activity (measured as pmol free phosphate) was detected at 1 h. Statistical analysis was performed using one-way ANOVA then Bonferroni's post-test (where § denotes a significant effect of OA on on TNFα-induced effects). (B-E) A549 cells were treated for 45 min with vehicle or 1 µM OA. Cells were washed before stimulation with TNFα (4 ng/ml). (B) IL-6 and (C) IL-8 mRNA expression was measured at 0, 1, 2, 4, 8, and 24 h (results expressed as fold increase compared to vehicle-treated cells at 0 h) and (D) IL-6 and (E) IL-8 protein secretion measured at 24 h. Statistical analysis was performed using two-way ANOVA then Bonferroni's post-test (where §denotes a significant effect of OA on TNFα-induced effects (P<0.05)). Data are mean±SEM values from n=3 independent experiments.

4.3.11 Specific knockdown of PP2A with siRNA reduces TNFα-induced PP2A protein levels and activity and results in increased TNFα-induced IL-8 and IL-6 mRNA expression and protein secretion

Finally, to demonstrate that these OA-mediated effects were specific to PP2A, A549 cells were transfected with scrambled control or siRNA against PP2A-C before TNFα stimulation. When PP2A-C was knocked down (confirmed by Western blotting in Figure 4.11A) we observed significant reduction in TNFα-induced PP2A enzymatic activity (Figure 4.11B) with a corresponding upregulation of TNFα-induced IL-6 and IL-8 mRNA expression and protein secretion from A549 cells (Figures 4.11C-4.11F, respectively: $P<0.05$)

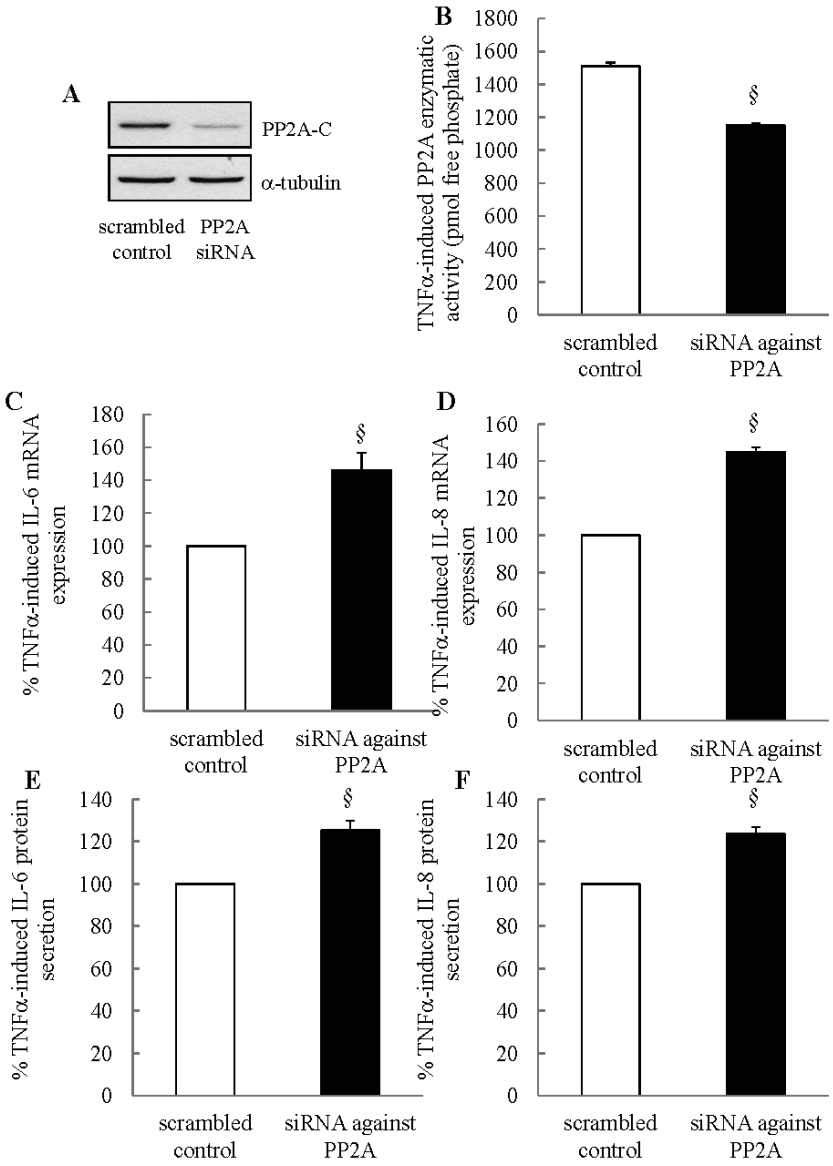


Figure 4.11 Specific knockdown of PP2A with siRNA reduces TNFα-induced PP2A protein levels and activity and results in increased TNFα-induced IL-8 and IL-6 mRNA expression and protein secretion. A549 cells transfected with scrambled control or siRNA against PP2A-C were stimulated with TNFα (4 ng/ml) before: (A) PP2A-C (36 kDa) knockdown at 1 h was confirmed by Western blotting (representative results are shown as cropped blots and full-length blots are presented in Supplementary Figure 4); (B) PP2A enzymatic activity detected at 1 h (measured as pmol free phosphate); (C) IL-6 and (D) IL-8 mRNA expression measured at 1 h (results expressed as % of TNFα-induced mRNA expression in scrambled control (designated as 100%)) ; and (E, F) IL-6 and IL-8 protein secretion measured at 24 h (results expressed as % of TNFα-induced protein secretion in scrambled control (designated as 100%)). Statistical analysis was performed using Student's unpaired t test (where § denotes a significant effect of siRNA against PP2A-C on TNFα-induced effects (P<0.05)). Data are mean±SEM values from n=3 independent experiments.

4.4 Discussion

To explore the role of basal PP2A in cytokine expression in human lung epithelial cells, we took multiple approaches to modulate PP2A phosphatase activity by using a PP2A inhibitor, OA; two PP2A activators, FTY720 and AAL(S); an expression plasmid to overexpress PP2A-C; and siRNA to knockdown PP2A-C. These studies reveal the important role played by PP2A in cytokine regulation in the context of airway inflammation and demonstrate the link between perturbation of PP2A activity and effects on functional outcomes such as cell signalling and cytokine expression.

Several studies have demonstrated that PP2A serves as a negative regulator of MAPKs (Sontag et al., 1993; Prickett and Brautigan, 2007; Junttila et al., 2008) and in support we found that inhibition of PP2A by OA upregulates p38 MAPK, ERK and JNK phosphorylation in A549 cells. These results demonstrate that the basal activity of PP2A in untreated cells restrains MAPK-mediated cell signalling in A549 cells. In our previous studies we demonstrated that IL-6 and IL-8 are the two major cytokines upregulated via MAPK-mediated pathways (Amrani et al., 2001; Henness et al., 2006; Quante et al., 2008; Moutzouris et al., 2010). JNK has been reported to be regulated by PP2A and play a role in IL-8 production in models of TNFα and lipopolysaccharide-induced lung inflammation, with an important consequence for severe asthma (Kobayashi et al., 2011; Kobayashi et al., 2012).

Notably, PP2A inhibition also has a significant impact on the critical anti-inflammatory protein - TTP. TTP is an mRNA destabilizing protein that targets numerous cytokines (Brooks and Blackshear, 2013), including those involved in asthmatic inflammation. Its expression and function are p38 MAPK-dependent (Mahtani et al., 2001; Sun et al., 2007). There is a dynamic equilibrium between unphosphorylated and phosphorylated forms of TTP. The unphosphorylated

form of TTP is active and capable to decay cytokine mRNA. However, this form of TTP is unstable and undergoes proteosomal degradation, whereas phosphorylated TTP is stable but unable to target cytokines mRNA for decay. Importantly, TTP is a direct target of PP2A; as PP2A phosphatase activity is responsible for dephosphorylation of TTP at two key serines (Ser^{52} and Ser^{178}) (Brook et al., 2006). In this way, PP2A shifts this balance of TTP towards unphosphorylated TTP via dephosphorylation (Brook et al., 2006; Sun et al., 2007). Our study demonstrates that inhibition of PP2A (with OA for 45 min) significantly induces TTP mRNA expression but the resulting TTP protein has electrophoretic mobility in immunoblots indicative of hyperphosphorylated forms of TTP. Our study concurs with previous reports that demonstrate that inhibition of PP2A phosphatase activity causes accumulation of hyperphosphorylated and stable TTP protein (Brook et al., 2006; Clement et al., 2011), suggesting that in the absence of PP2A activity, the balance is shifted towards stable, but inactive, phosphorylated forms of TTP.

Our study utilizing OA demonstrates the important anti-inflammatory control exerted by PP2A phosphatase basal activity in A549 lung epithelial cells. These studies were confirmed in BEAS-2B cells. Given that OA is a non-selective pharmacological inhibitor, we have directly implicated PP2A with PP2A-C overexpression as well as PP2A activators. A number of small molecules have been reported to activate PP2A (Perrotti and Neviani, 2013). To date the best known of these is the sphingosine analog FTY720 (fingolimod) and we have utilized it herein overcome OA-mediated repression of PP2A enzymatic activity and repress cytokine expression. But it is important to note that FTY720 is also an agonist/antagonist of the S1P pathway and we and others have shown that S1P is pro-inflammatory and pro-asthmatic (Ammit et al., 2001; Roviezzo et al., 2007; Nishiuma et al., 2008; Rahman et al., 2014). Therefore we utilized

AAL(S), a PP2A activator devoid of S1P agonism (Don et al., 2007; Collison et al., 2013a; Hatchwell et al., 2014) to confirm the anti-inflammatory effect of basal PP2A in A549 cells.

Our study demonstrates that basal PP2A activity restrains cytokine expression in a cellular model of asthmatic inflammation and highlights an important role for MAPKs and TTP. Moreover, PP2A plays an important role in cytokine expression in cells stimulated with TNFα; we show that inhibition of PP2A with OA, and more specifically with PP2A-C knockdown by siRNA, results in significant increases in cytokine production. Taken together our study has revealed, in part, the molecular mechanisms of PP2A anti-inflammatory function and highlight the potential of boosting the power of endogenous phosphatases as novel anti-inflammatory strategies to combat asthmatic inflammation.

Chapter 5
Activating Protein Phosphatase 2A (PP2A) Enhances Tristetraprolin (TTP) Anti-Inflammatory Function in A549 Lung Epithelial Cells

This work has been published in the article entitled "Activating Protein Phosphatase 2A (PP2A) Enhances Tristetraprolin (TTP) Anti-Inflammatory Function in A549 Lung Epithelial Cells. **Md. Mostafizur Rahman**, Nowshin N. Rumzhum, Jonathan C. Morris, Andrew R. Clark, Nicole M. Verrills and Alaina J. Ammit. *Cell Signal*. 2016 Apr;28(4):325-34."

Chapter 5
Activating PP2A Enhances TTP Anti-Inflammatory Function in A549 Lung Epithelial Cells

5.1 Introduction

In Chapter 4, we successfully demonstrate the anti-inflammatory role of basal PP2A and confirm the possible link with TTP activation. Besides, we propose that enhancing the power of endogenous PP2A may have potential to act as an alternative anti-inflammatory strategy in asthmatic inflammation. Based on the results of our studies and previously published reports (Perrotti and Neviani, 2008; Wallace et al., 2012) we hypothesize that activating PP2A may boost the anti-inflammatory function of TTP. We aim to confirm this hypothesis here in this study.

TTP is an mRNA destabilizing molecule that acts at the post-transcriptional level to inhibit the expression of many pro-inflammatory cytokines responsible for respiratory disease pathogenesis (reviewed in (Prabhala and Ammit, 2015)). TTP is regulated by phosphorylation at a number of levels; gene expression, mRNA and protein stability is p38 MAPK-dependent (Mahtani et al., 2001; Ross et al., 2015) and its anti-inflammatory action is controlled by the phosphorylation status of TTP protein at Ser^{52} and Ser^{178} (Mahtani et al., 2001; Marchese et al., 2010; Ross et al., 2015). There is a dynamic equilibrium between unphosphorylated and phosphorylated TTP. 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., 2006; Ross et al., 2015). When TTP is phosphorylated, the protein is stabilized (Tchen et al., 2004; Brook et

al., 2006; Hitti et al., 2006), but this phosphorylated form of TTP is unable to cause mRNA decay, and therefore is inactive (Marchese et al., 2010).

When active, TTP has a substantial repressive impact on airway inflammation (Ross et al., 2015). However, under respiratory disease conditions, although TTP is present, it is likely held in a dormant, inactive form due to the high levels of p38 MAPK phosphorylation (Renda et al., 2008). Therefore developing a strategy to activate TTP under inflammatory conditions could be therapeutically useful. This dynamic equilibrium provides us with important points of regulatory control that we propose to exploit to enhance TTP function. However, blocking p38 MAPK with inhibitors in an attempt to promote greater amounts of unphosphorylated (active) TTP doesn't work because TTP is a p38 MAPK-responsive gene and this will result in less TTP mRNA expression (Mahtani et al., 2001; Prabhala et al., 2015). A better approach is to allow TTP to be expressed and then activate its mRNA destabilizing function. This is the promise of PP2A, a serine/threonine phosphatase responsible for dephosphorylation of TTP at Ser^{52} and ser^{178} (Sun et al., 2007); amongst other molecules (Shanley et al., 2001; Miskolci et al., 2003; Junttila et al., 2008). PP2A is a ubiquitously expressed enzyme and in a recent publication (Rahman et al., 2015) we demonstrated the important regulatory role exerted by PP2A under basal (unstimulated conditions) in the A549 lung epithelial cell line. When PP2A basal enzymatic activity was removed, i.e. with the PP2A inhibitor okadaic acid or via specific knockdown of PP2A-C with siRNA, TTP was hyper-phosphorylated and rendered non-functional (Rahman et al., 2015). Hence, proinflammatory cytokine production ensued. Therefore it follows that if TTP is switched off when PP2A activity is reduced, the corollary of these results is that TTP will be switched on by PP2A activators. This was the case under unstimulated conditions (Rahman et al., 2015), but

whether PP2A activators can switch TTP on and repress cytokine production under inflammatory conditions mimicked *in vitro* was currently unknown.

We address this herein by utilizing a cellular model of airway inflammation using A549 lung epithelial cells (Cornell et al., 2009; King et al., 2009b; Rahman et al., 2015) stimulated with TNFα to mimic pro-inflammatory conditions *in vitro* (Cornell et al., 2009; Rahman et al., 2015). We measure the impact on two key TTP targets implicated in respiratory disease, IL-8 and IL-6, as a functional outcome. We examine the ability of the sphingosine analog, FTY720, and the chiral analog of FTY720 devoid of S1P agonism, AAL(S) (Don et al., 2007; Collison et al., 2013a; Hatchwell et al., 2014; Rahman et al., 2015) to activate PP2A and repress TNFα-induced cytokine production in a TTP- dependent manner. Thus, in this study we address the hypothesis that PP2A activators can switch TTP on and repress cytokine production in an *in vitro* model of airway inflammation, and show for the first time that the PP2A activators FTY720 and AAL(S) repress TNFα-induced IL-8 and IL-6 mRNA expression and protein secretion in a TTP-dependent manner.

5.2 Materials and Methods

5.2.1 Chemicals

FTY720 was purchased from the Cayman Chemical Company (Ann Arbor, MI) and AAL(S) was synthesized (Don et al., 2007; Collison et al., 2013a; Hatchwell et al., 2014; Rahman et al., 2015). TNFα is from R&D Systems (Minneapolis, MN). Unless otherwise specified, all chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO).

5.2.2 Cell culture

Please see Chapter 4.2.2

5.2.3 PP2A activity assay

For detailed description, see Chapter 2.2.8.

5.2.4 Real-time RT-PCR

Please see Chapter 4.2.5

5.2.5 ELISA

IL-8 and IL-6 ELISAs were performed according to the manufacturer's instructions (BD Biosciences Pharmingen, San Diego, CA). For detailed description, see Chapter 2.2.5.

5.2.6 Western blotting

TTP was measured by Western blotting using rabbit antisera against TTP (Sak21) (Mahtani et al., 2001). Detection of α-tubulin was used as the loading control (mouse monoclonal IgG_1, DM1A: Santa Cruz Biotechnology, Santa Cruz, CA). Primary antibodies were detected with goat anti-rabbit and anti-mouse HRP-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA) and visualized by enhanced chemiluminescence (PerkinElmer, Wellesley, MA). Densitometry analysis was performed using ImageJ**.**

5.2.7 siRNA

A549 cells (5×10^5 cells/well) were transfected with siRNA against TTP, or scrambled control, by reverse transfection with RNAiMAX according to the manufacturer's protocols (Invitrogen, NY, USA). Untransfected controls were also performed. Specifically, for each well of 6-well plates, 800 ng of ON-Target plus Control Non-targeting siRNA (scrambled control) or ON-target plus SMART pool Human TTP siRNA (aka Zfp36: both from Dharmacon, Thermo-Fisher Scientific, Waltham, MA) was diluted in 500 μL of Opti-MEM Reduced Serum Media (Invitrogen). This was followed by the addition of 5 μL of RNAiMAX reagent (Invitrogen) into each well and incubation at room temperature for 20 min. After transfection, cells were cultured for 24 h in media without antibiotics, and then growth-arrested for 16 h in Ham's F-12K supplemented with 0.1% BSA, supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), before stimulation with TNFα (4 ng/ml).

5.2.8 Statistical analysis

Statistical analysis was performed using either the Student's unpaired *t* test, one-way or two-way ANOVA followed by Bonferroni's post-test. *P* values <0.05 were sufficient to reject the null hypothesis for all analyses. Data are mean+SEM of n≥3 independent replicates.

5.3 Results

5.3.1 PP2A activators, FTY720 and AAL(S), enhance TNFα-induced PP2A phosphatase activity in A549 cells

PP2A is a ubiquitous enzyme that exerts basal phosphatase activity. In confirmation of our earlier study (Rahman et al., 2015), we show in Figure 5.1 that this basal activity can be

significantly increased by stimulation for 1 h with TNFα ($P<0.05$). We then examined the impact of two PP2A activators: 1) the classical PP2A activator - FTY720; 2) the novel PP2A activator and chiral analog of FY720 devoid of S1P agonism – AAL(S). Notably, both PP2A activators can significantly augment TNFα-induced PP2A activity (Figures 5.1A & 5.1B: $P<0.05$), but are without effect when added to unstimulated cells.

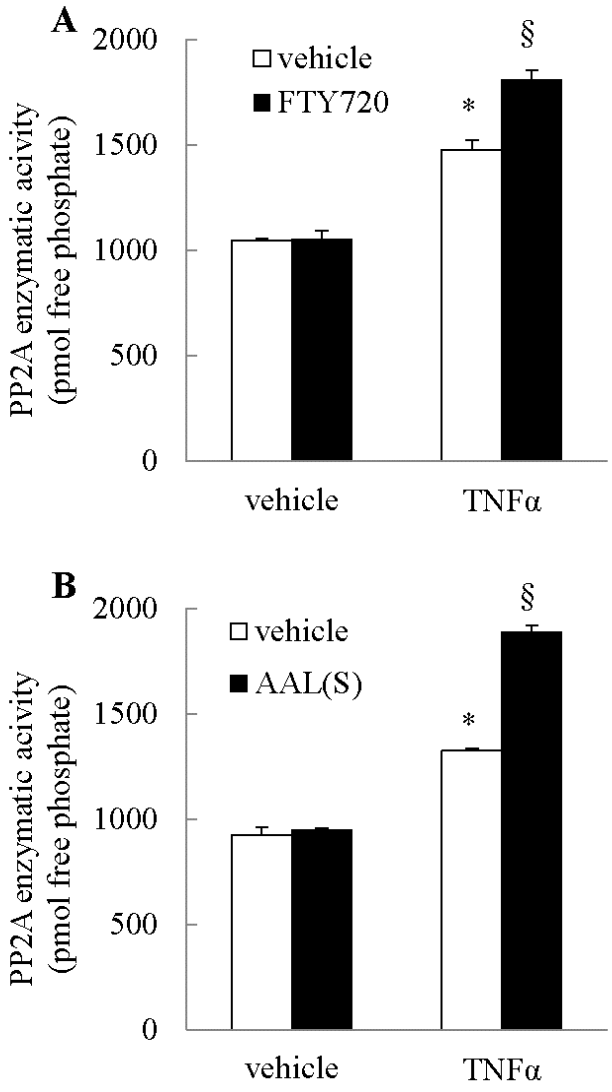


Figure 5.1 PP2A activators, FTY720 and AAL(S), enhance TNFα-induced PP2A phosphatase activity in A549 cells. A549 cells were pre-treated with (A) 2.5 μM FTY720 or (B) 2.5 μM AAL(S) for 6 h, compared to vehicle (2.5 μM DMSO)-treated controls. Cells were treated with vehicle or TNFα (4 ng/ml) and then PP2A enzymatic activity (measured as pmol free phosphate) detected at 1 h. Statistical analysis was performed using one-way ANOVA then Bonferroni's post-test (where * denotes a significant effect of TNFα compared to vehicle-treated cells, and § denotes a significant effect of PP2A activators on TNFα-induced PP2A activity (P<0.05)). Data are mean+SEM values from n=3 independent experiments.

5.3.2 PP2A activators, FTY720 and AAL(S), significantly repress TNFα-induced IL-8 and IL-6 mRNA expression and protein secretion

We then wished to examine the impact of PP2A activation with FTY720 and AAL(S) on TNFα-induced expression kinetics of two TTP targets - IL-8 (Figure 5.2) and IL-6 (Figure 5.3). As shown in Figure 5.2, TNFα significantly increased IL-8 mRNA expression (Figures 5.2A & 5.2C) and protein secretion (Figures 5.2B & 5.2D) from A549 cells ($P<0.05$). Significant levels of IL-8 mRNA expression was first observed at 1 h and remained elevated throughout the 24 h period ($P<0.05$). Protein production ensued with significantly elevated levels of IL-8 secretion detected from 4 h ($P<0.05$). Notably, TNFα-induced cytokine production could be repressed by pretreatment with PP2A activators. FTY720 significantly repressed TNFα-induced IL-8 mRNA expression (Figure 5.2A) and cytokine secretion (Figure 5.2B) ($P<0.05$). IL-8 mRNA expression (Figure 5.2C) and cytokine secretion (Figure 5.2D) was similarly repressed by pretreatment with AAL(S). To extend these observations, we also examined the impact of PP2A activators on another pro-inflammatory cytokine implicated in chronic respiratory disease - IL-6 - and results shown in Figure 5.3 confirm that PP2A activators significantly repress TNFα-induced IL-6 mRNA expression (Figures 5.3A & 5.3C) and protein secretion (Figures 5.3B & 5.3D) from A549 cells ($P<0.05$).

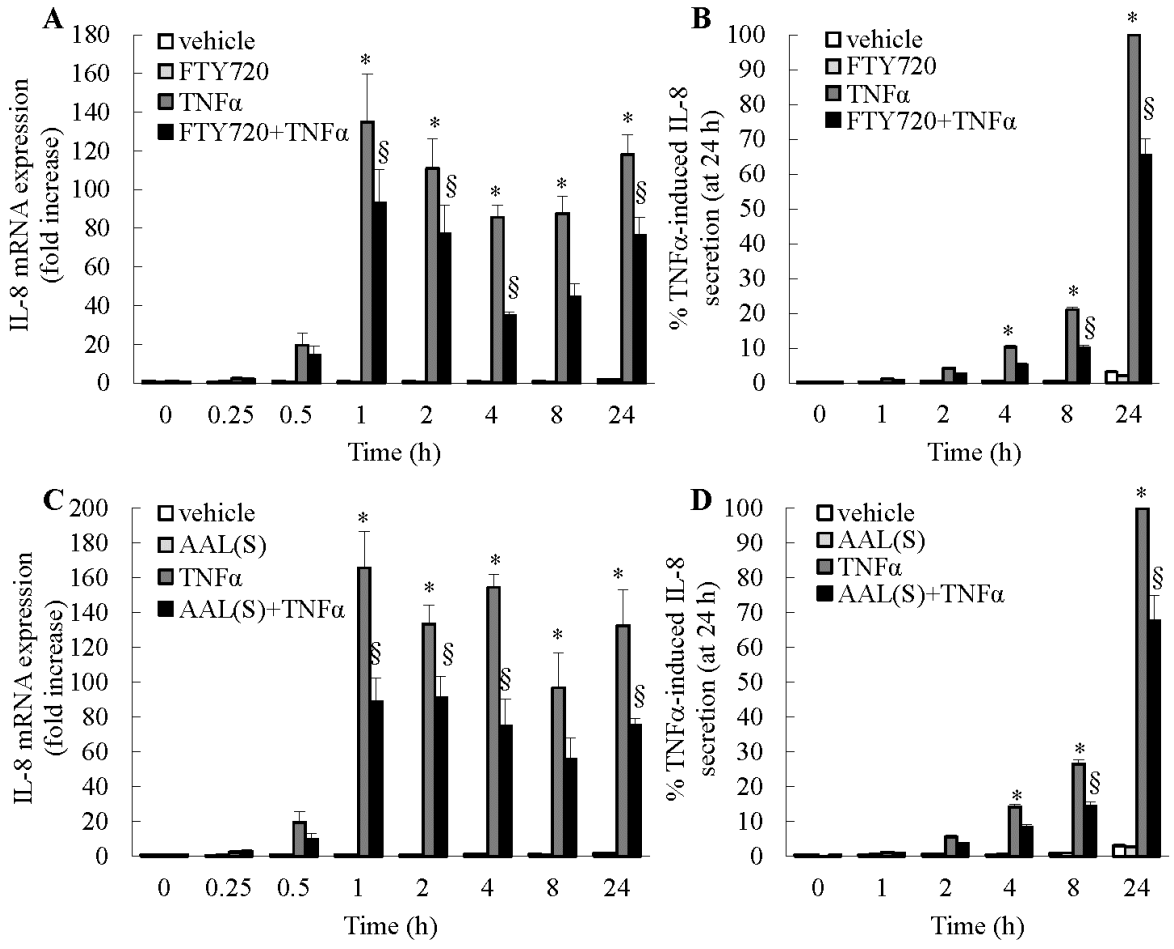


Figure 5.2 PP2A activators, FTY720 and AAL(S), significantly repress TNFα-induced IL-8 mRNA expression and protein secretion. A549 cells were pre-treated with (A, B) 2.5 μM FTY720 or (C, D) 2.5 μM AAL(S) for 6 h, compared to vehicle (2.5 μM DMSO)-treated controls. Cells were treated with vehicle or TNFα (4 ng/ml) and then (A, C) IL-8 mRNA expression (results expressed as fold increase compared to vehicle-treated cells at 0 h) and (B, D) IL-8 protein secretion measured (results expressed as a percentage of TNFα-induced IL-8 secretion at 24 h) at the indicated times. Statistical analysis was performed using two-way ANOVA then Bonferroni's post-test (where * denotes a significant effect of TNFα compared to vehicle-treated cells, and § denotes significant repression of TNFα-induced cytokine production by PP2A activators (P<0.05)). Data are mean+SEM values from n=4-5 independent experiments.

5.3.3 Specific knockdown of TTP with siRNA increases TNFα-induced IL-8 and IL-6 mRNA expression and protein secretion

So far we have shown that PP2A activators - FTY720 and AAL(S) - increase PP2A activity and significantly inhibit TNFα-induced IL-8 and IL-6 mRNA expression and cytokine secretion (Figures 5.1-5.3). We hypothesize that PP2A activators mediate their anti-inflammatory effect in a TTP-dependent manner. Towards testing this link, we first needed to show the impact of TTP on cytokine production under inflammatory conditions. To do this, we transiently transfected A549 cells with siRNA against TTP and stimulated cells with TNFα, compared to relevant controls (Figure 5.4). As expected, TNFα significantly increased TTP mRNA and TTP protein expression after just 1 h (P<0.05). As shown in Figure 5.4A, siRNA against TTP significantly repressed TNFα-induced TTP mRNA expression (P<0.05). TNFα-induced TTP protein production was similarly knocked down by siRNA against TTP as demonstrated by Western blot (Figure 5.4B). Importantly, when TTP was reduced by siRNA, there was a corresponding increase in IL-8 and IL-6 mRNA expression (Figures 5.4C & 5.4E) and cytokine secretion (Figures 5.4D & 5.4F) from A549 cells (P<0.05). These results demonstrate that TTP represses cytokine production in airway inflammatory conditions modeled *in vitro.*

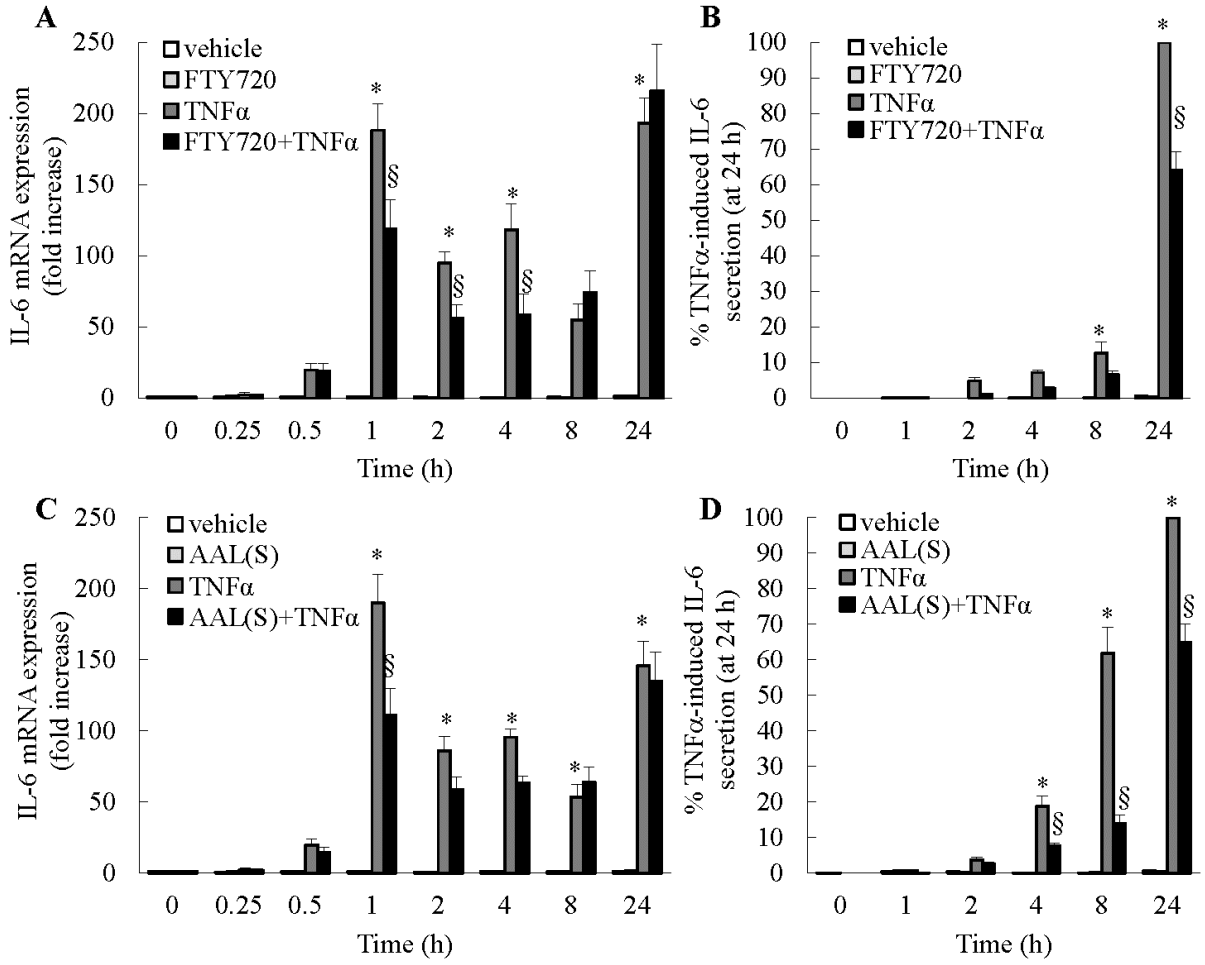


Figure 5.3 PP2A activators, FTY720 and AAL(S), significantly repress TNFα-induced IL-6 mRNA expression and protein secretion. A549 cells were pre-treated with (A, B) 2.5 μM FTY720 or (C, D) 2.5 μM AAL(S) for 6 h, compared to vehicle (2.5 μM DMSO)-treated controls. Cells were treated with vehicle or TNFα (4 ng/ml) and then (A, C) IL-6 mRNA expression (results expressed as fold increase compared to vehicle-treated cells at 0 h) and (B, D) IL-6 protein secretion measured (results expressed as a percentage of TNFα-induced IL-6 secretion at 24 h) at the indicated times. Statistical analysis was performed using two-way ANOVA then Bonferroni's post-test (where * denotes a significant effect of TNFα compared to vehicle-treated cells, and § denotes significant repression of TNFα-induced cytokine production by PP2A activators (P<0.05)). Data are mean+SEM values from n=4-5 independent experiments.

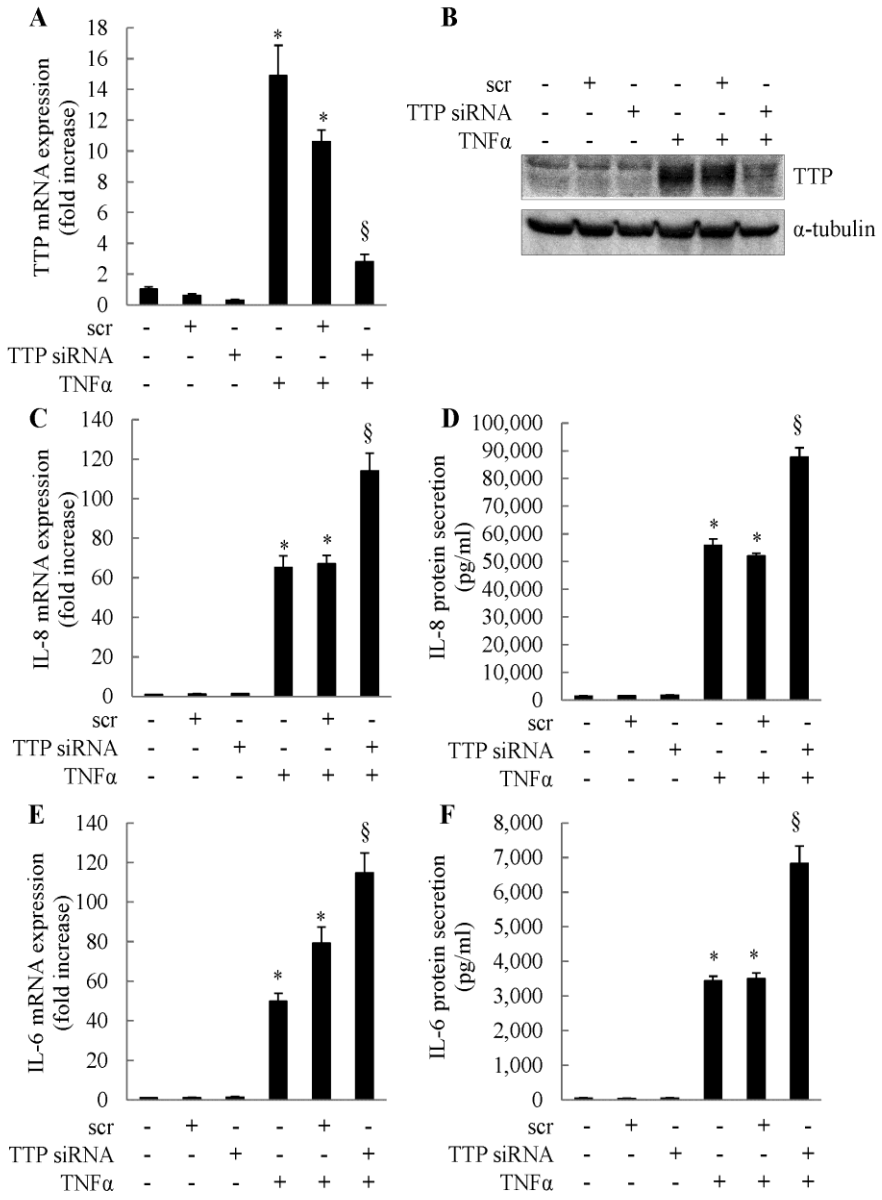
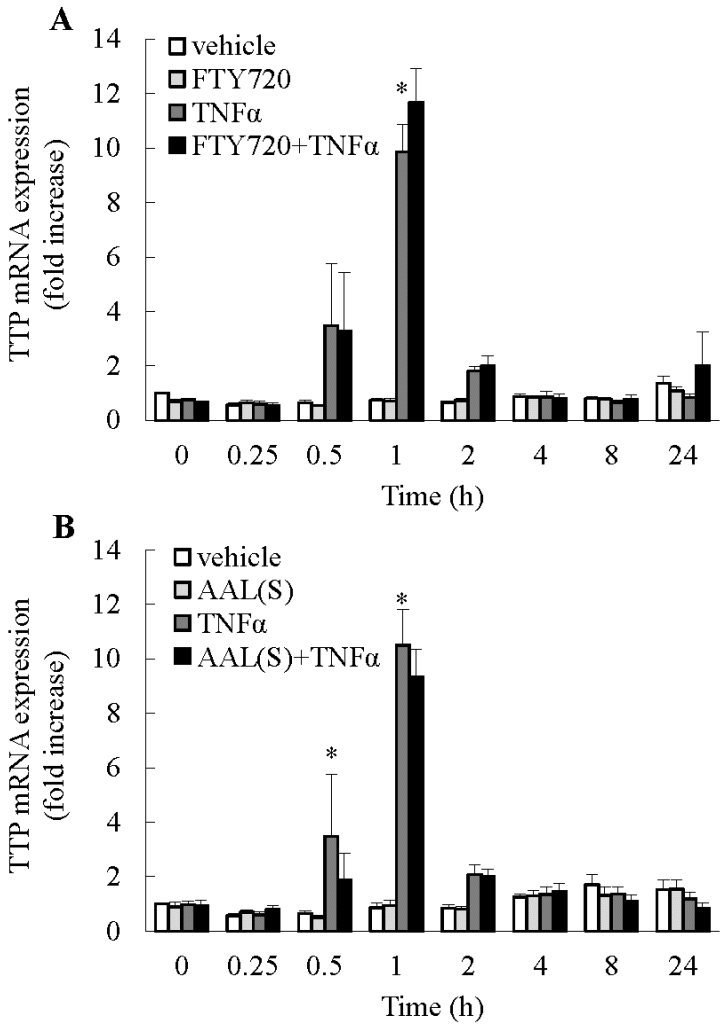


Figure 5.4 Specific knockdown of TTP with siRNA increases TNFα-induced IL-8 and IL-6 mRNA expression and protein secretion. A549 cells were either left untransfected or transfected with scrambled control (scr) or siRNA against TTP (siRNA), then treated with vehicle or TNFα (4 ng/ml) before: (A, B) knockdown of TTP mRNA and TTP protein (43 to 49 kDa) expression confirmed at 1 h by (A) RT-PCR and (B) Western blotting (compared to α-tubulin as loading control); (C, E) IL-8 and IL-6 mRNA expression measured at 1 h (results expressed as fold increase compared to untransfected vehicle-treated controls); and (D, F) IL-8 and IL-6 protein secretion measured at 24 h. Statistical analysis was performed using Student's unpaired *t* test (where * denotes a significant effect of TNFα compared to untransfected vehicle-treated controls, and § denotes a significant effect of siRNA against TTP compared to untransfected controls treated with TNFα ($P<0.05$)). Data are mean+SEM values from n=3 independent experiments.

5.3.4 PP2A activators do not enhance TTP mRNA expression; rather they modify TTP's dynamic equilibrium

Given we showed that PP2A regulates TTP (Rahman et al., 2015), and that PP2A activators inhibited IL-6 and IL-8 production (Figures 5.2 & 5.3), we next sought to determine if PP2A activators affected TTP expression/activity. We demonstrate that it was not via an increase in TTP mRNA expression. As shown in Figure 5.5A, TNFα significantly increased TTP mRNA expression with a peak at 1 h ($P<0.05$); however, this was unaffected by pre-treatment with FTY720. Pre-treatment with AAL(S) was similarly without effect on TNFα-induced TTP mRNA expression (Figure 5.5B). We then wished to examine the effect of PP2A activators on TTP protein levels. We initially established the temporal kinetics of TTP protein upregulation induced by TNFα. TTP's anti-inflammatory function is controlled by phosphorylation and there is a dynamic equilibrium between unphosphorylated/active and phosphorylated/active TTP. As shown in Figure 5.5C, and in confirmation of earlier studies in A549 (King et al., 2009b; Rahman et al., 2015) and other cell types (Mahtani et al., 2001; Prabhala et al., 2015), the rabbit antisera Sak21 detects different phospho-forms of TTP with immunoreactive bands at higher molecular weight indicating phosphorylated TTP (inactive), while lower bands are unphosphorylated (active). TTP is an immediate-early gene (Carballo et al., 1998) and can be detected at 1 h in both phosphorylated and unphosphorylated forms. By 2 h, phosphorylated TTP predominates and reduces over time (Figure 5.5C). In order to examine the effect of PP2A activators on these phospho-forms of TTP we focused in on the 1 h (Figure 5.5D) and 2 h time points (Figure 5.5E). As demonstrated by Western blotting and quantitated by densitometry, PP2A activators reduced the amount of TTP protein upregulated in response to TNFα. This is seemingly counterintuitive, but we offer an explanation that is supported by recent *in vivo* studies

(Ross et al., 2015; Smallie et al., 2015). We propose that PP2A activators affect TTP's dynamic equilibrium; shifting the equilibrium from phosphorylated (inactive) towards unphosphorylated (active) TTP. Because the phosphorylation of TTP both inactivates TTP and protects it from proteasome-mediated degradation, we predicted that PP2A agonists that promoted TTP dephosphorylation would decrease total TTP protein levels. This was exactly the outcome observed in Figures 5.5D & 5.5E.



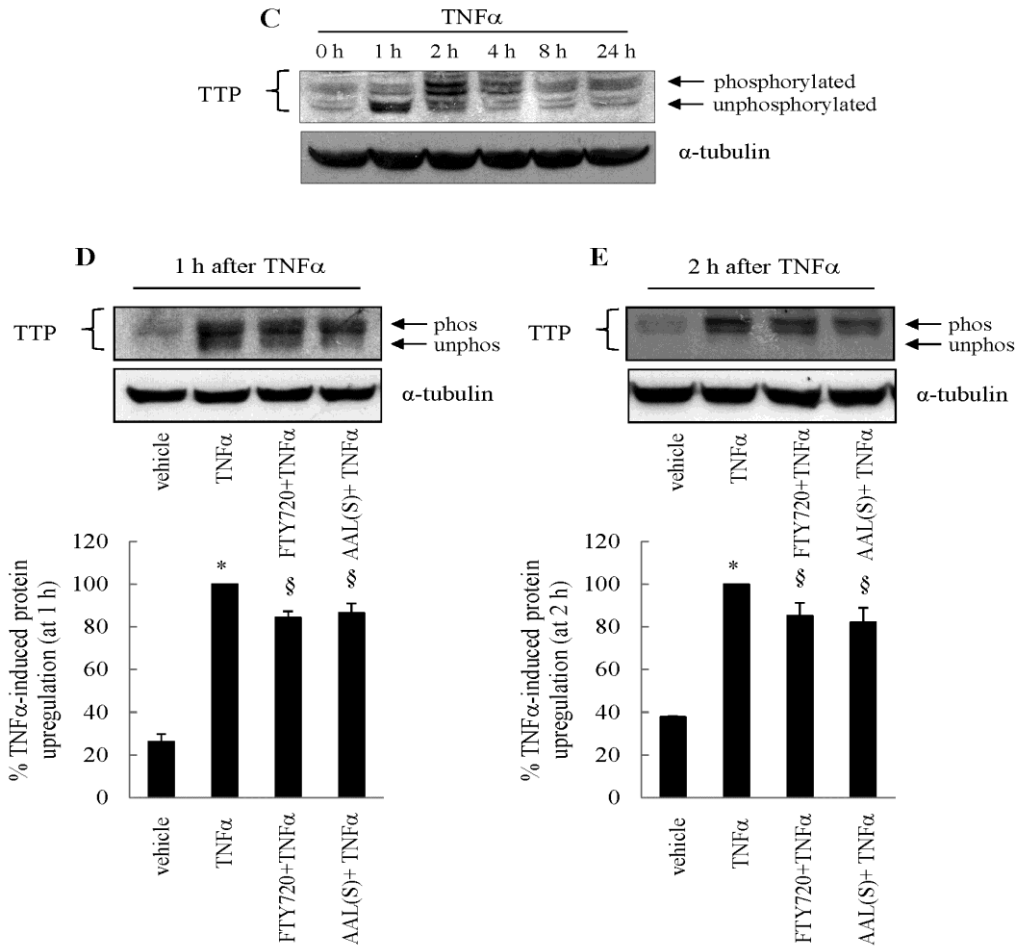


Figure 5.5 PP2A activators do not enhance TTP mRNA expression; rather they modify TTP's dynamic equilibrium. (A, B) A549 cells were pre-treated with (A) 2.5 μM FTY720 or (B) 2.5 μM AAL(S) for 6 h, compared to vehicle (2.5 μM DMSO)-treated controls. Cells were treated with vehicle or TNFα (4 ng/ml) and then TTP mRNA expression measured (results expressed as fold increase compared to vehicle-treated cells at 0 h) at the indicated times. Statistical analysis was performed using two-way ANOVA then Bonferroni's post-test (where * denotes a significant effect of TNFα compared to vehicle-treated cells (P<0.05)). Data are mean+SEM values from n=4-5 independent experiments. (C-E) A549 cells were either: (C) treated with TNFα (4 ng/ml) for 0, 1, 2, 4, 8, 24 h; or (D, E) pre-treated with vehicle, 2.5 μM FTY720 or 2.5 μM AAL(S) for 6 h, then treated with vehicle or TNFα (4 ng/ml) for 1 h or 2 h. Western blotting for TTP (43 to 49 kDa) was performed (compared to α-tubulin as loading control). Please note two bands of immunoreactivity for TTP: bands at higher molecular weight indicate stable phosphorylated TTP (inactive), while lower bands are unphosphorylated (active) but unstable. Results are representative Western blots or densitometry analysis (normalized to α-tubulin and expressed as % TNFα-induced TTP protein upregulation at either 1 h (D) or 2 h (E)). Statistical analysis was performed using one-way ANOVA then Bonferroni's post-test (where * denotes a significant effect of TNFα, and § denotes a significant effect of PP2A activators on TNFα-induced effects (P<0.05)). Data are mean+SEM values from n=3-4 independent experiments.

5.3.5 Specific knockdown of TTP with siRNA reverses repression of TNFα-induced IL-8 and IL-6 mRNA expression and protein secretion by the PP2A activator FTY720

Finally, we demonstrated a link between PP2A activation and TTP-mediated cytokine repression. We examined whether FTY720-mediated repression was TTP-dependent by knocking-down TTP with siRNA and observing the resultant effect on cytokine production. Figure 5.6A confirms that siRNA against TTP reduces TTP mRNA expression. In confirmation of earlier data, FTY720 significantly repressed TNFα-induced IL-8 mRNA expression (Figure 5.6B) and protein secretion (Figure 5.6C) ($P<0.05$). IL-6 mRNA expression and protein secretion was also significantly repressed by FTY720 (Figures 5.6D & 5.6E: $P<0.05$). Notably, specific knockdown of TTP with siRNA reverses repression of IL-8 and IL-6 mRNA expression and protein secretion by FTY720 (Figures 5.6B-5.6E). These data support our hypothesis that PP2A activators mediate their anti-inflammatory effect in a TTP-dependent manner.

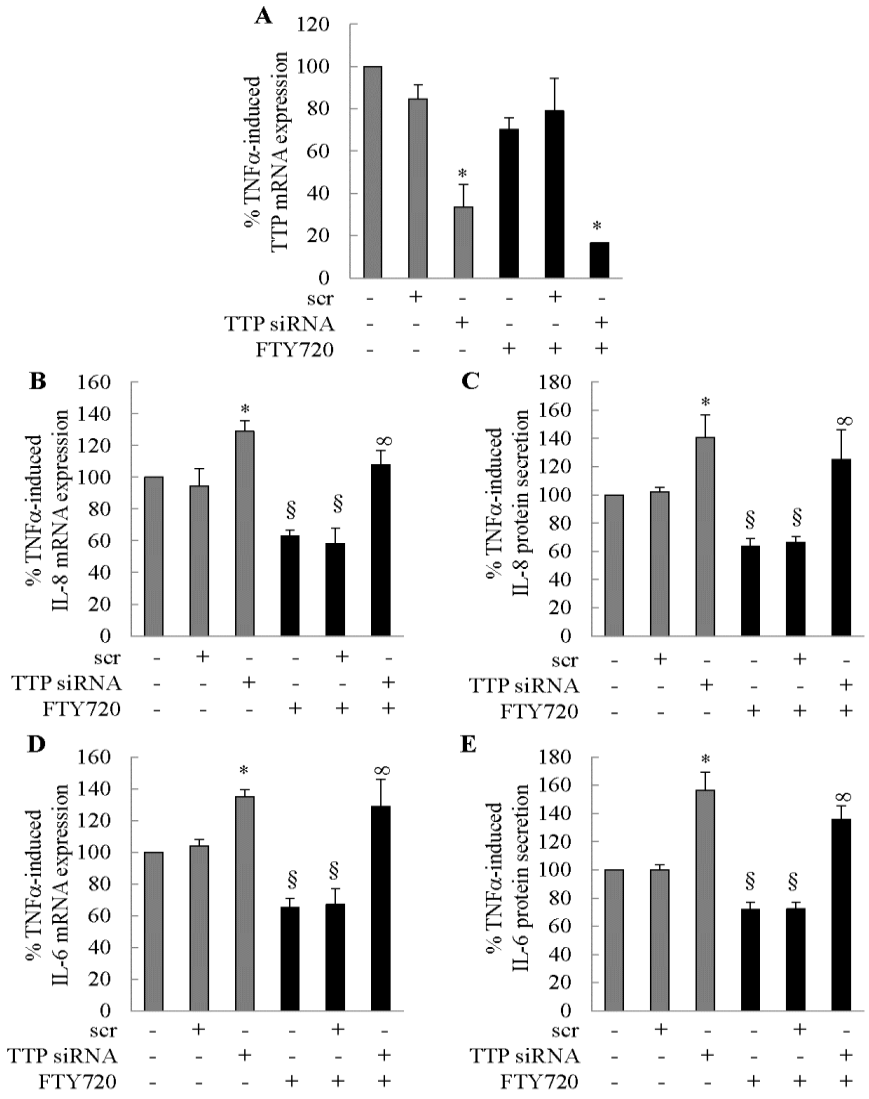


Figure 5.6 Specific knockdown of TTP with siRNA reverses repression of TNFα-induced IL-8 and IL-6 mRNA expression and protein secretion by the PP2A activator FTY720. A549 cells were either left untransfected or transfected with scrambled control (scr) or siRNA against PP2A-C, and then pre-treated for 6 h with vehicle (2.5 μM DMSO) or with 2.5 μM of FTY720. Cells were stimulated with TNFα (4 ng/ml) before: (A) knockdown of TTP mRNA expression confirmed or (B, D) IL-8 and IL-6 mRNA expression measured at 1 h (results expressed as a percentage of TNFα-induced mRNA expression in untransfected controls); (C, E) IL-8 and IL-6 protein secretion measured at 24 h (results expressed as a percentage of TNFα-induced protein secretion in untransfected controls). Statistical analysis was performed using Student's unpaired *t* test (where: * denotes significant effect of TTP against siRNA and § significant repression of TNFα-induced cytokine production by FTY720 (compared to untransfected controls); ∞ denotes that specific knockdown of TTP with siRNA reverses repression of TNFα-induced IL-8 and IL-6 mRNA expression and protein secretion by FTY720 (compared to untransfected controls) (P<0.05). Data are mean+SEM values from n=3 independent experiments.

5.4 Discussion

PP2A is a tri-molecular holoenzyme responsible for dephosphorylating (and hence activating) TTP, amongst many other targets. Small molecule PP2A activators have been developed and in this study we use two key examples – FTY720 and AAL(S) – to show that PP2A activators can enhance TTP's anti-inflammatory function to inhibit cytokine production in a cellular model of airway inflammation *in vitro*.

PP2A is best known for its role as a tumour suppressor responsible for the regulation of a range of cellular signalling pathways involved in proliferation, survival and migration in cancer (Perrotti and Neviani, 2013). Notably, PP2A expression/activity is reduced in a number of cancers (Roberts et al., 2010; Cristobal et al., 2014). Thus, restoration of PP2A tumour-suppression activity by PP2A activators has emerged as a novel therapy for treating cancer. Excitingly, the knowledge gained from understanding of PP2A in cancer has propelled research into its role and function in respiratory disease. PP2A levels (and therefore activity) were reduced in mouse models of allergen- and rhinovirus-induced asthma (Collison et al., 2013a) and could be reinstated pharmacologically with AAL(S) to repress hallmark features of airway disease (Collison et al., 2013a; Hatchwell et al.). Notably, defects in PP2A cause corticosteroid insensitivity in severe asthma (Kobayashi et al., 2011). Reduced PP2A activity induces corticosteroid resistance and this can be reversed by the long-acting β_2-agonist formoterol (Kobayashi et al., 2012). Cornell *et al*. (Cornell et al., 2009) showed that blocking PP2A robustly increased IL-8 expression in A549 cell via post-transcriptional mRNA stabilization; TTP was implicated but not directly examined. With these studies, we and others have underscored the importance of investigations into the regulation and pharmacological modulation of PP2A in airway inflammation. But PP2A dephosphorylates a number of kinases that drive inflammatory

pathways (Shanley et al., 2001; Miskolci et al., 2003; Junttila et al., 2008); whether TTP was involved remained an open question.

We address this herein. PP2A is the phosphatase responsible for TTP dephosphorylation (Sun et al., 2007). Our earlier studies have shown the impact of PP2A inhibitors on TTP. Brook *et al.* (Brook et al., 2006) used a non-specific PP2A inhibitor calyculin A and demonstrated accumulation of the hyperphosphorylated in active form of TTP. More recently (Rahman et al., 2015), we showed that when PP2A is reduced (either by a non-specific pharmacological inhibitor okadaic acid, or more specifically with siRNA against PP2A-C) TTP is hyperphosphorylated and rendered inactive; hence the homeostatic restraint that the ubiquitous enzyme PP2A exerts under basal conditions is lost and cytokine secretion ensues. In the current study we build upon these observations and exploit the capacity of known PP2A activators to enhance PP2A phosphatase activity; effectively shifting the equilibrium from phosphorylated/inactive towards unphosphorylated/active TTP.

A number of small molecules have been reported to activate PP2A (Perrotti and Neviani, 2013). To date the best known of these is the sphingosine analog FTY720 (fingolimod; Gilenya (Novartis)). However FTY720 has other targets. FTY720 is also a functional antagonist of the S1P pathway. FTY720 becomes phosphorylated (by sphingosine kinase 2 (Spk2)) to FTY720-P. FTY720-P is then released from cells to then act back on S1P receptors (S1PR1, 3, 4, 5, but not S1PR2), internalizing the receptors and preventing them from being activated again by native ligand S1P (functional antagonism). In the context of respiratory disease, this is not desirable as we have shown that S1P is elevated in asthma (Ammit et al., 2001) and drives development of a pro-inflammatory phenotype, including IL-8 and IL-6 expression *in vitro* (Ammit et al., 2001; Rahman et al., 2014). Accordingly, there is a compelling need to develop PP2A activators that

are devoid of S1P agonism. To this end, a chiral analog of FTY720 that does not bind S1P receptors, called AAL(S) was developed (Don et al., 2007), and we showed that it is just as effective as FTY720 at activating PP2A complexes *in vitro* and significantly represses airways disease in mouse models *in vivo* (Collison et al., 2013a). AAL(S) is an improvement on FTY720, being devoid of S1P agonism, and we now utilize these PP2A activators in A549 lung epithelial cells to show that although they do not activate PP2A when added alone, they increased TNFα-induced PP2A activity to inhibit epithelial cell cytokine production in a TTP-mediated manner. Further understanding of the molecular mechanism of PP2A regulation in respiratory inflammation is required, but it could be speculated that PP2A activators may repress inflammation under inflamed (mimicked here with TNFα), rather than non-inflamed conditions.

Taken together our study confirms that PP2A is a druggable target in respiratory disease (Collison et al., 2013a; Hatchwell et al.) and establishes a link with TTP. Use of PP2A activators underscores the potential of enhancing TTP function with small molecules to reduce airway inflammation in respiratory disease. This new knowledge advances our understanding of the mechanistic basis of airway inflammation and suggests molecular strategies and avenues for future pharmacotherapeutic approaches based on exploiting the anti-inflammatory function of TTP

Chapter 6
TLR2 ligation induces corticosteroid insensitivity in A549 lung epithelial cells: anti-inflammatory impact of PP2A activators

This work is currently under review in *The International Journal of Biochemistry & Cell Biology* entitled "TLR2 ligation induces corticosteroid insensitivity in A549 lung epithelial cells: anti-inflammatory impact of PP2A activators. **Md. Mostafizur Rahman**, Pavan Prabhala, Nowshin N. Rumzhum, Brijeshkumar S. Patel, Thomas Wickop, Nicole M. Verrills and Alaina J. Ammit

Chapter 6
TLR2 ligation induces corticosteroid insensitivity in A549 lung epithelial cells: anti-inflammatory impact of PP2A activators

6.1 Introduction

In Chapter 4 and 5 we successfuly demonstrate the anti-inflammatory role of PP2A and establish a link with TTP. These studies corroborate the potential of PP2A activators, as alternative anti-inflammatory strategies in asthmatic inflammation. We therefore aim to explore the role of PP2A activators, known (FTY720) and novel (Theophylline) in the context of corticosteroid insensitivity. At the same time, we aim to explore the underlying molecular mechanisms of mimic bacterial infection, Pam3CSK4-induced conticosteroid insensitivity.

Corticosteroids are front-line anti-inflammatory therapies that are widely used to treat people with chronic lung disease. In asthma, although corticosteroids have proven clinical efficacy, it is increasingly recognized that their anti-inflammatory effectiveness can vary widely depending on disease context (such as when people with asthma are experiencing respiratory infections). This is known as corticosteroid insensitivity. In severe asthma, the degree of insensitivity increases to become corticosteroid resistance; a steroid-refractory condition experienced by 10% of people with asthma. Thus, the impact of corticosteroid insensitivity is increasingly recognized as a major problem limiting the efficacy of anti-inflammatory therapy in chronic respiratory disease (reviewed in (Ammit, 2013; Chung, 2013)). Moreover, corticosteroids are much less effective in COPD than in asthma, and this is considered due to relative corticosteroid insensitivity that exists in COPD (Marwick and Chung, 2010). To combat inflammation, steroid dose can be

increased, but this is not ideal. Thus, alternative anti-inflammatory strategies that could effectively treat inflammation when corticosteroid insensitivity exists are urgently required.

Cellular models of corticosteroid insensitivity are invaluable *in vitro* tools in our quest to develop novel and efficacious pharmacotherapeutic strategies to treat respiratory disease in the future. They are an essential first step towards elucidating the mechanisms responsible for respiratory infections and the exacerbation of chronic lung diseases. This was highlighted in a recent review by Saturni *et al*. (Saturni et al., 2015), and demonstrated by Papi *et al.* (Papi et al., 2013), where A549 lung epithelial cells exposed *in vitro* to rhinoviral infection resulted in corticosteroid insensitivity. The molecular mechanisms responsible were explored and shown to be due to reduced nuclear translocation of the receptor for corticosteroids – the GR (Papi et al., 2013). Ligand-activated GR translocation is a critical step in corticosteroid function; without this, GRs are unable to interact in a *cis*- or *trans*-manner to exert transcriptional control. Although GR has two isoforms GRα and GRβ, to be functionally active GRβ has to be more abundant than GRα. However no cells in asthmatics contain higher levels of GRβ than GRα (Gagliardo et al., 2001). Notably, upregulation of a critical, corticosteroid-induced, anti-inflammatory protein –MKP-1 - was attenuated by rhinoviral infection and contributed to corticosteroid insensitivity. While the impact of respiratory viruses on corticosteroid insensitivity have begun to be uncovered (reviewed in (Saturni et al., 2015)), the influence of bacterial infection warrants further investigation.

Accordingly, we have established an *in vitro* cellular model of bacterial exacerbation utilizing the synthetic bacterial lipoprotein Pam3CSK4 (Manetsch et al., 2012c; Hirota et al., 2013; Alkhouri et al., 2014). The impact of this TLR2 ligand on corticosteroid insensitivity and the role played by GR translocation and MKP-1 upregulation was previously unknown. We address this

herein and show that Pam3CSK4 induces corticosteroid insensitivity in A549 lung epithelial cells. Interestingly, the mechanism is not via retardation of GR translocation into the nucleus. Instead we reveal that while the total amount of anti-inflammatory MKP-1 produced in response to corticosteroid in the context of TLR2 ligation was unaffected, a proportion of MKP-1 was rendered inactive by oxidation of the catalytic cysteine. MKP-1 is one of the major ways in which corticosteroids achieve anti-inflammatory action (Issa et al., 2007; Quante et al., 2008; King et al., 2009a; Manetsch et al., 2012c; Che et al., 2014; Rahman et al., 2014). Given that this anti-inflammatory pathway is less effective under *in vitro* conditions mimicking bacterial exacerbation, we were compelled to uncover alternative, non-steroidal anti-inflammatory strategies. Notably, we show that PP2A activators can repress inflammation when corticosteroid insensitivity exists.

6.2 Material and Methods

6.2.1 Chemicals

Pam3CSK4 was purchased from InVivoGen (San Diego, CA), TNFα was from R&D Systems (Minneapolis, MN) and FTY720 was from Cayman Chemical Company (Ann Arbor, MI). Unless otherwise specified, all chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO).

6.2.2 Cell culture

Please see Chapter 4.2.2

6.2.3 ELISA

IL-6 ELISA was performed according to the manufacturer's instructions (BD Biosciences Pharmingen, San Diego, CA).

6.2.4 Western blotting

To examine GR translocation, cytoplasmic and nuclear protein extraction was performed using NE-PER nuclear and cytosolic extraction kit according to the manufacturer's instructions (Thermo Fisher Scientific, Rockford, IL). Western blotting was performed using rabbit polyclonal IgG antibodies raised against GR (E-20: Santa Cruz Biotechnology, Santa Cruz, CA), compared to α-tubulin (mouse monoclonal IgG_1, DM1A: Santa Cruz Biotechnology) and a rabbit polyclonal antibody to lamin A/C (Cell Signalling Technology, Danvers, MA) as a loading control for the cytosolic and nuclear fractions, respectively. To measure MKP-1upregulation in whole cell lysates, MKP-1 was quantified by Western blotting using a rabbit polyclonal antibody against MKP-1 (C-19: Santa Cruz Biotechnology) with α-tubulin utilized as the loading control. Primary antibodies were detected with goat anti-rabbit and anti-mouse HRP-conjugated secondary antibodies (Cell Signalling Technology) and visualized by enhanced chemiluminescence (PerkinElmer, Wellesley, MA).

6.2.5 MKP-1 oxidation

The method used to measure oxidized MKP-1 was an adaptation of the PROP (purification of reversibly oxidized proteins) technique developed by Templeton *et al.* (Templeton et al., 2010; Victor et al., 2012). A549 cells were subjected to oxidative stress conditions (see experimental text) before commencing the PROP procedure. Briefly, the non-oxidized thiols were blocked

using N-ethylmaleimide, then the oxidized thiols were reduced with dithiothrietol and eluted with thiol affinity chromatography (thiopropyl sepharose 6B: GE Healthcare, Little Chlafont, UK). Oxidized MKP-1 was then measured using Western blot and the degree of oxidation compared to native MKP-1 measured in whole cell lysates run in parallel.

6.2.6 Statistical analysis

Statistical analysis was performed using either the Student's unpaired *t* test, one-way or two-way ANOVA followed by Bonferroni's post-test. *P* values <0.05 were sufficient to reject the null hypothesis for all analyses. Data are mean+SEM of n≥3 independent replicates.

6.3 Results

6.3.1 TLR2 ligand Pam3CSK4 induces corticosteroid insensitivity in A549 cells

Hererin we adapt a clinically-relevant model of corticosteroid insensitivity (Papi et al., 2013) to mimic bacterial exacerbation *in vitro* and demonstrate that exposure to a synthetic bacterial lipoprotein (TLR2/TLR1 ligand; Pam3CSK4) induces relative corticosteroid insensitivity. This is demonstrated in Figure 6.1, where we show that the dose-dependent repression of TNFα-induced IL-6 secretion achieved with dexamethasone is significantly altered by Pam3CSK4 pre-treatment (*P*<0.05). This is particularly notable at 0.01-1 μM dexamethasone, where the inhibitory impact of dexamethasone on cytokine secretion from A549 cells is significantly attenuated by Pam3CSK4 (Figure 6.1: *P*<0.05).

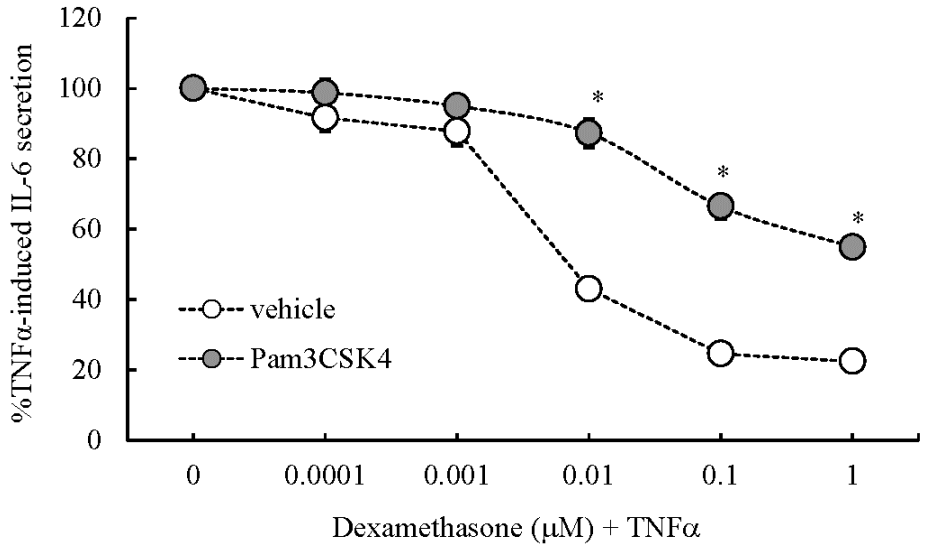


Figure 6.1 TLR2 ligand Pam3CSK4 induces corticosteroid insensitivity in A549 cells. A549 cells were pre-treated for 30 min with either vehicle (0.0001-1 μM DMSO) or Pam3CSK4 (1 μg/ml), followed by vehicle or dexamethasone (0.0001-1 μM) for 30 min. After stimulation with TNFα (4 ng/ml) for 24 h, IL-6 was measured by ELISA. Statistical analysis was performed using two-way ANOVA then Bonferroni's post-test (where * denotes a significant reduction of corticosteroid-mediated repression by Pam3CSK4 (P<0.05)). Data are mean+SEM values from n=9 independent experiments.

6.3.2 Corticosteroid insensitivity induced by Pam3CSK4 is not due to altered translocation of GR into the nucleus

We then wished to explore the molecular mechanism responsible for corticosteroid insensitivity exerted by the TLR2 ligand. Corticosteroids mediate their actions via interaction with the cytosolic GR that then undergoes translocation into the nucleus. As this is an essential step necessary for corticosteroid action, we examined whether the molecular basis of corticosteroid insensitivity established by Pam3CSK4 is due to a retardation of GR translocation. We address this in Figure 6.2, where the effect of Pam3CSK4 pre-treatment on GR translocation induced by

dexamethasone (0.0001-1 μM) was assessed under two experimental conditions: i.e. in the absence (Figure 6.2A) or presence of stimulation with TNFα (Figure 6.2B). Cytoplasmic and nuclear protein extracts were prepared and nuclear entry of GR determined by Western blotting, compared with α-tubulin and lamin A/C as loading controls for the cytosolic and nuclear fractions, respectively. As shown in Figure 6.2A, GR resides in the cytosol and is undetectable in the nucleus under unstimulated conditions. After treatment with dexamethasone however, GR undergoes translocation in a concentration-dependent manner. Notably, dexamethasone-induced GR translocation is unaffected by Pam3CSK4 (Figure 6.2A). These results were independent of stimulation with TNFα; as confirmatory experiments (performed in Figure 6.2B) show a similar pattern of GR translocation in cells that have been treated with TNFα. Taken together these experiments demonstrate that corticosteroid insensitivity induced by Pam3CSK4 is not due to altered translocation of GR into the nucleus.

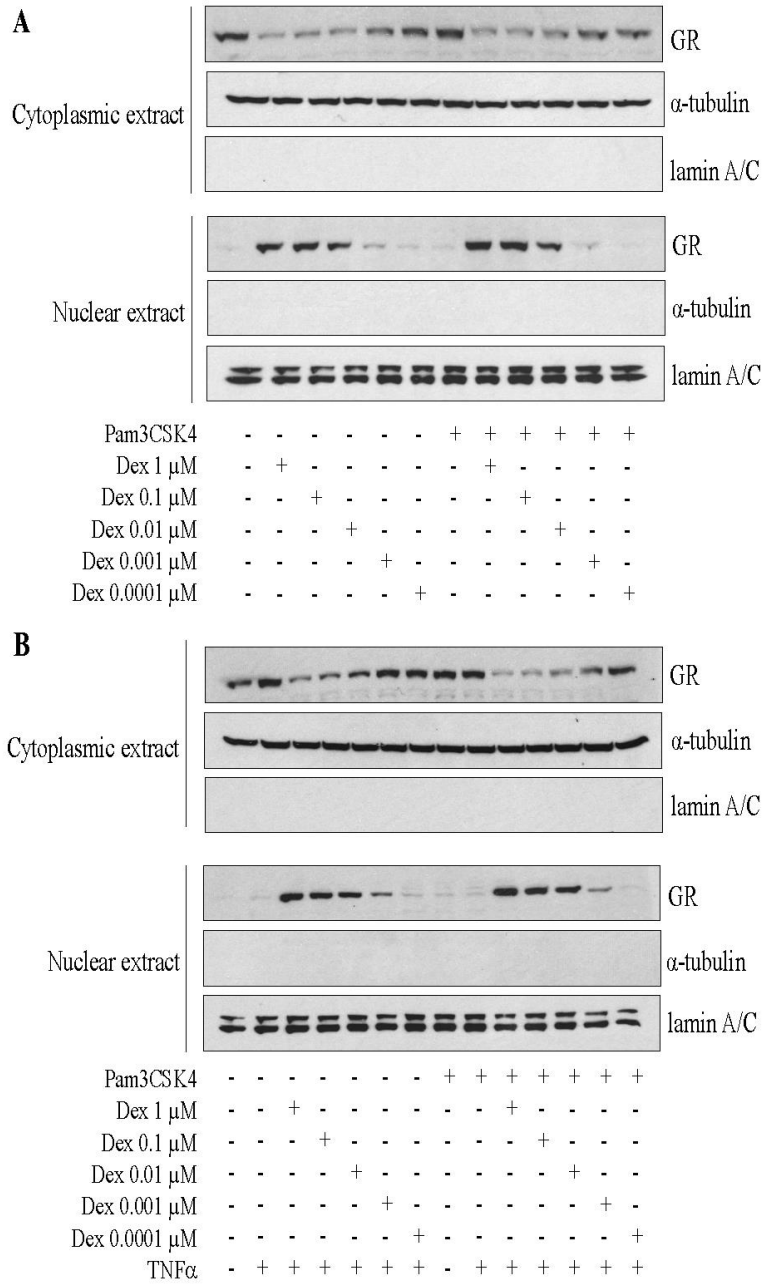


Figure 6.2 Corticosteroid insensitivity induced by Pam3CSK4 is not due to altered translocation of GR into the nucleus. A549 cells were pre-treated for 30 min with either vehicle or Pam3CSK4 (1 μg/ml), followed by vehicle (0.0001-1 μM DMSO) or dexamethasone (0.0001-1 μM) for 30 min. GR translocation was examined in the (A) absence or (B) or presence of stimulation with TNFα (4 ng/ml) for 1 h. Cytoplasmic and nuclear protein extracts were prepared and nuclear entry of GR (86 kDa) determined by Western blotting, compared with α-tubulin (55 kDa) and lamin A/C (45, 50 kDa) as loading controls for the cytosolic and nuclear fractions, respectively. Data are representative blots from n=3 independent experiments.

6.3.3 Pam3CSK4 does not affect corticosteroid-induced upregulation of anti-inflammatory MKP-1

Thus far we have shown that Pam3CSK4 reduces the anti-inflammatory efficacy of corticosteroids on TNFα-induced cytokine production without affecting GR translocation. We (Quante et al., 2008; Manetsch et al., 2012c; Che et al., 2014; Rahman et al., 2014) and others (Issa et al., 2007; King et al., 2009a) have shown that a major mechanism responsible for the anti-inflammatory actions of corticosteroids is via the upregulation of MAPK-deactivating phosphatase – MKP-1; thus, we investigated whether impaired MKP-1 upregulation was responsible for corticosteroid insensitivity induced by bacterial ligand Pam3CSK4. This is explored in Figure 6.3, where MKP-1 upregulation in whole cell lysates of A549 cells was compared by Western blotting (Figure 6.3A) and analysed by densitometry (Figure 6.3B). We demonstrate that TNFα induces significant upregulation of MKP-1 protein (3.2±0.5-fold compared to vehicle-treated cells (designated as 1) (P<0.05)); in alignment with previous studies in A549 cells (King et al., 2009a). MKP-1 is corticosteroid-inducible (King et al., 2009a) and accordingly, TNFα-induced MKP-1 in A549 cells pre-treated with dexamethasone is significantly enhanced at 0.01-1 μM (Figure 6.3B: P<0.05). However, when we examine the influence of Pam3CSK4 pre-treatment on MKP-1 upregulation there was no significant effect; as demonstrated by Western blotting (Figure 6.3A) and confirmed by densitometry analysis (Figure 6.3B). Thus, our hypothesis that the bacterial ligand Pam3CSK4 attenuated upregulation of MKP-1 protein was not supported.

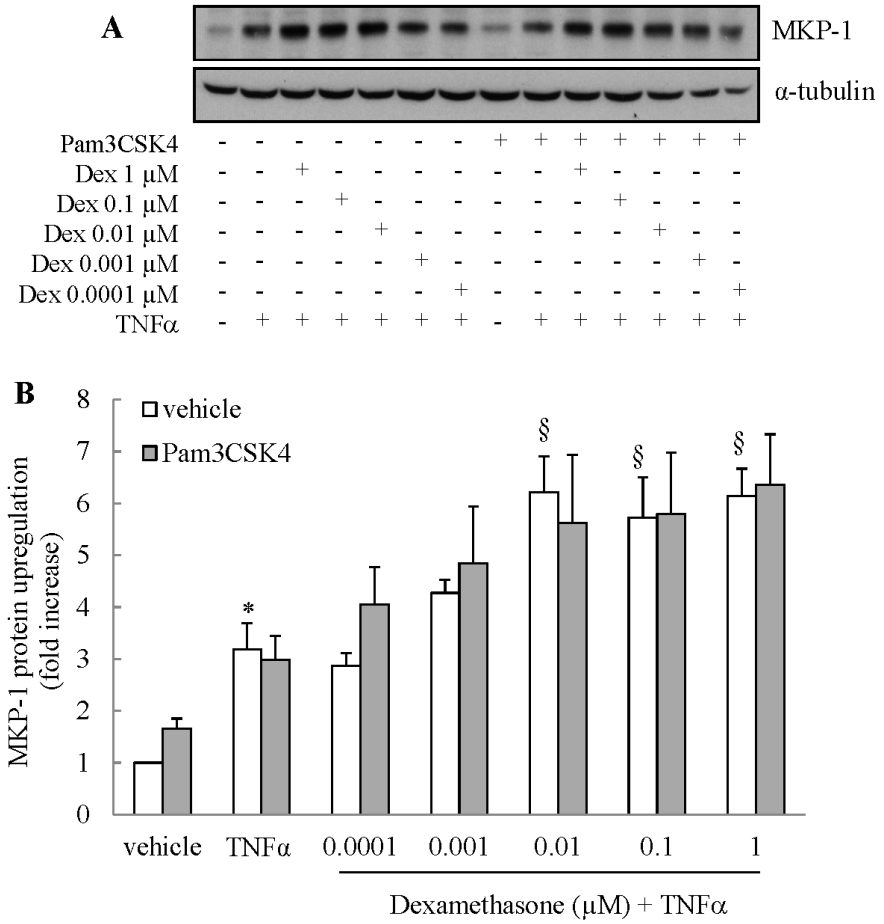


Figure 6.3 Pam3CSK4 does not affect corticosteroid-induced upregulation of anti-inflammatory MKP-1. A549 cells were pre-treated for 30 min with either vehicle or Pam3CSK4 (1 μg/ml), followed by vehicle (0.0001-1 μM DMSO) or dexamethasone (0.0001-1 μM) for 30 min. Cells were treated without and with TNFα (4 ng/ml) for 1 h and the impact on MKP-1 (40 kDa) upregulation compared by Western blotting (with α-tubulin as the loading control). (A) Results are representative Western blots and (B) densitometry analysis of MKP-1 protein upregulation (normalized to α-tubulin and expressed as fold increase compared to vehicle-treated cells). Statistical analysis was performed using Student's unpaired *t* test or one-way ANOVA then Fisher's post-hoc multiple comparison test (where * denotes significant increase in MKP-1 protein upregulation induced by TNFα, and § denotes significant potentiation of TNFα-induced MKP-1 by dexamethasone ($P<0.05$); there was no effect of Pam3CSK4). Data are mean+SEM values from n=3 independent experiments.

6.3.4 Corticosteroid insensitivity may be due to Pam3CSK4-induced oxidization of MKP-1

However, MKP-1 protein is subject to redox regulation (Kamata et al., 2005; Hou et al., 2008) as the catalytic cysteine responsible for phosphatase action can be oxidized and thus reduce anti-inflammatory function (Bonham and Vacratsis, 2009). Notably, Pam3CSK4 can increase oxidative stress in a TLR2-mediated manner and thus we were intrigued to examine whether MKP-1 expressed under these conditions may be oxidized.

To do this, we utilized a technique referred to as PROP (purification of reversibly oxidized proteins) (Templeton et al., 2010; Victor et al., 2012). A549 cells were treated under conditions established above to induce corticosteroid insensitivity (dexamethasone (at 10 nM) + TNFα without/with Pam3CSK4) in order to ask the question - is the MKP-1 produced in cells that have been pre-treated with Pam3CSK4 oxidized? In order to show that Pam3CSK4 increases MKP-1 oxidation, experiments were performed in parallel, and then either prepared as whole cell lysates to measure native MKP-1, or subjected to the PROP technique to detect oxidized MKP-1. As shown in Figure 6.4A and in confirmation with our earlier data (Figure 6.3B), Pam3CSK4 had no significant effect on the dexamethasone + TNFα-induced upregulation of MKP-1 detected in whole cell lysates (see native MKP-1 (WCL): Figure 6.4A). In contrast, there was a significant (P<.0.05) increase in oxidized MKP-1 detected by the PROP technique. In cells pre-treated with the widely-used positive control for oxidative stress – H_20_2 (hydrogen per oxide) (Kamata et al., 2005; Templeton et al., 2010; Victor et al., 2012) before TNFα, we can confirm that the PROP technique detects increases in oxidized MKP-1. Taken together these results suggest that MKP-1 oxidation induced by Pam3CSK4 may be a contributing factor in corticosteroid insensitivity.

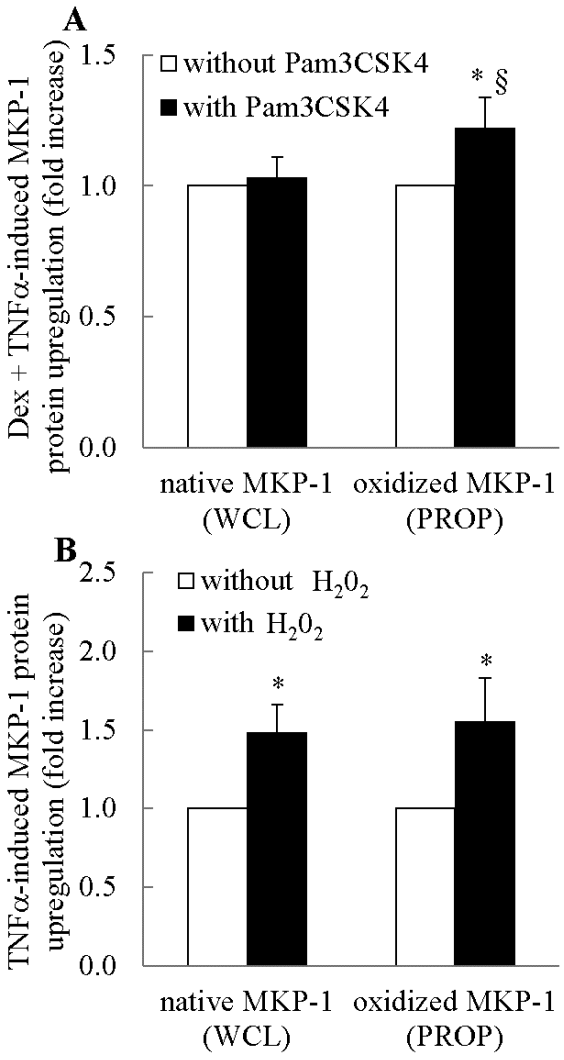


Figure 6.4 Corticosteroid insensitivity may be due to Pam3CSK4-induced oxidization of MKP-1. (A) A549 cells were pre-treated for 30 min with either vehicle or Pam3CSK4 (1 µg/ml), followed by dexamethasone (0.01 µM) for 30 min then TNFα (4 ng/ml) for 1 h. In order to show that Pam3CSK4 increases MKP-1 oxidation, experiments were performed in parallel, and then either prepared as whole cell lysates (WCL) to measure native MKP-1, or subjected to the PROP technique to detect oxidized MKP-1. (B) As a positive control for oxidative stress, A549 cells were pre-treated for 30 min without and with 100 µM H_2O_2 before stimulation with TNFα (4 ng/ml) for 1 h. MKP-1 was detected by Western blotting and densitometry analysis performed (results expressed as fold increase compared to cells treated without (A) Pam3CSK4 or (B) H_2O_2). Statistical analysis was performed using one-way ANOVA then Fisher's post-hoc multiple comparison test (where * denotes significant increase in MKP-1 upregulation, and § denotes significant increase in oxidized MKP-1 (P<0.05)). Data are mean+SEM values from n=3-4 independent experiments.

6.3.5 Alternative anti-inflammatory approaches to overcome corticosteroid insensitivity: known and novel PP2A activators

By modeling bacterial exacerbation *in vitro* we have demonstrated that exposure to a bacterial ligand induces corticosteroid insensitivity. We revealed, in part, a contributing molecular mechanism (oxidation of MKP-1) and show that nuclear translocation of GR is unaffected. We now turn to exploring alternative anti-inflammatory approaches and hypothesize that PP2A activators, being non-steroidal, may prove to inhibit cytokine production when corticosteroid insensitivity exists. To address this we examine the impact of two compounds: FTY720 and theophylline. FTY720 is a well-established activator of PP2A (Perrotti and Neviani, 2013); while theophylline has only been recently reported by us (Patel et al., 2015) to function as a novel activator of PP2A. We show that FTY720 (2.5 μM) in combination with dexamethasone (0.001-1 μM) achieves significant repression of IL-6 with lower concentrations of corticosteroids (Figure 6.5A), and inhibits IL-6 secretion in cells where corticosteroid insensitivity has been established by exposure to the bacterial ligand Pam3CSK4 (Manetsch et al., 2012c; Alkhouri et al., 2014) (Figure 6.5B) ($P<0.05$). At a concentrtaion of 10 μM, theophylline is anti-inflammatory and can repress TNFα-induced IL-6 secretion (Figure 6.6A); in confirmation of our earlier publication (Patel et al., 2015). It also appeared to have an additive repressive effect together with corticosteroids, although this difference was not significant. However, theophylline can still significantly repress IL-6 secretion even when corticosteroid insensitivity exists (Figure 6.6B: $P<0.05$). Thus, PP2A activators are a non-steroidal anti-inflammatory alternative and/or corticosteroid-sparing approach in respiratory inflammation where corticosteroid insensitivity exists.

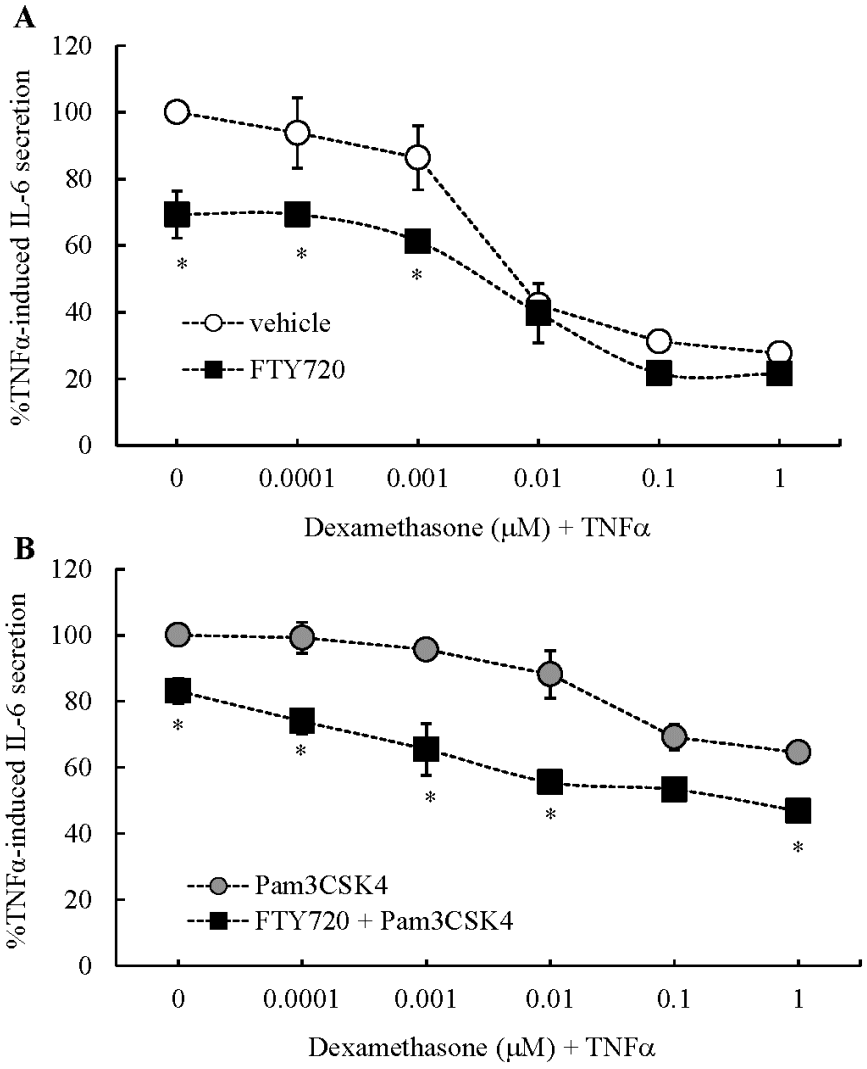


Figure 6.5 FTY720 is corticosteroid-sparing and overcomes corticosteroid insensitivity. A549 were pre-treated for 6 h with vehicle (0.0001-1 μM DMSO) or FTY720 (2.5 μM), before 30 min with (A) vehicle or (B) bacterial mimic Pam3CSK4 (1 μg/ml), then dexamethasone (0.0001-1 μM) for 30 min. After 24 h with TNFα (4 ng/ml), IL-6 was measured by ELISA. Statistical analysis was performed using two-way ANOVA then Bonferroni's post-test (where * denotes a significant effect of FTY720 (P<0.05)). Data are mean+SEM values from n=3 independent experiments.

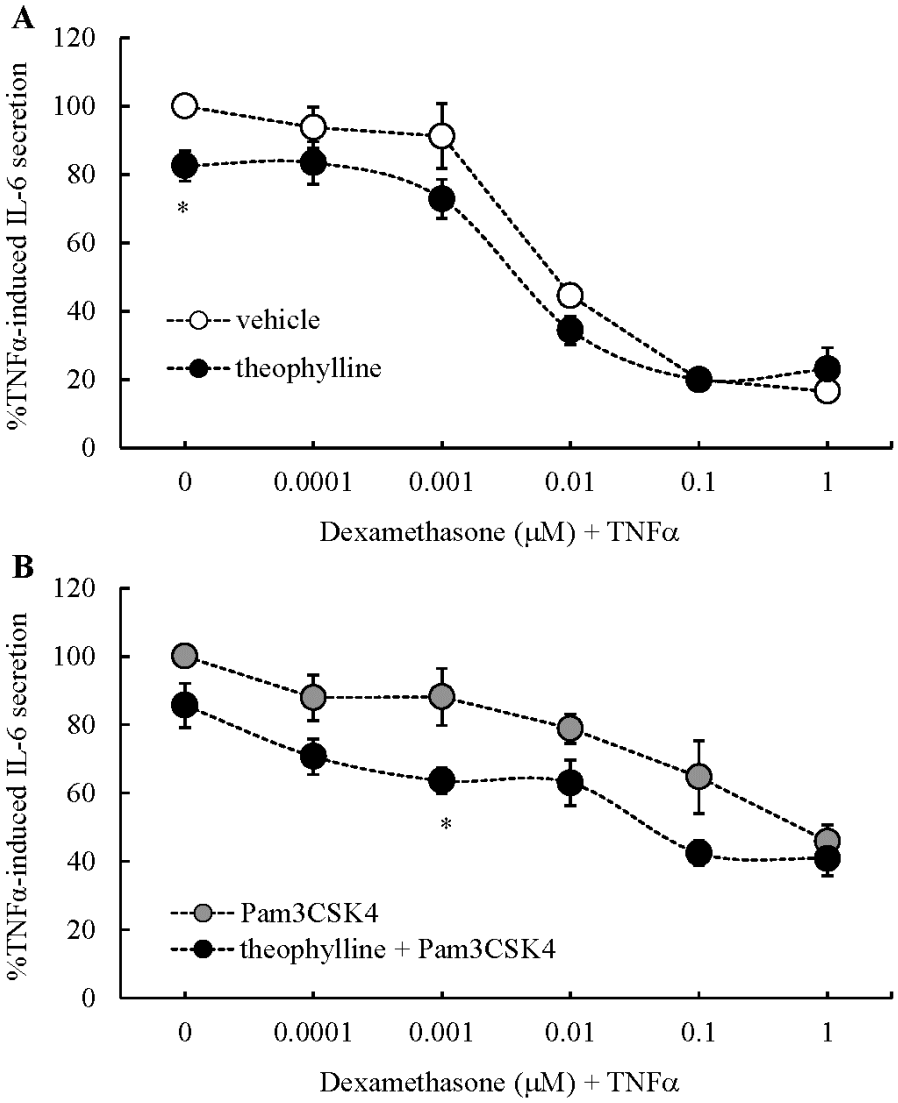


Figure 6.6 Theophylline is anti-inflammatory and can overcome corticosteroid insensitivity. A549 were pre-treated for 30 min with (A) vehicle (0.0001-1 μM DMSO) or (B) bacterial mimic Pam3CSK4 (1 μg/ml), then dexamethasone (0.0001-1 μM) for 30 min, without or with theophylline (10 μM). After 24 h with TNFα (4 ng/ml), IL-6 was measured by ELISA. Statistical analysis was performed using two-way ANOVA then Bonferroni's post-test (where * denotes a significant effect of theophylline (P<0.05)). Data are mean+SEM values from n=3 independent experiments.

6.4 Discussion

Corticosteroid insensitivity is a major factor limiting treatment of chronic respiratory disease today. The relative responsiveness to corticosteroids can be influenced by a range of endogenous and exogenous factors, including respiratory infections. In this study we modelled bacterial infection *in vitro* and showed for the first time that ligation of the TLR2 receptor with a synthetic bacterial lipoprotein induces corticosteroid insensitivity. We then explored the cellular mechanisms responsible and demonstrate that, unlike rhinoviral-induced corticosteroid insensitivity, Pam3CSK4 did not perturb GR nuclear translocation. Notably, while the overall amount of native MKP-1 upregulation protein produced under these conditions was unaffected by Pam3CSK4 pre-treatment, a significant proportion of MKP-1 was oxidized and consequently may be rendered inactive. Finally, we explored alternative modalities to repress cytokine production and demonstrate the utility of PP2A activators as non-steroidal anti-inflammatory alternatives and/or corticosteroid-sparing approaches in respiratory inflammation where corticosteroid insensitivity exists.

When a person with chronic lung disease (such as asthma and COPD) experiences an infection their lung function deteriorates and they require greater amounts of their anti-inflammatory medicines to treat their respiratory symptoms. This is because viral or bacterial infection changes the molecular pathways by which respiratory medicines act; making them less sensitive. These changes are due to interaction of viral and bacterial products with pattern recognition receptors, particularly TLRs expressed on airway cells (reviewed in (Saturni et al., 2015)). We have focused on mimicking bacterial infection *in vitro* with Pam3CSK4 and have previously shown that TLR2 ligation robustly increased TNFα-induced secretion of cytokines implicated as playing a role in exacerbation (Manetsch et al., 2012c). Moreover, TLR2 activation causes

tachyphylaxis to bronchodilatory β_2-agonists *in vitro* and *ex vivo* (Alkhouri et al., 2014). In the current study, we reveal an additional detrimental consequence of bacterial infection beyond amplification of inflammation and β_2-adrenergic desensitization; namely, we show that Pam3CSK4 induces corticosteroid insensitivity.

However, the molecular mechanisms responsible differ from those reported for corticosteroid insensitivity induced in A549 cells by rhinoviral infection (Papi et al., 2013). Corticosteroids initiate their effects on gene expression by interacting with cytoplasmic GRs. Translocation of GR into the nucleus is a requisite step to allow GR to act as a ligand-dependent transcription factor. Once in the nucleus, ligand-activated GRs then repress inflammation by: (i) activation of glucocorticoid response elements and upregulation of anti-inflammatory genes, including MKP-1, glucocorticoid-inducible leucine zipper 1; (ii) inhibitory interactions with proinflammatory DNA-binding transcription factors, such as NF-κB; and (iii) effects on recruitment of transcriptional co-activators, co-repressors and the chromatin machinery (reviewed in (Ammit, 2013)). Papi *et al.* (Papi et al., 2013) showed that rhinoviral infection reduced GR nuclear translocation, thus levels of MKP-1 upregulation was lower, Additionally, they demonstrated induction of MAPK signalling pathways (JNK in particular) and NF-κB activation. In contrast, we show that GR translocation was unaffected by Pam3CSK4 and that the upregulation of MKP-1 in whole cell lysates was unchanged.

However, just examining the overall protein levels of MKP-1 may not reveal the entire story. It is increasingly recognized that the MAPK-deactivating ability of dual-specificity phosphatases (with MKP-1, aka DUSP1, being a founding member of this family) can be regulated post-translationally by oxidation (Bonham and Vacratsis, 2009). The catalytic cysteine of MKP-1 can be modified by oxidation, thereby reducing its function as phosphatase. Redox regulation of

MKP-1 has been demonstrated *in vitro* (Kamata et al., 2005; Hou et al., 2008) and highlighted as a potential mechanism responsible for corticosteroid insensitivity in our recent *in vivo* study exposing MKP-1 wild-type and knock-out mice to ozone (Pinart et al., 2014). Notably, Pam3CSK4 can increase oxidative stress in a TLR2-mediated manner (Singh et al., 2015) and therefore we utilized the PROP technique to show that a proportion of MKP-1 expressed under these conditions is oxidized. Taken together, these results suggest that Pam3CSK4-mediated oxidation of MKP-1 may contribute to corticosteroid insensitivity.

Finally, we explored alternative means of repressing cytokines when corticosteroid insensitivity exists. We focused on small molecules that are known and novel activators of the ubiquitous serine-threonine phosphatase –PP2A. PP2A dephosphorylates a number of kinases that drive inflammatory cell signaling, including MAPKs and NF-κB (Shanley et al., 2001; Miskolci et al., 2003; Junttila et al., 2008; Rahman et al., 2015). A number of small molecules can activate PP2A (Perrotti and Neviani, 2013). The best known of these is FTY720, and we recently showed that it activates TNFα-induced PP2A enzymatic activity and represses cytokine production in A549 cells (Rahman et al., 2015). In the current study we examine its efficacy when used as an adjunct therapy with corticosteroids *in vitro* and show that FTY720 can act additively in a corticosteroid-sparing manner. Most importantly in the context of the bacterial exacerbation, FTY720 can significantly repress cytokine production when corticosteroid insensitivity exists. Lastly, we explored the potential effects of theophylline. Theophylline has beneficial effects in asthma and COPD and acts via a number of different molecular pathways (reviewed in (Barnes, 2013)). We recently reported a new anti-inflammatory function for theophylline; it increases TNFα-induced PP2A enzymatic activity in A549 cells, as well as primary cultures of airway smooth muscle. Thus, we examined its impact on corticosteroid insensitivity and although the impact was not as

great as FTY720, theophylline was able overcome corticosteroid insensitivity. It is important to note however, that we believe that these repressive effects on cytokine function exerted by FTY720 and theophylline are anti-inflammatory effects acting via alternative, PP2A-dependent pathways, rather than restoration of corticosteroid sensitivity.

Collectively, our study shows that, like rhinoviral infection modelled *in vitro*, bacterial ligands exert corticosteroid insensitivity. These models allow us to explore the molecular mechanisms responsible and highlight potential therapeutic strategies to combat inflammation in infectious exacerbation. We reveal that oxidation of anti-inflammatory MKP-1, rather than retardation of nuclear translation, occurs via TLR2-mediated pathways. Finally, we suggest that activating PP2A may prove to be efficacious, non-steroidal anti-inflammatory alternatives and/or corticosteroid-sparing approaches in the context of bacterial exacerbation.

Chapter 7
Individual Cell type variation and different functional outcomes

Chapter 7
Individual Cell type variation and different functional outcomes

7.1 Introduction

In Chapter 3 - Chapter 6, we clearly demonstrated the role of S1P mediated MAPKs in neutrophilic asthma, the anti-inflammatory role of basal PP2A, the link between PP2A and TTP and, most importantly, our studies reveal that PP2A activators have the potential to function as alternative anti-inflammatory strategies that are effective even when corticosteroid insensitivity exists. We performed most of our PP2A studies using A549 cells (partly using BEAS-2B). Therefore in this study we aim to perform some key experiments using BEAS-2B and ASM cells to further confirm our findings. However, due to individual cell type variability we fail to demonstrate a clear conclusion.

Individual cell types are distinguished based on the morphology or the phenotype of cells within a species. Multicellular organisms consist of diverse and specialised cell types including structural cells, muscle cells and skin cells in humans. Although these cells are genetically the same, they are distinct both in appearance and functions.

Cellular models (cell lines - a population of cells with same genetic makeup and derived from a single cell) were developed in the mid 20th century and gained robust acceptance in Molecular Biology research (Masters, 2002). To date, two types of cellular models are successfully employed in Biomedical research; immortal cell lines (cells that via intentionally induced mutation can undergo division indefinately while keeping their cellular features unchanged), and

primary cell lines (derived directly from the parent tissue containing the same karyotype and chromosome number as those in the original tissue) (Hayflick). Both models have been extensively used in Molecular Biology research not only for their easy accessibility and but also as they can be employed in almost all types of experimental techniques.

According to evolutionary principle it is believed that all organisms share some degree of relatedness and genetic resemblance due to common ancestry (Hendry et al., 2011). Therefore *in vivo* experimental models (animal models including mouse, rat and guinea pig) are considered as more authentic experimental models. However *in vivo* models are not suitable with certain experimental techniques such as siRNA or overexpression plasmid, whereas *in vitro* cellular models suit these techniques very well. Further, immortal cells undergo a significant mutation to become immortal and which may possibly alter their biology as well. This is why we use primary cell lines.

Although there are options for selecting experimental models based on experimental need, individual variability is still the key issue we need to resolve. Here in this chapter we aim to address how individual cell type variability impacts on experimental outcomes using BEAS-2B and ASM cells.

7.2 Materials and Methods

7.2.1 Cell culture

For detailed description, see Chapter 2.2.1

7.2.2 Chemicals

FTY720 was purchased from Cayman Chemical Company (Ann Arbor, MI) and AAL(s) was synthesized (Don et al., 2007; Collison et al., 2013a; Hatchwell et al., 2014). TNFα is from R&D Systems (Minneapolis, MN). Unless otherwise specified, all chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO).

7.2.3 PP2A activity assay

For detailed description, see Chapter 2.2.8

7.2.4 ELISAs

Please see Chapter 5.2.5

7.2.5 Real-time RT-PCR

Please see Chapter 4.2.5

7.2.6 siRNA transfection

Please see Chapter 5.2.7

7.2.7 Statistical analysis

Statistical analysis was performed using either the Student's unpaired *t* test, one-way or two-way ANOVA followed by Bonferroni's post-test. *P* values <0.05 were sufficient to reject the null hypothesis for all analyses. Data are mean±SEM of $n \geq 3$ independent replicates.

7.3 Results

7.3.1 PP2A activators, FTY720 and AAL(S) increase TNFα-induced IL-6 mRNA expression and protein secretion in BEAS-2B cells.

Respiratory epithelial cells are an important source of both IL-6 and IL-8 (Cromwell et al., 1992), which are implicated in asthmatic inflammation. In our previous studies, we found that both IL-6 (Figure 5.3) and IL-8 (Figure 5.2) are significantly increased by the treatment of TNFα (4 ng/ml) in A549 cells and are significantly repressed by the pre-treatment of PP2A activators, FTY720 and AAL(S) (Figure 5.2, 5.3). To obtain further confirmation we wish to check TNFα-induced expression of IL-6 and their suppression by PP2A activators using another respiratory epithelial cell line, BEAS-2B. Interestingly, in BEAS-2B cells we find that TNFα increased IL-6 mRNA (Figure 7.1A, 7.1B) and protein (Figure 7.1B, 7.1D) expression; however, PP2A activators FTY720 and AAL(S) do not repress their expression. Instead expression further increased over TNFα.

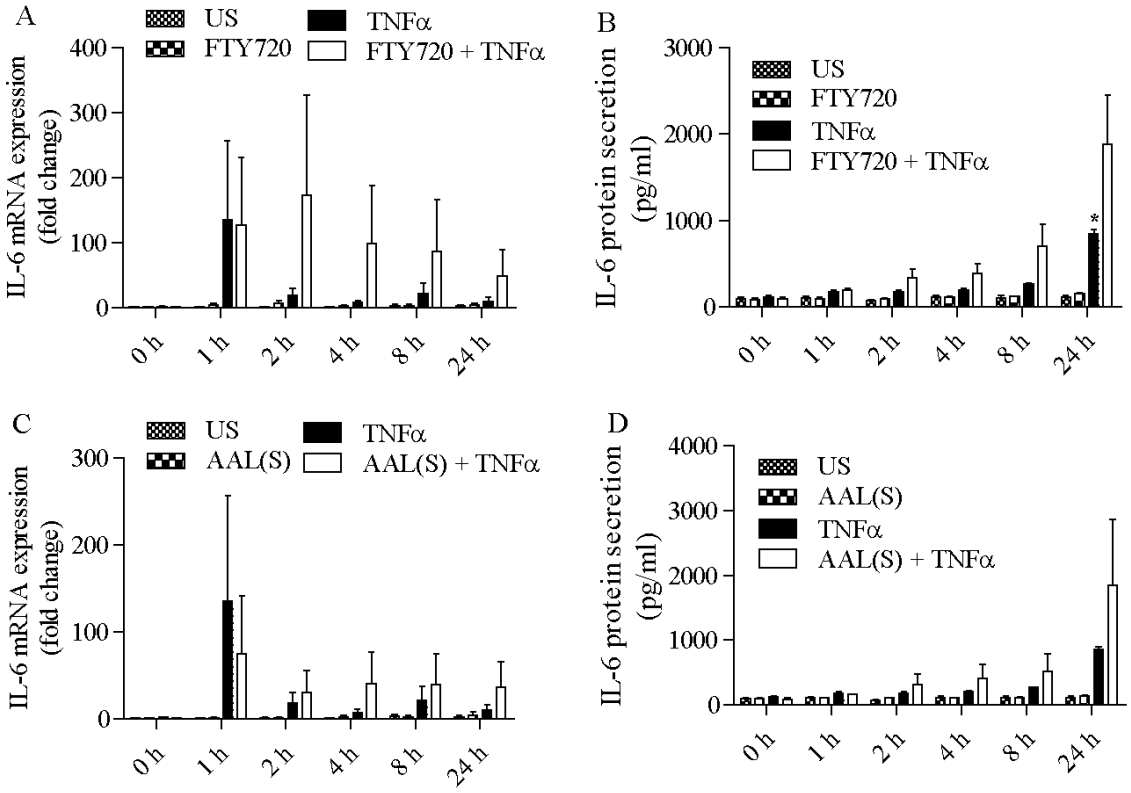


Figure 7.1 PP2A activators, FTY720 and AAL(S), increase TNFα-induced IL-6 mRNA expression and protein secretion. BEAS-2B cells were pre-treated with (A, B) 2.5 μM FTY720 or (C, D) 2.5 μM AAL(S) for 6 h, compared to vehicle-treated controls. Cells were treated with vehicle (1 μM DMSO) or TNFα (4 ng/ml) and then (A, C) IL-6 mRNA expression (results expressed as fold increase compared to vehicle-treated cells at 0 h) and (B, D) IL-6 protein secretion measured at the indicated times. Statistical analysis was performed using two-way ANOVA then Bonferroni's post-test (where * denotes a significant effect of TNFα compared to vehicle-treated cells, and § denotes significant repression of TNFα-induced cytokine production by PP2A activators (P<0.05)). Data are mean+SEM values from n=4-5 independent experiments.

7.3.2 PP2A activators, FTY720 and AAL(S), significantly repress TNFα-induced IL-8 mRNA expression and protein secretion in BEAS-2B cells.

We then intend to check IL-8 mRNA and protein expression in BEAS-2B cells with similar treatment conditions. Our data show that TNFα significantly increases IL-8 mRNA (Figure 7.2A, 7.2C) and protein (Figure 7.2B, 7.2D) expression. Interestingly PP2A activators significantly

repressed (though the repression is very little) both mRNA at early time point (1 h and 2h) and protein expression at a later time point (4 h, 8 h and 24 h), which match with the data of A549 cells (Figure 5.2).

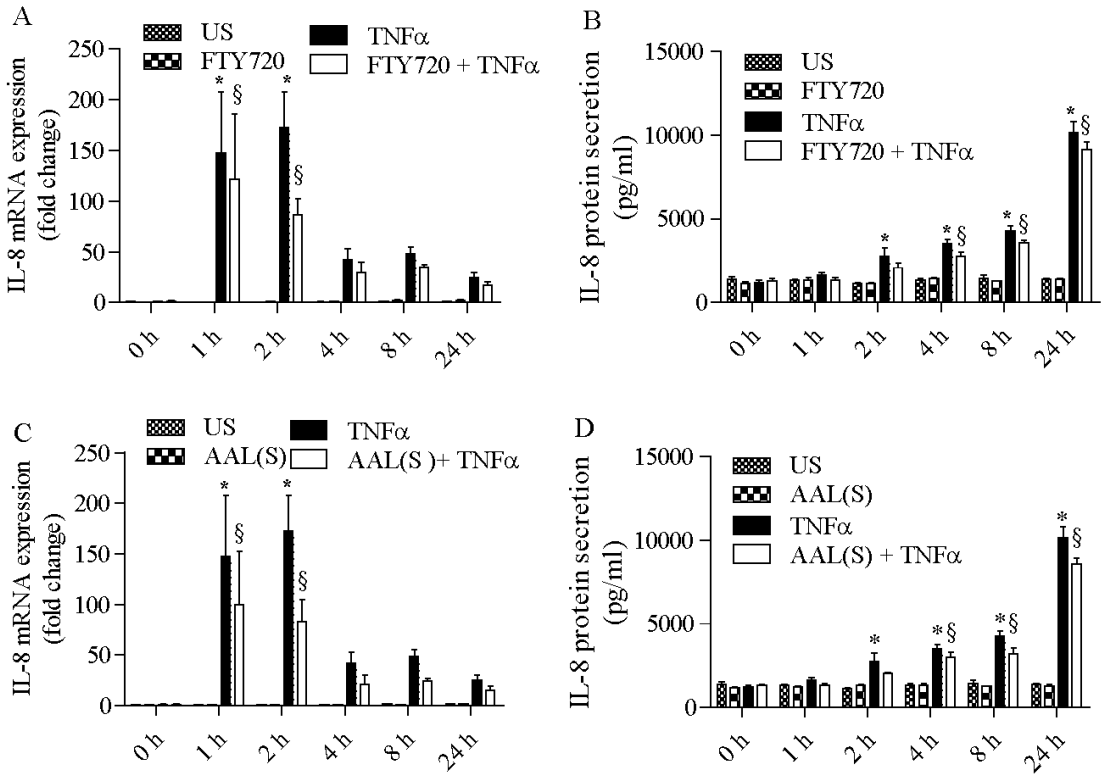


Figure 7.2 PP2A activators, FTY720 and AAL(S), significantly repress TNFα-induced IL-8 mRNA expression and protein secretion. BEAS-2B cells were pre-treated with (A, B) 2.5 μM FTY720 or (C, D) 2.5 μM AAL(S) for 6 h, compared to vehicle (2.5 μM DMSO)-treated controls. Cells were treated with vehicle or TNFα (4 ng/ml) and then (A, C) IL-8 mRNA expression (results expressed as fold increase compared to vehicle-treated cells at 0 h) and (B, D) IL-8 protein secretion measured at the indicated times. Statistical analysis was performed using two-way ANOVA then Bonferroni's post-test (where * denotes a significant effect of TNFα compared to vehicle-treated cells, and § denotes significant repression of TNFα-induced cytokine production by PP2A activators (P<0.05)). Data are mean+SEM values from n=4-5 independent experiments.

7.3.3 PP2A activators FTY720 and AAL(S) do not affect TNFα-induced TTP mRNA but increased IL-6 and IL-8 mRNA and protein expression in ASM cells.

In the BEAS-2B cell line, TNFα-induced IL-8 expression and its repression by PP2A activators consistent with A549 cells but not IL-6. We therefore aim to check IL-6 and IL-8 cytokines expression in ASM cells. Data in ASM cells is rather more complex than that of BEAS-2B (Figure 7.1, 7.2). In ASM cells, TNFα significantly increased IL-6 and IL-8 mRNA (Figure 7.3B, 7.3D) and protein (Figure 7.3C, 7.3E) expression and PP2A activators further increased (though not significantly) both IL-6 and IL-8 expression. However, TTP mRNA (Figure 7.3A) expression was unaffected with the treatment with PP2A activators.

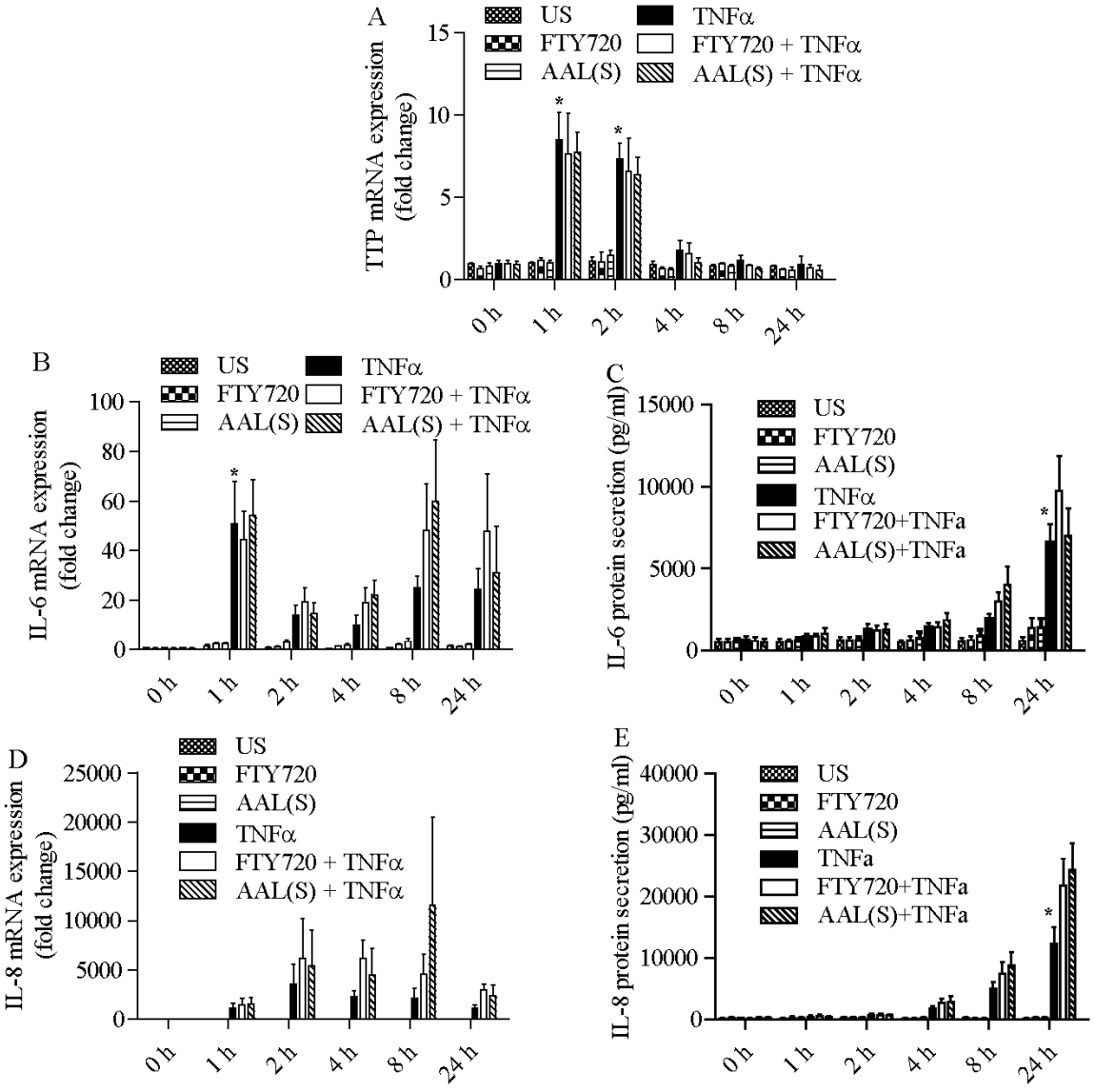
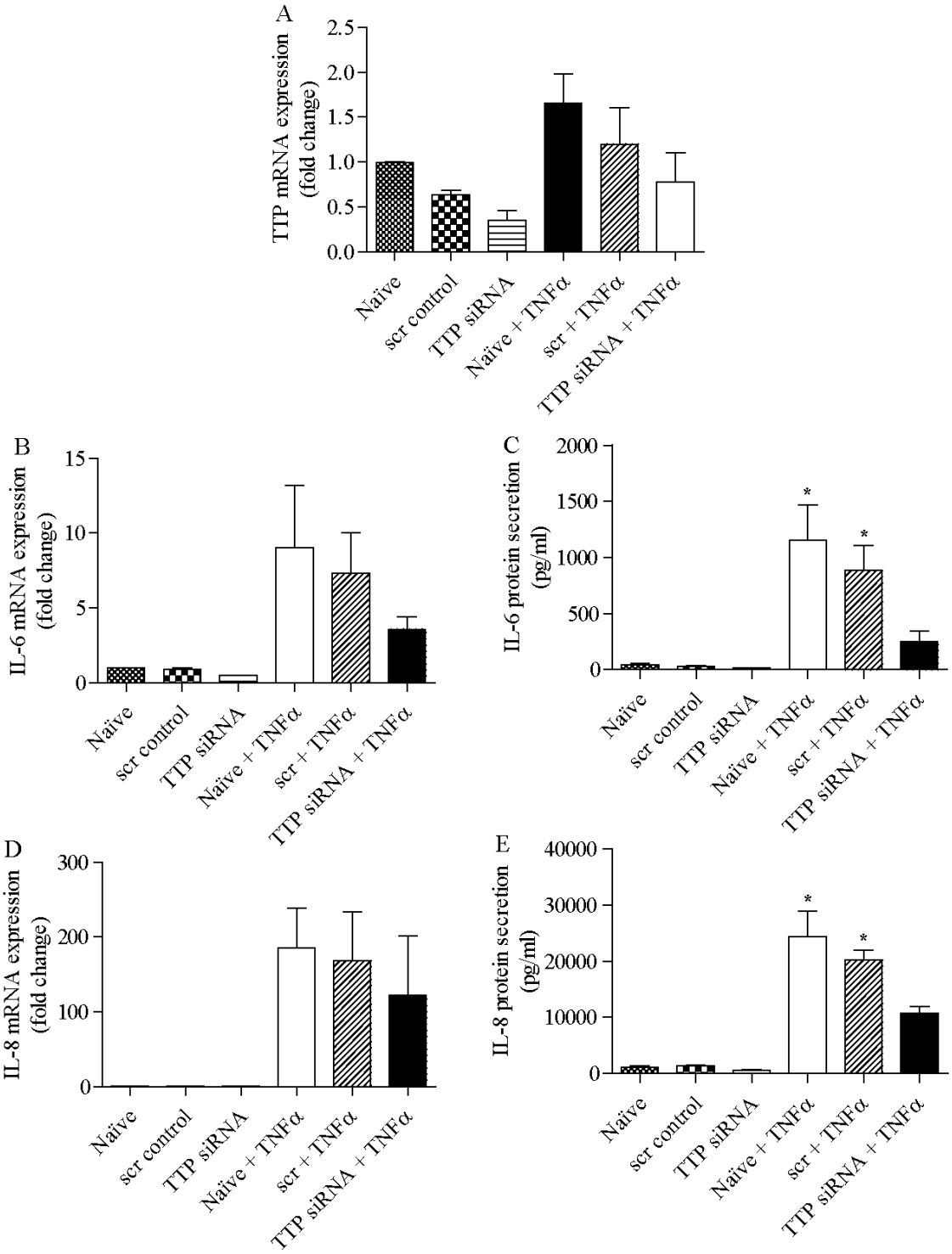


Figure 7.3 PP2A activators, FTY720 and AAL(S), do not affect TNFα-induced TTP mRNA but increased IL-6 and IL-8 mRNA and protein expression in ASM cells. Primary ASM cells were pre-treated with 2.5 μM of FTY720 or AAL(S) for 6 h, compared to vehicle (2.5 μM DMSO)-treated controls. Cells were treated with vehicle or TNFα (4 ng/ml) and then TTP mRNA (A), and IL-6 and IL-8 mRNA (B, D) and protein (C, E) expression measured at the indicated times. Statistical analysis was performed using two-way ANOVA then Bonferroni's post-test (where * denotes a significant effect of TNFα compared to vehicle-treated cells, and § denotes significant repression of TNFα-induced cytokine production by PP2A activators (P<0.05)). Data are mean+SEM values from n=4-5 independent experiments.

7.3.4. Specific knockdown of TTP with siRNA decreases TNFα-induced IL-6 and IL-8 mRNA expression and protein secretion in BEAS-2B cells.

Figure 7.1 and 7.2 both clearly demonstrate that PP2A activators (FTY720 and AAL(S)) have variable effects depending on the cell type. To confirm that this variability is not limited to certain experimental protocols, we decided to check the effect of TTP siRNA in the presence or absence of PP2A activators using BEAS-2B cells. Data in A549 (Figure 5.4) show that specific knock down of TTP with siRNA significantly increased TNFα-induced IL-6 and IL-8 mRNA and protein expression over TNFα. Also, in chapter 5, we demonstrated that activating PP2A can enhance the anti-inflammatory function of TTP in A549 cells. To the confirm efficacy of TTP siRNA we first of all checked TTP mRNA expression (Figure 7.4A). The data clearly demonstrates that TTP siRNA is working well in our experimental conditions. We then checked IL-6 and IL-8 mRNA (Figure 7.4B, 7.4D) and protein (7.4C, 7.4E). The results show that TNFα increased both cytokines mRNA and protein expression. However, in the absence of TTP, cytokines level was considerably reduced, which demonstrates the very unusual but inflammatory function of TTP. We also checked the effect of PP2A activators, FTY720 and AAL(S) in the absence of TTP. As shown in Figure 7.4F and 7.4H, TNFα-induced IL-6 mRNA and protein expression were further increased by FTY720 but AAL(S) significantly decreased IL-6 mRNA but did not affect IL-6 protein secretion; although in our previous experiments (Figure 5.4) we did find further increase of these cytokines expression. Data also show that PP2A activators reduced TNFα-induced IL-8 mRNA (7.4G; significantly) and protein (7.4I; statistically not significant) expression and more interestingly the absence of TTP further reduced IL-6 and IL-8 mRNA and proteins. These results further demonstrate cell type variability and it appears that in BEAS-2B cells TTP does not show anti-inflammatory functions.


A
TTP mRNA expression (fold change)
2.5
2.0
1.5
1.0
0.5
0.0
Naïve
scr control
TTP siRNA
Naïve + TNFα
scr + TNFα
TTP siRNA + TNFα
B
IL-6 mRNA expression (fold change)
15
10
5
0
C
IL-6 protein secretion (pg/ml)
2000
1500
1000
500
0
*
D
IL-8 mRNA expression (fold change)
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200
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E
IL-8 protein secretion (pg/ml)
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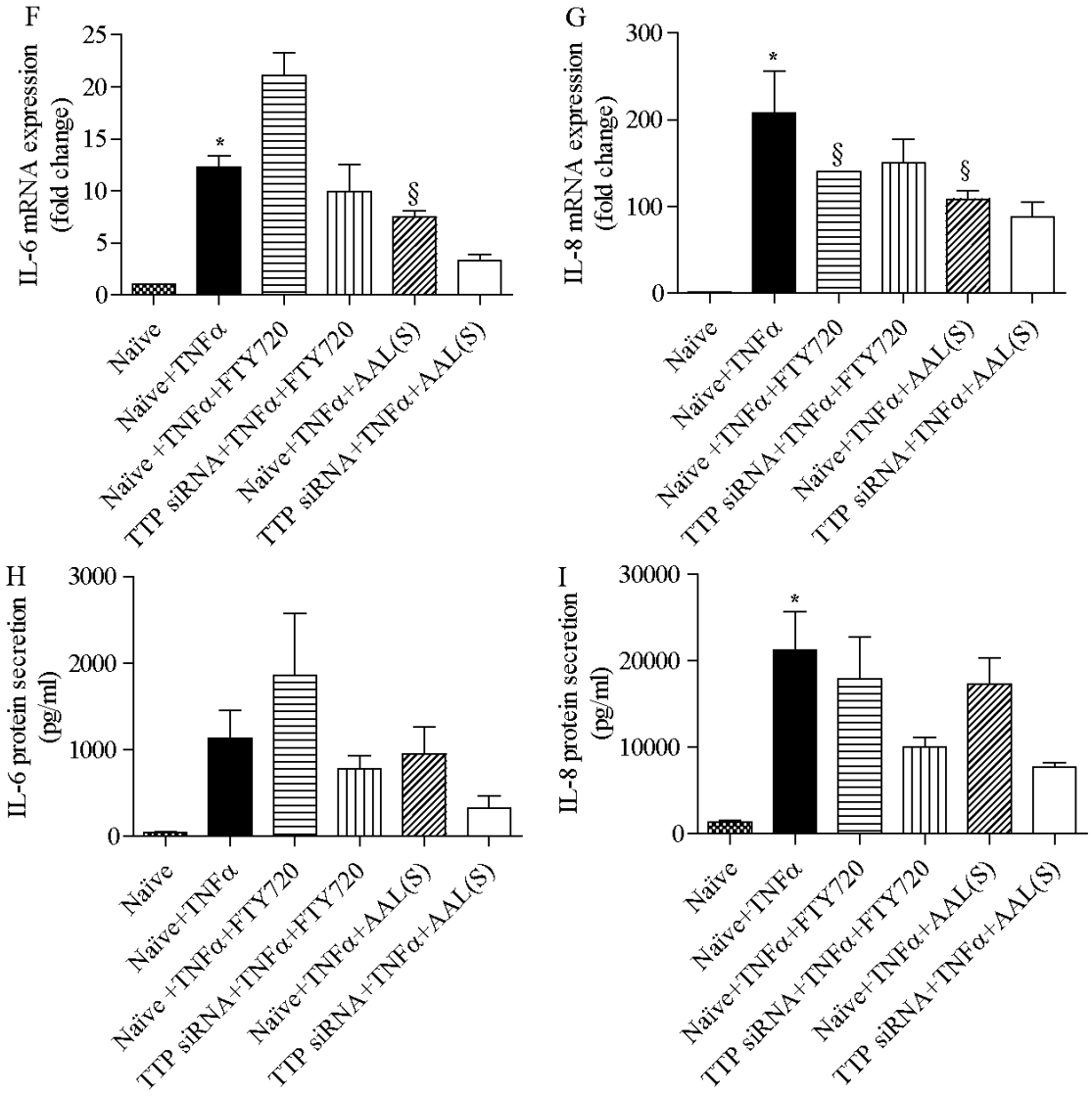

Figure 7.4 Specific knockdown of TTP with siRNA decreases TNFα-induced IL-6 and IL-8 mRNA expression and protein secretion. BEAS-2B cells were either left untransfected or transfected with scrambled control (scr) or siRNA against TTP, then treated with vehicle or TNFα (4 ng/ml) before: (A) knockdown of TTP mRNA expression confirmed at 1 h by RT-PCR (results expressed as fold increase compared to untransfected vehicle-treated controls); and IL-6 and IL-8 mRNA (B, D) and protein (C, E) secretion measured at 1 h and 24 h, respectively. Effect of AAL(S) and FTY720 on IL-6 and IL-8 mRNA (F, H) and protein (G, I) were measured at 1 h and 24 h, respectably in presence or absence of TTP siRNA. Statistical analysis was performed using Student's unpaired *t* test (where * denotes a significant effect of TNFα compared to untransfected vehicle-treated controls, and § denotes a significant effect of siRNA against TTP compared to untransfected controls treated with TNFα ($P<0.05$)). Data are mean+SEM values from n=3 independent experiments.

7.3.5 Steroid inhibited TNFα-induced IL-6 and IL-8 secretion was not affected by FTY720 and AAL(S)

Our studies confirm that normal BEAS-2B and primary ASM cellular models do not corroborate the role of PP2A in asthmatic inflammation due to cell type variability. We then aimed to check whether these cellular models are suitable in the context of corticosteroid insensitivity. Firstly we checked the role of PP2A activators in the presence of corticosteroid (particularly Dexamethasone) in the BEAS-2B cell line. As shown in Figure 7.5A, AAL(S) further inhibited IL-6 protein secretion but not FTY720. Moreover, PP2A activators fail to demonstrate any effect on IL-8 protein secretion in the presence of Dexamethasone (Figure 7.5B).

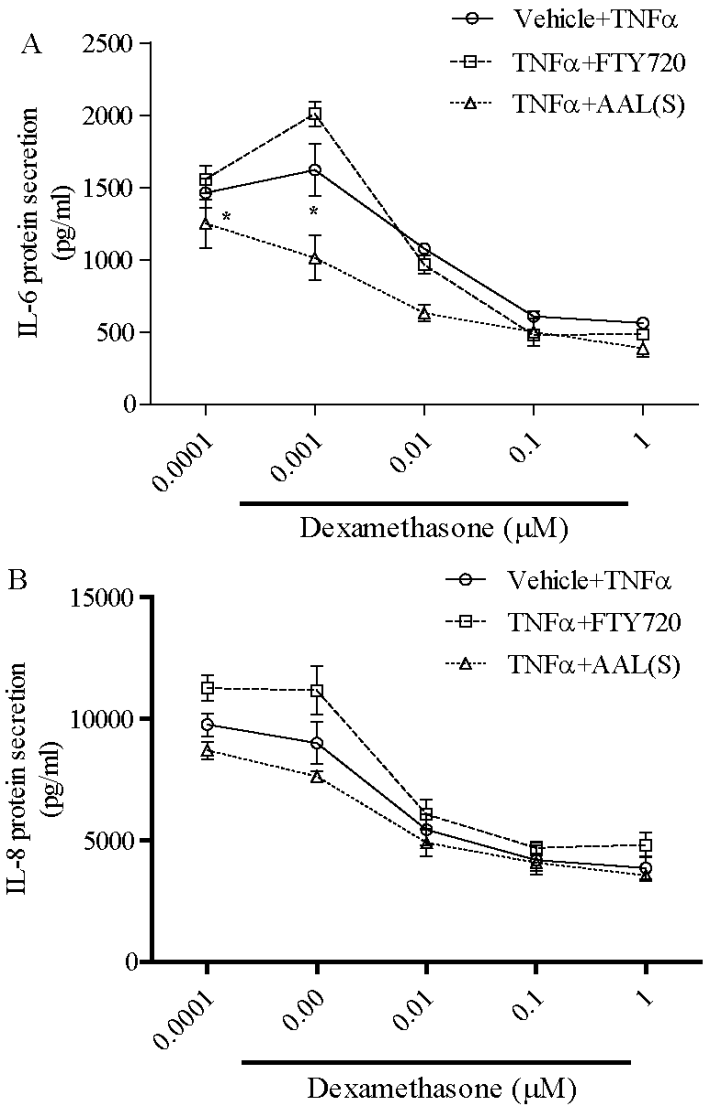

Figure 7.5 Steroid inhibited TNFα-induced IL-6 and IL-8 secretion was not affected by FTY720 and AAL(S). BEAS-2B cells were pre-treated with 2.5 μM FTY720 or 2.5 μM AAL(S) for 6 h, compared to vehicle (0.0001-1 μM DMSO)-treated controls or Dexamethasone (0.0001, 0.001, 0.01, 0.1 or 1 μM) for 30 min. Cell supernatants were harvested after 24 h of TNFα (4 ng/ml) stimulation. IL-6 (A) and IL-8 (B) protein secretion were measured by ELISA. Statistical analysis was performed using two-way ANOVA then Bonferroni's post-test. Data are mean±SEM values from n=3 independent experiments.

7.3.6 Steroid inhibited TNFα-induced IL-6 and IL-8 secretion was not repressed by pam3CSK4 or reversed by PP2A activators

In A549 cells we clearly demonstrate that mimicking bacterial infection (Pam3CSK4) significantly repressed Dexamethasone inhibited IL-6 protein secretion (Figure 6.1). To further confirm this result, we perfomed similar experiment using BEAS-2B cells. As shown in Figure 7.6A and 7.6B, Pam3CSK4 failed to demonstrate its effect in BEAS-2B cells and both IL-6 (Figure 7.6A) and IL-8 (Figure 7.6B) protein secretion are unaffected by PP2A activators. These data clearly show that BEAS-2B cells are not suitable models to check corticosteroid insensitivity.

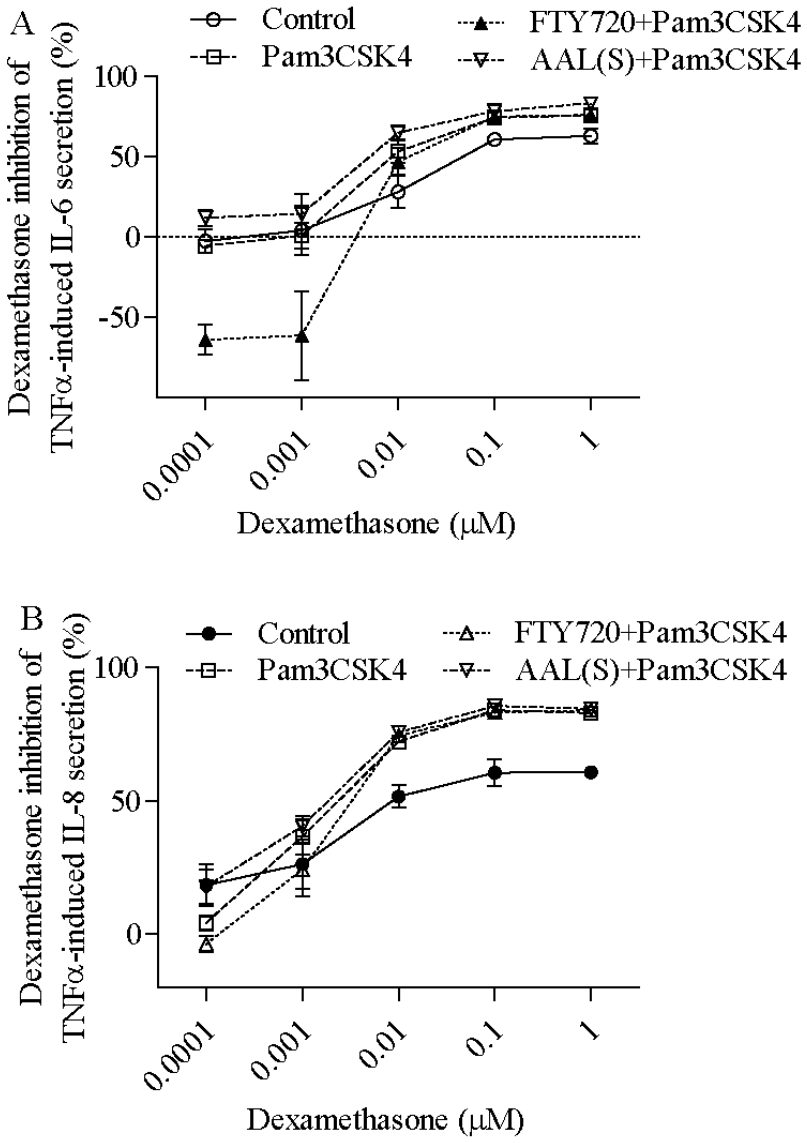

Figure 7.6 Steroid inhibited TNFα-induced IL-6 and IL-8 secretion were not repressed by pam3CSK4 or reversed by PP2A activators. BEAS2B cells were pre-treated with Pam3CSK4 (1 μg/ml) for 1 h or Dexamethasone (0.0001, 0.001, 0.01, 0.1 or 1 μM) for 30 min or 2.5 μM FTY720 or 2.5 μM AAL(S) for 6 h. Cell supernatants were harvested after 24 h of TNFα (4 ng/ml) stimulation. IL-6 (A) and IL-8 (B) protein secretion were measured by ELISA. Statistical analysis was performed using two-way ANOVA then Bonferroni's post-test (where * denotes a significant effect compared to TNFα-treated cells and § denotes a significant effect compared to Dexamethasone+TNFα-treated cells (P<0.05)). Data are mean±SEM values from n=3 independent experiments.

7.3.7 Steroid inhibited TNFα-induced IL-6 and IL-8 secretion was not affected by pam3CSK4

Our data regarding BEAS-2B cells clearly demonstrate that Pam3CSK4 does not effect Dexamethasone inhibited IL-6 and IL-8 protein secretion. We then planned to check the effect of Pan3CSK4 in ASM cells. Interestingly, we obtained consistent results in ASM cells such as BEAS-2B. Both IL-6 (Figure 7.7A) and IL-8 (Figure 7.7B) are unaffected with the treatment of Pam3CSK4. Overall the results in normal BEAS-2B and primary ASM cells are consistent. These results ultimately led us to check PP2A enzymatic activity level with the treatment of PP2A activators. As shown in Figure 7.7C, PP2A activators do have any effect on PP2A enzymatic activity.

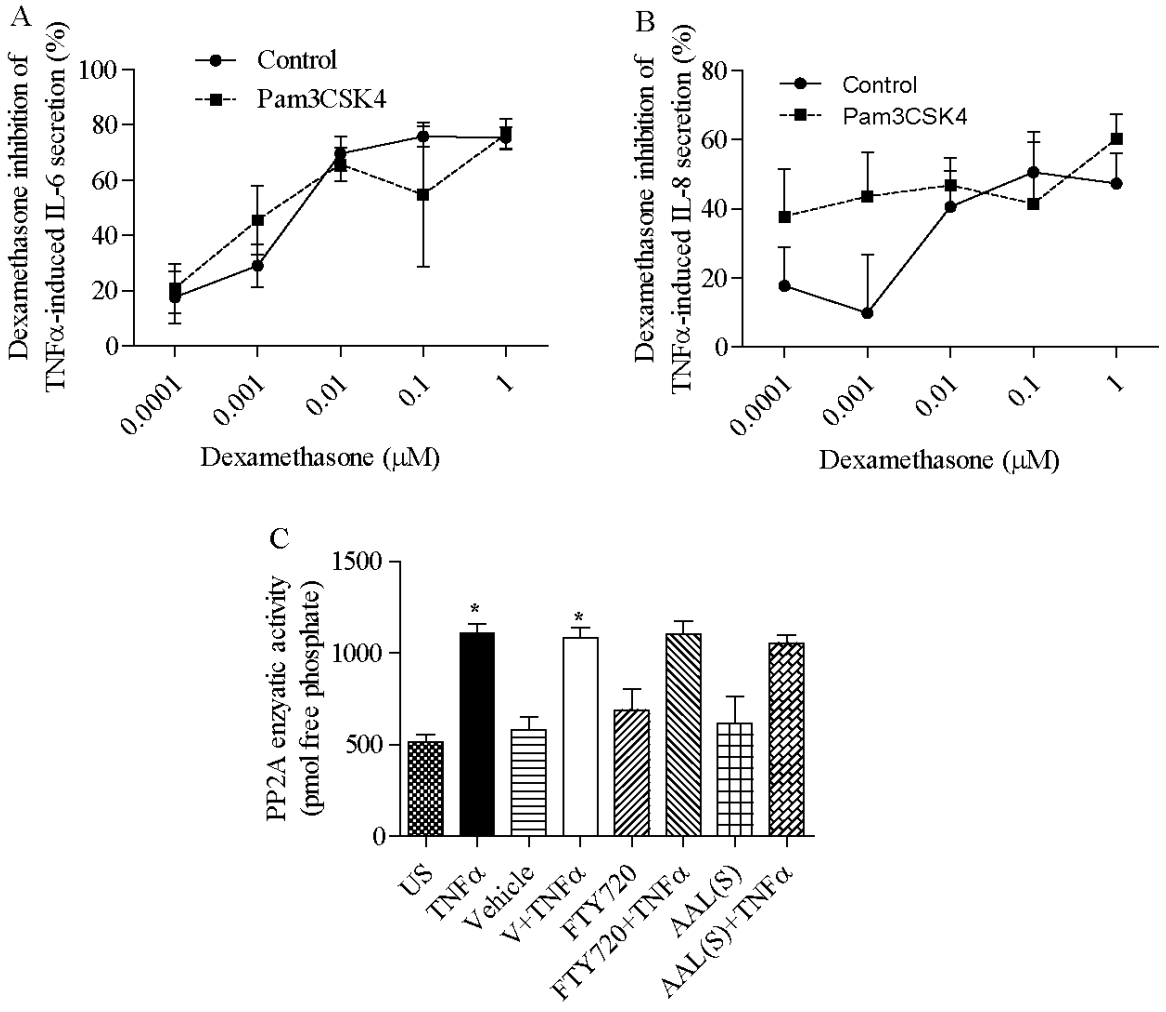

Figure 7.7 Steroid inhibited TNFα-induced IL-6 and IL-8 secretion was not affected by pam3CSK4. ASM cells were pre-treated with Pam3CSK4 (1 μg/ml) for 1 h or Dexamethasone (0.0001, 0.001, 0.01, 0.1 or 1 μM) for 30 min. Cell supernatants were harvested after 24 h of TNFα (4 ng/ml) stimulation. IL-6 (A) and IL-8 (B) protein secretion were measured by ELISA. PP2A activity (C) was measured at 1 h after TNFα stimulation followed by 6 h pre-treatment with 2.5 μM of FTY720 and AAL(S) or 1 h with Pam3CSK4 (1 μg/ml). Statistical analysis was performed using two-way ANOVA then Bonferroni's post-test (where * denotes a significant effect compared to TNFα-treated cells and § denotes a significant effect compared to Dexamethasone+TNFα-treated cells (P<0.05)). Data are mean±SEM values from n=3 independent experiments.

7.4 Discussion

Individual variability is one of the key issues in selecting experimental models. Despite similarities between cellular models, it is not unusual that very different functional outcomes can occur. Evidence supports that certain drugs (especially anti-cancer drugs) may be effective in some patients but not as effective in other patients (Schilsky, 2010). This individual variability ultimately leads to the concept personalized medication, which has recently emerged as an effective therapeutic approach to treat cancer.

In this project, we successfully demonstrate that endogenous PP2A effectively combats asthmatic inflammation and PP2A activators are potent even under corticosteroid insensitive conditions using a widely used cellular model (A549). A549 is an immortalized cellular model derived from lung carcinoma tissue. To obtain further confirmation of our findings we wished to employ another widely used respiratory epithelial cells, BEAS-2B (immortalized but isolated from normal human bronchial epithelium) and primary ASM cells to further corroborate the role of PP2A in asthmatic inflammation.

PP2A activators (FTY720 and AAL(S)) significantly repress TNFα-induced IL-6 and IL-8 mRNA and protein expression in A549 cells, whereas in BEAS-2B cells PP2A activators fail to repress (rather increased over TNFα) IL-6 mRNA and protein, though expression of IL-8 is repressed a little. Results using BEAS-2B corroborate the concept that treatment with IL-6 can repress PP2A enzymatic activity in Rat-1 fibroblast and human hepatoma Hep3B cells (Choi et al., 1998; de la Torre et al., 2005). It is reported that IL-6 binds with specific receptors on the cell surface and causes inactivation of PP2A via phosphorylation of tyrosine on its catalytic subunit, which in turn prolongs the activation of protein kinases (Chen et al., 1992; Wera and Hemmings,

1995). However, in ASM cells both IL-6 and IL-8 mRNA and protein expression are further increased over TNFα with the treatment of PP2A activators.

A growing body of evidence supports TTP as an mRNA binding protein targeting cytokines mRNA, which are implicated in asthmatic inflammation (Lai et al., 2006; Tudor et al., 2009). In A549 cells we show that blocking TTP using OA and siRNA against TTP significantly increased TNFα-induced cytokines expression over TNFα alone. Notably, our studies reveal that while blocking TTP using siRNA, PP2A activators lose their potential to suppress cytokines expression, which demonstrates that PP2A works via enhanching TTP anti-inflammatory function. However studies in BEAS-2B show opposite functional outcomes than that of A549 cells.

We modeled A549 to study Pam3CSK4-induced corticosteroid insensitivity and showed that the PP2A activator, FTY720, can bypass steroid insensitive conditions in the context of IL-6 protein expression. However, Pam3CSK4 does not cause steroid insensitivity in BEAS-2B and ASM cells and ultimately the PP2A activator failed to demonstrate its potential. More interestingly, PP2A activators fail to augment PP2A enzymatic activity in ASM cells. Results obtained using BEAS-2B and ASM cells suggest that PP2A activators may not be equally effective in all cell types. In addition, TTP fails to target cytokines mRNA both in BEAS-2B and ASM cells. This may be the reason for the ineffectiveness of the PP2A activator.

PP2A is highly regulated at multiple levels and associated with MAPKs and TTP as shown in Figure 1.8. We and others have reported that MKP-1 is regulated by MAPKs (especially p38 MAPK) (Franklin and Kraft, 1997; Manetsch et al., 2012b). A wide range of inflammatory stimuli are responsible for p38 MAPK phosphorylation and hence activation (Prabhala et al., 2015). Upon activation p38 triggers MKP-1 expression, which in turn downregulates p38

phosphorylation via negative feedback mechanisms (Manetsch et al., 2012b). TTP is also predominantly regulated by p38 activation. By regulating MAPKs, both PP2A and MKP-1 shift the TTP balance towards the unphosphorylated – active – from. Although the link between MKP-1 and PP2A is not yet clear, based on our studies in this project we deduce that they might be interlinked.

Further, PP2A is the key controller of MAPKs and at the same it is itself a target of several endogenous inhibitory proteins (Goldberg, 1999) including SET, CIP2A, α4, SV40 ST and PyMT (Figure 1.6). These complex regulatory mechanisms and the inter relationship between PP2A, TTP, MKP-1 and MAPKs may be the reason behind the individual variability. Extensive studies are required to explore the underlying molecular mechanisms, which may eventually lead to better understanding of individual variability.

Chapter 8
General discussion and conclusion

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Overview: Asthma is a widespread noncommunicable chronic health problem that affects millions of people around the globe. The prevalence of asthma has increased very significantly in recent decades and most alarmingly it is still an increasing trend. Inflammation is the major driving force of asthma pathogenesis, which is associated with the structural remodelling of the airway wall. Long-term exposure to pro-inflammatory mediators, including cytokines, results in remodelling or thickening of the airway. Development of remodelled airways is correlated with deterioration of lung function. Reducing inflammation thus remains a key goal in asthma therapy to ameliorate long term decline in patient health. The corticosteroids are the major class of anti-inflammatories currently used in the treatment of asthma. However, despite their efficacy as powerful anti-inflammatories, the array of side-effects following chronic corticosteroid use causes significant unwanted effects. Besides, many asthmatic inflammatory conditions (e.g. viral or bacterial infection, exacerbation) are not responsive to them. Corticosteroid insensitivity can range from relatively mild insensitivity to steroid resistance, as seen in severe asthma (Ammit, 2013; Chung, 2013). Therefore the challenge for asthma treatment today is to identify alternative anti-inflammatory strategies with reduced side-effects and greater effectiveness in steroid resistant conditions.

Substantial research on asthmatic inflammation over the last couple of years has increased our understanding of the mechanisms of airway inflammation and has ultimately led to a number of possible therapeutic targets. Notably, some alternative strategies such as sputum guided treatment and fractional concentration of FeNO have been evaluated, mainly in severe or

difficult-to-treat asthma, for adjusting asthma treatment. However sputum guided treatment is only beneficial when the patients required secondary care (Haldar et al., 2008) and FeNO has not generally been found to be effective (Petsky et al., 2012). In this project we therefore aimed to explore the potential of an endogenous protein phosphatase, PP2A, which offers great promise in the context of asthmatic inflammation.

Our approaches: The role of inflammation in asthma pathogenesis and airway remodelling is well studied. In searching for an alternative anti-inflammatory strategy, firstly we aimed to explore the role of the bioactive sphingolipid S1P in the context of neutrophilic asthma, which is relatively unexplored to date. A growing body of evidence supports the role of S1P in asthmatic inflammation and airway remodelling (Lai et al., 2011; Che et al., 2012). In our previous study we reported elevated levels of S1P in the broncho-alveolar lavage fluid of allergic asthmatics (Ammit et al., 2001). Mast cells are considered the major sources of S1P in allergic asthma (Jolly et al., 2002; Olivera and Rivera, 2011) and interestingly in asthmatic patients mast cells are micro-localized in ASM cells, airway mucous glands and bronchial epithelium (Oskeritzian et al., 2007). S1P potently induces secretion of IL-6 from ASM (Ammit et al., 2001; Che et al., 2013) and IL-8 from alveolar epithelial cells (Milara et al., 2009). This suggests that the bioactive sphingolipid S1P can orchestrate cytokine production in airway inflammation. In line with these studies, our present study shows that stimulation of primary ASM cells with S1P results in IL-8 gene expression and protein secretion (Figure 3.1A, 3.2A). Recently we demonstrated that corticosteroid-induced MKP-1 attenuated S1P-induced IL-6 secretion by dephosphorylating p38 MAPK and also the ERK-mediated activation of MSK1 and histone H3 phosphorylation (Che et al., 2014). Besides, we propose that S1P-induced IL-8 may be

controlled in a similar MSK1-dependent manner. In this study we reveal that S1P-induced IL-8 secretion is p38 MAPK- and ERK-dependent (Figure 3.6) and that these key phosphoproteins act on the downstream effector MSK1 to control secretion of the neutrophil chemoattractant cytokine IL-8 (Figure 3.7). To demonstrate the functional relevance of our *in vitro* data we perform neutrophil chemotaxis assays and show that S1P-induced effects can be significantly attenuated by pre-treatment with dexamethasone, pharmacological inhibition of p38 MAPK- or ERK-mediated pathways, or by knocking down MSK-1 with siRNA (Figure 3.7). These studies partly reveal the molecular mechanisms of S1P in the development of IL-8-driven neutrophilia in airway inflammation and suggest further studies into the causal role played by S1P in the neutrophilic asthma.

Findings of our first approach:

1. The inflammatory mediator, S1P, upregulates IL-8 secretion from primary ASM cells and contributes to the development of airway inflammation.
2. S1P-induced IL-8 secretion is p38 MAPK and ERK-dependent.
3. MSK1 controls the secretion of the neutrophil chemoattractant cytokine IL-8.
4. The corticosteroid dexamethasone significantly represses S1P-induced IL-8 expression.

PP2A is a master controller of multiple inflammatory signalling pathways. PP2A dephosphorylates a number of kinases that drive inflammatory cell signalling (Shanley et al., 2001; Junttila et al., 2008). Notably, PP2A can dephosphorylate members of the MAPK superfamily, including p38 MAPK (Junttila et al., 2008). The role of PP2A as a tumour suppressor is well studied and thus PP2A activator has emerged as a novel therapy for treating cancer. Excitingly, the knowledge gained from understanding of PP2A in cancer has propelled

research into its role and function in respiratory disease and more recently it has emerged as a druggable target in asthmatic inflammation; although the underlying molecular mechanisms remain relatively unexplored. In order to unveil the molecular mechanisms of PP2A in asthmatic inflammation we aimed to explore the role of basal PP2A in cancer patient derived respiratory epithelial cells (A549) and some confirmatory experiments were performed using normal respiratory epithelial BEAS-2B cells. Mounting evidence supports the role of PP2A as a negative regulator on MAPKs and in line with these studies we find that inhibition of basal PP2A by OA upregulates p38 MAPK, ERK and JNK phosphorylation in A549 cells (Figure 4.2). To demonstrate the role of basal PP2A in respiratory epithelial cells we employ multiple approaches including modulation of PP2A phosphatase activity by using a PP2A inhibitor, OA (Figure 4.1); two PP2A activators, FTY720 (Figure 4.7A) and AAL(S) (Figure 4.8A); an expression plasmid to overexpress the catalytic subunit of PP2A (PP2A-C) (Figure 4.9A & B); and siRNA to knockdown PP2A-C (Figure 4.11A & B). We and others previously reported that IL-6 and IL-8 are the two major cytokines upregulated via MAPK-mediated pathways. Studies performed in this project confirm the role of basal PP2A in restraining cytokines (especially IL-6 and IL-8) expression and demonstrate the link in asthmatic inflammation. Most importantly, in support with previous reports (Brook et al., 2006; Clement et al., 2011), in this study we have found that PP2A inhibition has a significant impact on the critical anti-inflammatory protein, TTP, which demonstrates the potential of endogenous phosphatases as novel anti-inflammatory strategies in asthmatic inflammation.

Findings of our second approach:

1. PP2A dephosphorylates MAPKs that regulate inflammatory cell signalling.

2. Inhibition of basal PP2A activity releases restraint on MAPKs and increases MAPKs-mediated gene expression, such as cytokines IL-6 and IL-8.
3. TTP mRNA expression ensues but resultant protein is rendered inactive by MAPKs.
4. Basal protein phosphatase 2A activity restrains cytokine expression and thereby regulates TTP functions.

TTP is a mRNA destabilizing protein that targets numerous cytokines (Brooks and Blackshear, 2013), including those which are implicated in asthmatic inflammation. TTP is controlled by phosphorylation and has a dynamic equilibrium between unphosphorylated and phosphorylated forms. Unphosphorylated forms of TTP are active and are capable of targeting cytokines mRNA. However, the activated form is unstable and undergoes proteosomal degradation, whereas phosphorylated TTP is stable but inactive and unable to target cytokines mRNA for decay. It is reported that P38 MAPK targets two important serines (Ser^{52} and Ser^{178}) of TTP (Brook et al., 2006), causes phosphorylation of TTP and most importantly PP2A targets the same serines and shifts the balance toward the unphosphorylated active form. In our previous study we showed that under unstimulated conditions inhibition of PP2A switch off TTP (Rahman et al., 2015); but whether PP2A activators can switch TTP on and repress cytokine production under inflammatory conditions mimicked *in vitro* is currently unknown. As a continuation of our previous study here we find that during TNFα-induced inflammatory conditions PP2A activators can enhance PP2A enzymatic activity (Figure 5.1), which effectively shifts the equilibrium from phosphorylated/inactive towards unphosphorylated/active TTP. It is reported that blocking PP2A robustly increases IL-8 expression in A549 cells via post-transcriptional mRNA stabilization (Cornell et al., 2009); TTP was implicated but not directly examined. Besides, PP2A dephosphorylates a number of kinases that drive inflammatory pathways (Shanley et al., 2001;

Miskolci et al., 2003; Junttila et al., 2008). Whether TTP was involved remained an open question. In support of these studies here we show that PP2A is the phosphatase responsible for TTP dephosphorylation (Figure 5.6); hence boosting PP2A activity offers a promising therapeutic avenue in asthmatic inflammation.

Findings of our third approach:

1. PP2A is responsible for dephosphorylating (and hence activating) TTP.
2. Small molecule PP2A activators, FTY720 and AAL(S) can enhance TTP's anti-inflammatory function to inhibit cytokine production.
3. PP2A activators ensure the potential to act as alternative anti-inflammatory strategies to reduce airway inflammation in respiratory disease.

More recently defective PP2A is linked with corticosteroid insensitivity in severe asthma (Kobayashi et al., 2011). In addition, it is reported that reduced PP2A activity induces corticosteroid resistance and this can be reversed by the long-acting β_2-agonist formoterol (Kobayashi et al., 2012). These studies clearly demonstrate the role of PP2A in corticosteroid insensitivity; whether enhancing PP2A activity can overcome this condition remains unclear. To explore this we modelled bacterial infection *in vitro* to show for the first time that ligation of the TLR2 receptor with Pam3CSK4 induces corticosteroid insensitivity (Figure 6.1). Notably, unlike rhinoviral-induced corticosteroid insensitivity (Papi et al., 2013), Pam3CSK4 does not affect GC nuclear translocation or the overall amount of native MKP-1. However, in support of Singh *et al* (Singh et al., 2015) we find a significant proportion of MKP-1 is oxidized and consequently may be rendered corticosteroid inactive. Most importantly, we explore in our study that PP2A activators known (FTY720) and novel (theophylline, recently we reported as PP2A activator (Patel et al., 2015)) can significantly repress cytokine production when corticosteroid

insensitivity exists (Figure 6.5 & Figure 6.6); although we believe that these repressive effects on cytokine function exerted by FTY720 and theophylline are anti-inflammatory effects acting via alternative, PP2A-dependent pathways, rather than restoration of corticosteroid sensitivity. Hence we propose that activating PP2A may prove to be efficacious, non-steroidal anti-inflammatory alternatives and/or corticosteroid-sparing approaches in the context of bacterial infection.

Findings of our fourth approach:

1. TLR2 ligation with a synthetic bacterial lipoprotein (Pam3CSK4) induces corticosteroid insensitivity.
2. Unlike rhinoviral-induced corticosteroid insensitivity, Pam3CSK4 does not perturb GR nuclear translocation.
3. The overall amount of native MKP-1 upregulation protein is unaffected by Pam3CSK4, but a significant proportion of MKP-1 was oxidized and consequently may be rendered corticosteroid inactive.
4. PP2A activators function as non-steroidal anti-inflammatory alternatives and/or corticosteroid-sparing approaches in respiratory inflammation where corticosteroid insensitivity exists.

In order to obtain further confirmation of our above findings we performed some key experiments using normal BEAS-2B and primary ASM cells. However, due to individual cell type variability, we failed to demonstrate further confirmation but explored new possibilities to uncover the reasons for this result.

Conclusion: In this project we further confirm the role of inflammatory mediators such as S1P, TNFα and Pam3CSK4 in cytokines expression and a novel role of one important corticosteroid,

dexamethasone, in neutrophilic asthma using primary ASM cells. We successfully demonstrate that basal PP2A restrains cytokines, especially IL-6 and IL-8 expression via MAPKs mediated signalling pathways. Our study also reveals that blocking PP2A activity causes TTP phosphorylation and hence inactivation. Further, our study confirms that PP2A is directly linked with TTP anti-inflammatory functions and PP2A activators can work effectively during Pam3CSK4-induced corticosteroid insensitive condition. Therefore we propose that enhancing PP2A activity can serve as an alternative anti-inflammatory strategy to combat asthmatic inflammation.

Future directions: Here in this thesis we mainly focus on the exploration of alternative anti-inflammatory strategies to combat asthmatic inflammation in a safer and more effective way. Though our studies successfully reveal that PP2A activators can act as alternative anti-inflammatory agents, further understanding of the molecular mechanism of PP2A regulation in respiratory inflammation is required. Based on the findings of this project and previous reports it could be speculated, however, that PP2A activators may repress inflammation under inflamed (mimicked here with TNFα), rather than non-inflamed conditions. Most importantly, the underlying molecular mechanism involved in corticosteroid insensitivity is as yet unclear and warrants extensive study. In addition, individual cell type variability is an important issue to explore. In this project we demonstrate the role of PP2A in asthmatic inflammation and corticosteroid insensitivity using mainly respiratory epithelial cells (mostly A549 cancer cells and some confirmatory experiments in normal BEAS-2B cells), using an *in vivo* model. TTP/PP2A knock-in and/or TTP/PP2A knock-out mouse models are especially needed for greater understanding.

Moreover, some specific questions are needed to be addressed, such as:

a) How endogenous PP2A is regulated?

b) How TNFα increases PP2A enzymatic activity?

c) How Pam3CSK4 induces corticosteroid insensitivity?

d) How PP2A activators bypass Pam3CSK-4-induced steroid insensitivity?

e) Why cell type variability affects functional outcomes?

We do believe addressing these questions will certainly increase our understanding, which will ultimately lead to the creation of a novel therapeutic avenue (alternative anti-inflammatory strategy) to combat asthma safely and effectively.

Several conditions, including infectious exacerbation, severe asthma and COPD induce corticosteroid insensitivity. Among them COPD has emerged as a major public health concern worldwide and a leading cause of morbidity and mortality. Notably, the burden of COPD is predicted to increase due to continued exposure to COPD risk factors including tobacco smoke, outdoor air pollution and indoor noxious particles and gases from cooking and heating. The prevalence, morbidity and mortality of COPD are increasing in many westernised countries and cigarette smoking is the main cause. In our thesis we mainly focuse on Pam3CSK4-induced corticosteroid insensitivity and demonstrate the potential of PP2A activators as alternative anti-inflammatory strategies in this condition. Hence the role of PP2A activators as alternative anti-inflammatory strategy in the context of COPD will be a logical extention of our study for better understanding and to check whether PP2A activators are able to bypass the COPD mediated corticosteroid insensitivity. To achieve this target the following approaches can be addressed using both *in vitro* and *in vivo* models:

1. Confirm whether active TTP is capable to reduce inflammation in COPD.

2. Investigate the molecular mechanisms of PP2A regulation and pharmacological modulation in the context of COPD mediated corticosteroid insensitivity.
3. Investigate the role of PP2A activators as alternative anti-inflammatory strategy to bypass corticosteroids insensitivity in COPD.

Our studies in this project successfully demonstrate that PP2A activators offer great promise as alternative anti-inflammatory strategies and druggable targets. To date a number of small molecules are reported to activate PP2A enzymatic activity (Perrotti and Neviani, 2013). Among these small molecules the sphingosine analog, FTY720 is well-studied. FTY720 is a non-specific PP2A activator and hence it has other targets. Most notably in the context of respiratory disease, FTY720 can induce S1P signalling. To overcome S1P agonism a chiral analog of FTY720, AAL(S), has recently been developed. AAL(S) is as equally potent as FTY720 in activating PP2A complexes *in vitro* and significantly represses airways disease in mouse models *in vivo* (Collison et al., 2013b). Although AAL(S) is an improved analog of FTY720, there is scope to improve it furher for greater efficacy. Thus, development of new and improved PP2A activators, examination of their mechanisms of action and demonstration that they repress cytokines in COPD *in vitro* and *in vivo* via enhanching TTP activity may offer better therapeutic avenues of alternative anti-inflammatory strategies to combat asthmatic inflammation in the future.

Chapter 9
References

Chapter 9
References

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