EFFECTS OF ANDROGRAPHOLIDE AND 14-DEOXY-11,12-DIDEHYDROANDROGRAPHOLIDE IN OBSTRUCTIVE RESPIRATORY DISEASE MOUSE MODELS

GUAN SHOU PING

BSc (Hons)

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHARMACOLOGY

NATIONAL UNIVERSITY OF SINGAPORE

2012

DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

_____

GUAN SHOU PING

26 September 2012

ACKNOWLEDGEMENTS

First and foremost, I would like to extend my most sincere appreciation to my supervisor Professor Wong Wai-Shiu Fred for his guidance that is so instrumental in my Ph.D studies. Without his encouragement and assistance, I would never have completed my studies and overcome all the obstacles in this project.

I also like to thank Professor Shen Han Ming, Dr. Leung Pui Lam, Bernard and Dr Gautam Sethi for their kind willingness to be my thesis advisory committee. Their invaluable advice assisted on my research works.

I especially want to thank Mr. Koh Hock Meng, Alan who always being very resourceful all this years.

I would also like to extend my gratitude for my senior Dr Bao Zhang and Dr Liao Wu Peng Winston for their guidance during my early PhD studies.

I would also like to thanks Dr Cheng Chang, Ryan Wu Song Lian, Kong Li Ren, Chan Tze Khee, Wilson Tee, Samantha Li, David Ng Shen Wen and all other colleague in the lab for their industrious effort in the various studies that we have gone through together, shared experience and supported me.

I would like to thank NUS for offering me a Scholarship that makes my study on this project possible.

Finally, I would like to thank my parent and my wife for their endless love, support, and patience all along.

Guan Shou Ping

TABLE OF CONTENTS

SUMMARY

Chronic obstructive pulmonary disease (COPD) and asthma account for most obstructive lung diseases that place a huge burden on health services and society. There are currently limited therapeutic options for severe asthmatic and COPD patients. Andrographolide and 14-deoxy-11,12-didehydroandrographolide (DDAG) are the main biologically active constituents isolated from *Andrographis paniculata*. Andrographolide has been shown to activate nuclear factor erythroid-2-related factor 2 (Nrf2). As Nrf2 activity is reduced in COPD, we hypothesize that andrographolide may have therapeutic value for COPD. Our group has also recently reported novel anti-inflammatory effects of andrographolide in a mouse asthma model as well. However, andrographolide has been shown to possess cytotoxic activity towards tumour cell lines. As DDAG is an analogue of andrographolide, we hypothesized that DDAG retains the anti-inflammatory effects for asthma but is devoid of cytotoxicity.

Contrary to andrographolide, DDAG did not elicit any cytotoxic activity in A549 and BEAS-2B human lung epithelial cells and rat basophilic leukemia (RBL)-2H3 cells using a MTS assay. BALB/c mice sensitized and challenged with ovalbumin (OVA)-developed allergic airway inflammation. DDAG dose-dependently inhibited OVA-induced increases in total cell counts and eosinophil counts, IL-4, IL-5, and IL-13 levels in lavage fluid and serum OVA-specific IgE level in a mouse asthma model. In addition, DDAG attenuated OVA-induced airway eosinophilia, mucus production, mast cell degranulation, pro-inflammatory biomarker expression in lung tissues, and airway hyperresponsiveness (AHR) to methacholine in mice. DDAG also blocked p65 nuclear translocation and DNA-binding activity in the OVA-challenged lung and in TNF-α -stimulate d human lung epithelial cells.

Andrographolide suppressed cigarette smoke-induced increases in BAL fluid cell counts, levels of IL-1β, MCP-1, IP-10 and KC, and levels of oxidative biomarkers 8-

isoprostane, 8-OHdG and 3-nitrotyrosine in a dose-dependent manner. Andrographolide also promoted inductions of glutathione peroxidase (GPx) and glutathione reductase (GR) activities in lungs from cigarette smoke-exposed mice. In BEAS-2B cells, andrographolide markedly increased nuclear Nrf2 accumulation, promoted binding to antioxidant response element (ARE), and total cellular glutathione level in response to CSE. Andrographolide up-regulated ARE-regulated gene targets including glutamate-cysteine ligase catalytic (GCLC) subunit, GCL modifier (GCLM) subunit, GPx, GR and heme oxygenase-1 (HO-1) in BEAS-2B cells in response to CSE.

Taken together, current study demonstrated that andrographolide possesses anti-oxidative properties against cigarette smoke-induced lung injury probably via augmentation of Nrf2 activity. DDAG, on the other hand, retains the anti-inflammatory activities of andrographolide for asthma probably through the inhibition of NF-κB, and thus, DDAG may be considered as a safer analogue of andrographolide for the potential treatment of asthma.

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

3-NT	3-nitrotyrosine
8-OHdG	8-hydroxy-2-deoxyguanosine
α_1-AT	α1-Antitrypsin
AERD	Aspirin-exacerbated respiratory disease
AHR	Airway hyperresponsiveness
AM	Alveolar macrophages
AMCase	Acidic mamalian chitinase
AMV	Avian myeloblastosis virus
ANOVA	Analysis of variance
AP	Alkaline phosphatase
APC	Antigen presenting cell
ARE	Antioxidant response elements
ASM	Airway smooth muscle
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BCA	Bicinchonic acid
bFGF	Basic fibroblast growth factor
BHC	Hexachlorocyclohexane
BSA	Bovine serum albumin
c/EBP	CCAAT-enhancer-binding proteins
C5a	Complement component 5a
CAT	Catalase
CCL3	Macrophage inflammatory protein-1α
CCR	C-C chemokine receptor
CD 4	Cluster of differentiation 4
CD 13	Cluster of differentiation 13 or Alanine aminopeptidase
CD 117	Cluster of differentiation 117 or Proto-oncogene c-Kit
CDDO	2-cyano-3, 12-dioxooleana-1, 9-dien-28-oic acid
Cdyn	Dynamic compliance
COPD	Chronic obstructive pulmonary disease
COX-2	Cyclooxygenase-2/Prostaglandin-endoperoxide synthase 2
CRG-2	Cytokine Responsive Gene-2
CSE	Cigarette smoke extract
CSF	Colony-stimulating factor
CXCL8	Interleukin 8
CXCL10	Interferon-γ-inducible protein 10
CysLTs	Cysteinyl leukotrienes
DAMP	Damage-associated molecular patterns
DC	Dendritic Cell
DDAG	14-deoxy-11,12-didehydroandrographolide
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulfoxide

DTNB	5,5'-dithio-bis-2-nitrobenzoic acid
ECL	Enhanced chemiluminescent
ECM	Extracellular matrix
ECP	Eosinophil cationic protein
ecSOD	Extracellular superoxide dismutase
EDTA	Ethylenediaminetetraacetic acid
EPO	Eosinophil peroxidase
FBS	Fetal bovine serum
FcεRI	high-affinity IgE receptor
FEV_1	Forced expiratory volume in 1 second
FIZZ1	Found in inflammatory zone-1
FOXP3	Forkhead box P3
FVC	Forced vital capacity
GATA	GATA-binding protein (globin transcription factor)
GCLC	Glutamate-cysteine ligase, catalytic subunit
GCLM	Glutamate-cysteine ligase, modifier subunit
GCP-2	Granulocyte chemotactic protein 2
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
GPE1	GST-P enhancer-1
GPx	Glutathione peroxidise
GR	Glutathione reductase
GRO-α	Growth-related oncogene-α
GSH	Glutathione
GST	Glutathione S transferase
H&E	Haematoxylin and Eosin
HO-1	Heme oxygenase-1
HRP	Horseradish peroxidase
i.p.	Intraperitoneal injection
ICAM-1	Intercellular adhesion molecule 1
IFN-γ	Interferon-γ
IgE	Immunoglobulin E
IgG1	Immunoglobulin G1
IgG2a	Immunoglobulin G2a
IKK β	Inhibitory κB kinase-β
IL	Interleukin
IL1RL1	Interleukin 1 receptor-like 1
IM	Interstitial macrophages
iNOS	Inducible nitric oxide synthase
IP-10	Interferon-γ-inducible protein 10
iTreg	Inducible T regulatroy lymphocyte
KC	Keratinocyte-derived chemokine
KEAP1	Kelch-like ECH-associated protein 1
LPS	Lipopolysaccharide

LTB_4	Leukotriene B_4
LTC_4	Leukotriene C_4
Lyn	Tyrosine-protein kinase Lyn
M1	Classical activated macrophages
M2	Alternatively activated macrophages
mAb	Monoclonal antibody
MBP	Major basic protein
MCP-1	Monocyte chemotactic protein-1
MIP-1α	Macrophage inflammatory protein 1α
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
MyD88	Myeloid differentiation primary response gene (88)
NAC	*n*-acetylcysteine
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Neutrophil elastase
NFAT	Nuclear factor of activated T-cells
NF-HEV	Nuclear factor from high endothelial venules
NF-κB	Nuclear factor kappa B
NGF	Nerve growth factor
NHBE	Normal human bronchial epithelial cells
NLR	NOD-like receptor
NO	Nitric oxide
Nrf2	Nuclear erythroid-2-related factor 2
nTreg	Natural T regulatory lymphocyte
OVA	Ovalbumin
PAF	Platelet-activating factor
PAMP	Pathogen-associated molecular patterns
PAR	Protease-activated receptors
PAS	Peroidic acid Schiff
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Pinacyanol Erythrosinate
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
PGP	N-acetyl proline-glycine-proline
PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern recognition receptors
PVDF	Polyvinylidene difluoride
RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted
RBL	Rat basophilic leukemia

rcf	Relative Centrifugal Force
Rl	lung resistance
RNS	Reactive nitrogen species
RORγt	Receptor-related orphan receptor-γt
ROS	Reactive oxygen species
rpm	Revolutions per minute
SCF	Stem-cell factor
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SNP	Single-nucleotide polymorphisms
SOD	Superoxide dismutase
STAT6	Signal transducer and activator of transcription 6
Syk	Spleen tyrosine kinase
TAE	Tris-acetate-EDTA
TARC	Thymus and activation regulated chemokine
T-BET	T cell–specific T-box transcription factor
TBP	TATA binding protein
Tc	Cytotoxic T lymphocyte
TCR	T Cell Receptor
TEMED	Tetramethylethylenediamine
TGF-β	Tansforming growth factor-β
Th	T helper lymphocyte
TIMP	Tissue inhibitor of metalloproteinases
TLR	Toll-like receptor
TMB	3,3´,5,5´-tetramethylbenzidine
TNB	5-thios-2-nitrobenzoic acid
TNF-α	Tumour necrosis factor-α
Treg	Regulatory T lymphocytes
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VLA-4	Very late antigen 4

LIST OF PUBLICATIONS AND CONFERENCE ABSTRACTS

Publication

Guan S, Tee W, Ng D, Chan T, Peh H, Ho W, et al. (2013). Andrographolide protects against cigarette smoke-induced oxidative lung injury via augmentation of Nrf2 activity. *Br J Pharmacol* **168**(7): 1707-1718.

Guan SP, Mok YK, Koo KN, Chu KL, Wong WS (2009). Chitinases: biomarkers for human diseases. *Protein and peptide letters* **16**(5)**:** 490-498.

Bao Z, Guan SP, Cheng C, Wu S, Wong SH, Kemeny DM, *et al.* (2009). A novel antiinflammatory role for andrographolide in asthma via inhibition of the nuclear factor-kappaB pathway. *Am J Respir Crit Care Med* **179**(8)**:** 657-665.

Guan SP, Kong LR, Cheng C, Lim JC, Wong WS (2011). Protective role of 14-deoxy-11,12-didehydroandrographolide, a noncytotoxic analogue of andrographolide, in allergic airway inflammation. *Journal of natural products* **74**(6)**:** 1484-1490.

Cheng C, Ho WE, Goh FY, Guan SP, Kong LR, Lai WQ, *et al.* (2011). Anti-malarial drug artesunate attenuates experimental allergic asthma via inhibition of the phosphoinositide 3-kinase/Akt pathway. *PloS one* **6**(6)**:** e20932.

Ng FS, Wong KY, Guan SP, Mustafa FB, Kajiji TS, Bist P, *et al.* (2011). Annexin-1-deficient mice exhibit spontaneous airway hyperresponsiveness and exacerbated allergen-specific antibody responses in a mouse model of asthma. *Clin Exp Allergy* **41**(12)**:** 1793-1803.

Goh FY, Upton N, Guan SP, Cheng C, Shanmugam MK, Sethi G, *et al.* (2012). Fisetin, a bioactive flavonol, attenuates allergic airway inflammation through negative regulation of NF-kappaB. *Eur J Pharmacol* **679**(1-3)**:** 109-116.

Tang Y, Guan SP, Chua BY, Zhou Q, Ho AW, Wong KH, *et al.* (2012). Antigen-specific effector CD8 T cells regulate allergic responses via IFN-gamma and dendritic cell function. *J Allergy Clin Immunol* **129**(6)**:** 1611-1620 e1614.

Conference Abstract

Guan SP, Kong LR, Cheng C, Wong WS. (2010). An Anti-Inflammatory Role For 14-Deoxy-11,12-Didehydroandrographolide In Asthma. Am. J. Respir. Crit. Care Med; 181: A5673. (“*2010 ATS International Conference”*, May 14-19, 2010, New Orleans, Louisiana, USA)

Guan SP, Tee W, DSW Ng, TK Chan, HY Peh, EWX Ho, C Cheng, JC Mak and WSF Wong. (2012) Andrographolide Attenuates Cigarette Smoke Induce Lung Inflammation and Oxidative Stress By Augmenting Nrf2 and Attenuating NF-κB Response. (“*Yong Loo Lin School of Medicine Graduate Scientific Congress 2nd Annual Graduate Scientific Congress”*, 15 Feb 2012, NUHS Tower Block, Main Auditorium, Singapore)

1. INTRODUCTION

1.1 Obstructive Lung Disease

Respiratory disease is a common and important cause of illness and death around the world. Respiratory disease can be segregated into two major categories, which include obstructive lung disease and restrictive lung disorders. Obstructive lung diseases which are characterized by airway obstruction while restrictive lung disease is characterized by reduced lung volume due to restricted lung expansion. Obstructive lung diseases are far more common than restrictive diseases in a general population (Culver, 2011). To assess the pathophysiology of obstructive lung disease, spirometrical measurement which provides the initial lung function is commonly used. The main diagnostic criteria for a lung diseases lie in a diminished forced expiratory volume in 1 second (FEV_1)/ the total volume of air exhaled during a forced manoeuvre, the forced vital capacity (FVC) (Macintyre, 2009).

A person is said to have airway obstruction when the ratio of FEV_1/FVC is less than 70% (Seemungal *et al.,* 2008). Diseases resulting in obstructive pathophysiology primarily include asthma, chronic obstructive pulmonary disease (COPD), bronchiectasis, and bronchiolitis (Ryu *et al.,* 2001). Although a broad spectrum of disorders is associated with airflow obstruction, COPD and asthma account for most obstructive lung disease (Ryu *et al.,* 2001). Both diseases are characterized by airway obstruction, which is variable and reversible in asthma but is progressive and largely irreversible in COPD (Barnes, 2008c). As the incidence of asthma and COPD is increasing globally, both diseases are placing an increasing burden on health services in industrialized and developing countries (Mannino *et al.*, 2007; Pearce *et al.*, 2007). In asthma and COPD, there is chronic inflammation of the respiratory tract, and when the intensity of the inflammation increases, there are acute exacerbations in both diseases as well (Barnes, 2008c).

Nonetheless, there are marked differences in inflammation pattern in asthma and COPD that occur within the respiratory tract. The differences are linked to the recruitment of

inflammatory cell types and production of mediators that underlie both of the diseases. Hence, asthma and COPD also have different consequence to inflammation and respond differently to therapy (Ichinose, 2009). There have been several recent important advances in our understanding of the immunopathology of asthma and COPD (Murphy *et al.*, 2010; Yao *et al.*, 2009). The understanding of the similar and different immune mechanisms that are involved in both asthma and COPD has important implication for the development of new therapies for these troublesome diseases.

1.2 Asthma

Asthma is a chronic respiratory disease characterized by episodic attacks of impaired breathing (Akinbami *et al.*, 2011). According to Global Initiative for Asthma's (GINA) guidelines, asthma is defined as a chronic inflammatory disorder of the respiratory airways in which various cells and cellular elements play a role. The chronic inflammation is associated with AHR that leads to recurrent episodes of shortness of breath, coughing, wheezing, and chest tightness, particularly at night or in the early morning. These episodes are usually associated with widespread, but variable, airflow obstruction within the lung that is often reversible either spontaneously or with treatment (GINA, 2011). Asthma comprises a range of heterogeneous phenotypes that differ in presentation, aetiology and pathophysiology. Allergic (extrinsic) and non-allergic (intrinsic) asthma are 2 well characterized asthma phenotypes. People with allergic asthma develop the disease early in life. They are atopic (producing IgE specific to identifiable allergens) and have identifiable allergic triggers, and other allergic diseases such as rhinitis or eczema or a family history of allergic diseases. Non-allergic asthma develops later in life (after 40 years of age) and it is associated with aspirin-exacerbated respiratory disease (AERD) but not with allergic sensitization. Hence, non-allergic asthma is generally not as well understood. Other types of asthma phenotype include

exercise-induced asthma, obesity-related asthma and neutrophilic asthma. Nonetheless, in patients with asthma of any severity, up to 50% of them have a T-helper Type 2 (Th2)-predominant phenotype. Asthma that presents without evidence of Th2 immunity remains poorly understood (Wenzel, 2012). Although several types of asthma have been classified clinically, allergic asthma is the most common form of the disease (Mukherjee *et al.*, 2011). Allergic asthma is a chronic respiratory condition characterized by airway inflammation, mucus hypersecretion, and AHR (Galli *et al.*, 2008b). Increased mucus secretion and airway hyper-responsiveness leads to recurrent wheezing and shortness of breath (Moreira *et al.*, 2011).

1.2.1 Epidemiology and Burden of Asthma

Asthma is a widespread disease. There are 300 million people affected by asthma worldwide (Masoli *et al.*, 2004). Nearly 30 million people in the United State alone are affected by asthma, accounting for about 12% of children and 9% of adults in the US (Juhn, 2012). In Singapore, the prevalence of asthma has risen over the last 40 years but now appears to have stabilized, with approximately 5.5% of children in 1969 to 20% of children in 1994 affected by the disease (Wang *et al.*, 2004). Another recent survey estimated that almost 9% of school attending children have asthma and about quarter of them is inadequately controlled (Yang *et al.*, 2007). In the United States, 10.5 million school days were reported to be missed by asthmatic children aged 5-17 years in the year 2008 alone. Each day, an average of 9 persons in the United States dies from asthma. In addition, 2 out of 100 person with asthma are hospitalize and 8.4 out of 100 person with asthma have emergency department visit in 2009 (Akinbami *et al.*, 2012). The morbidity due to asthma, direct health care costs, indirect costs such as lost productivity, and mortality due to asthma continue to pose a high burden to the United States economy (Akinbami *et al.*, 2011).

1.2.2 Pathophysiology of Asthma

Asthma is a chronic inflammatory disease accompanied by remodelling of the airways and AHR that result in the clinical expression of airway obstruction that usually is reversible. The symptoms and physiological changes in asthma often involved airway narrowing. Several factors contribute to the development of airway narrowing in asthma. Airway smooth muscle (ASM) contraction responding to multiple bronchoconstrictor mediators and neurotransmitter is the predominant mechanism of airway narrowing and is largely reserved by brochodilators. During acute exacerbations, airway oedema, which is due to increased microvascular leakage in response to inflammatory mediators, also play a role in airway narrowing. Structural changes, often termed "remodelling", lead to airway thickening that may play an important role in more severe disease and is not fully reversible by current therapy. Mucus hypersecretion, which is a product of increased mucus secretion and inflammatory exudates, may lead to luminal occlusion that is also known as mucus plugging (GINA, 2011).

The structural changes are manifested by changes that involve all the layer of the respiratory airway wall. Changes in the epithelium include an increased number of goblet cells and epithelial detachment. Subepithelial fibrosis results from the development of increased deposition of extracellular-matrix molecules in the lamina reticularis which lies beneath the epithelial basement membrane and changes in fibroblasts with increased development of myofibroblasts and increased vascularity. Blood vessels in the airway walls proliferate under the influence of growth factors such as vascular endothelial growth factor (VEGF) and may contribute to increased airway wall thickness. The increased size (hypertrophy), number (hyperplasia) and function of ASM cells contribute to the increased thickness of the muscular layer of the airways wall (Figs 1.1 and 1.2) (Galli *et al.*, 2008b).

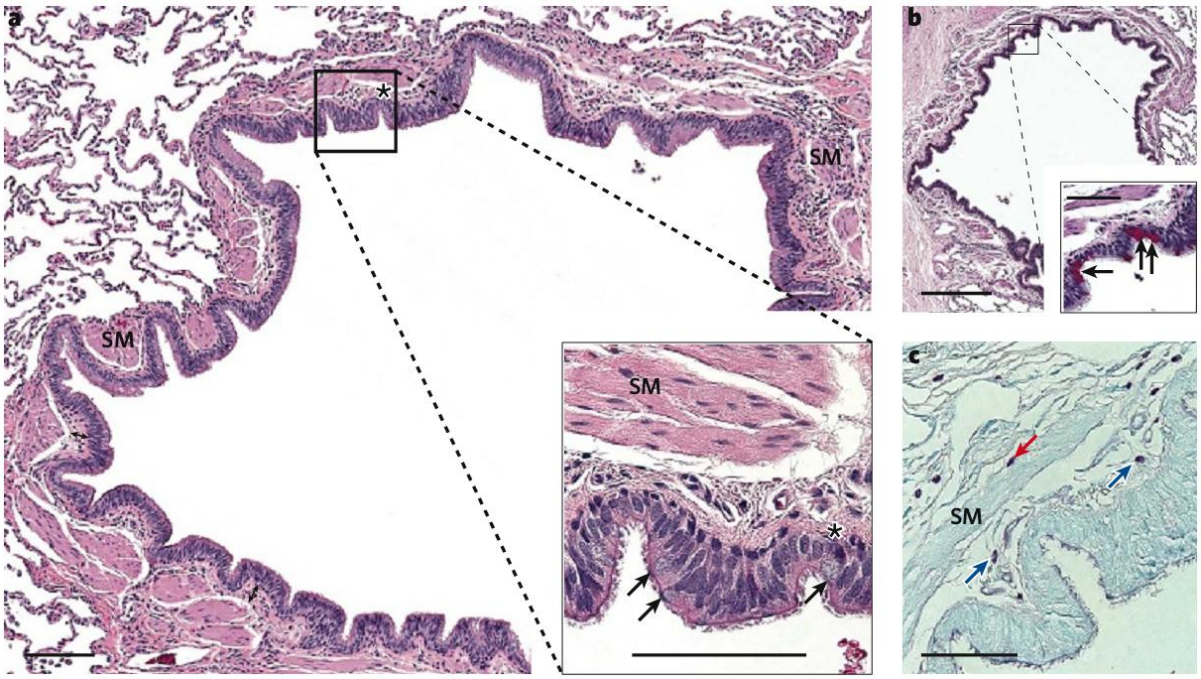


Figure 1.1. Tissue sections from the airway of a non-asthmatic person (a–c). A normal small bronchus stained with haematoxylin and eosin (H&E) in **a**. There are few goblet cells (black arrows in insets) in the epithelium. The basement membrane and underlying lamina reticularis (at asterisk in **a**) are normal. Sections in **b** were stained with periodic acid–Schiff (PAS) and diastase to stain mucus red. The submucosa (the length of the double-headed arrows in **a**) contains few leukocytes and the occasional mast cell (blue arrows in **c**), and the bronchial smooth muscle (SM) has few adjacent mast cells (red arrow in **c**) visualize by staining with pinacyanol erythrosinate (PE) which stain mast cells purple. Adapted from Galli (Galli *et al.*, 2008b)

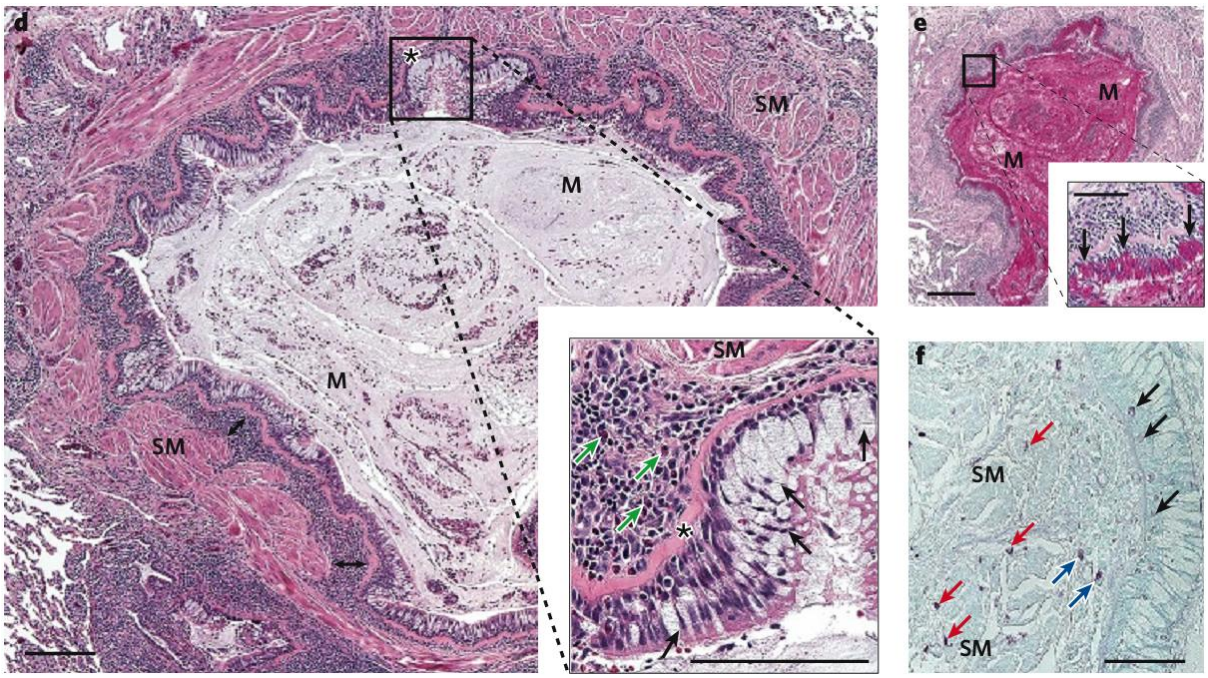


Figure 1.2. Tissue sections from the airway of a patient with severe asthma (d–f). A small bronchus from a patient with a history of severe asthma are stained with H&E in d, PAS and diastase in e and PE in f. Mucus (M) fills the airway lumen (d and e). There are many goblet cells (black arrows in insets) and the occasional intra-epithelial mast cell (black arrows in f). The lamina reticularis (asterisk in inset in d) is noticeably thickened. The submucosa (double-headed arrows in d) contains many eosinophils (green arrows in inset in d) and other leukocytes, as well as mast cells (blue arrows in f). There is more bronchial smooth muscle (SM) than in a–c in figure 1.1, and there are many mast cells (red arrows in f) among bundles of smooth muscle cells. Adapted from Galli (Galli et al., 2008b)

1.2.2.1 Inflammatory and Structural Cells

The inflammatory responses of allergic asthma are mediated by Th2 cells together with mast cells, B cells and eosinophils, as well as a number of inflammatory cytokines and chemokines (Galli *et al.*, 2008a).

T Lymphocytes

T lymphocytes are critical mediators of the allergic airway inflammation in asthma. Pathogenic allergen-specific T lymphocytes are generated in regional lymph nodes and are then recruited into the airway by chemoattractants produced by the asthmatic lung. These recruited effector T lymphocytes and their products then mediate the cardinal features of asthma that includes airway eosinophilia, mucus hypersecretion, and AHR.

Although T lymphocytes are important mediators of adaptive immune responses and are vital for host defence against infection, aberrant accumulation of T lymphocytes in the lung is seen in numerous non-infectious pulmonary inflammatory diseases such as asthma, where T lymphocytes in the lung are believed to orchestrate an abnormal inflammatory process. In asthma, the airways develop prominent inflammation with accumulation of activated effector T lymphocytes around the airways and in the airway lumen. It is thought that these cells are recruited into the lung and serve as the critical controllers of airway inflammation (Medoff *et al.*, 2008).

There are several subsets of T lymphocytes, each with a distinct function and these cell types can be distinguish based by the virtue that they express unique combinations of molecules in their membranes. Two major classes of T-lymphocytes are distinguished by the presence of either CD4, which presence on T helper (Th) and Regulatory (Treg) lymphocyte, or CD8, which presence on cytotoxic T lymphocyte (Tc). Different subtypes of T helper lymphocytes have been defined on the basis of the cytokines they secrete. The main cytokines

orchestrating T helper type 1 (Th1) and T helper type 2 (Th2) differentiations are IL-12 and IL-4, respectively (Lund *et al.*, 2003). In turn, Th1 cells produce interferon-γ (IFNγ), whereas Th2 lymphocytes primarily produce IL-4, IL-5 and IL-13.

Th2 lymphocyte plays a significant role in allergic asthma. Although clinical studies increasingly suggest that asthma is a heterogeneous disease, observational immunologic studies in human asthma, allergen challenge models in both human asthma and animal models, and substantial in vitro data has substantiated the notion that asthma is primarily a Th2 disease (Lloyd *et al.*, 2010). As an example, recent study has affirmed that Th2 driven inflammation defines major sub-phenotypes of asthma as Th2 biased response are seems to be detectable in 50% of individuals with asthma (Woodruff *et al.*, 2009). In asthma patients, the Th2 lymphocytes become exuberant and drive mast cell, and eosinophilic inflammation (Robinson, 2010). The notion that Th1 lymphocytes could have an inhibitory role in asthma as they can directly inhibit the development of Th2 lymphocytes has been substantiated by the deletion of the Th1 lymphocytes master transcription factor T-bet in mice results in the development of spontaneous AHR and IL-13-dependent eosinophilia (Finotto *et al.*, 2002). Nonetheless, when IFNγ, the signature Th1-type cytokine, is administered to the airways of patients with asthma, there is no improvement of disease symptoms (Boguniewicz *et al.*, 1995). The positive correlations between the numbers of Th2 lymphocytes present in the airways with disease severity further strengthen the suggestion that Th2 lymphocytes plays an essential role in human asthma.

Although atopic asthma has a substantial Th2 lymphocyte component, the disease is particularly heterogeneous, and the discovery of several of other T lymphocyte subsets, such as T helper type 17 (Th17) lymphocytes, T helper type 9 (Th9) lymphocytes and regulatory T (Treg) lymphocytes (Lloyd *et al.*, 2010) has suggested that other T lymphocytes also contribute to the development of asthma. The cytokine environment at the time of $CD4^+$ T

lymphocyte activation is a critical determinant in generating these effector subsets, owing to the ability of certain cytokine to activate specific transcription factors required for the differentiation of the Th subsets. In the case of Th1 and Th2 cells, this process was dependent on IL-12 acting on T-bet and IL-4 acting on GATA-binding protein 3 (GATA3), respectively (Murphy *et al.*, 2002). GATA3 is a transcription factor that is vital for the differentiation of uncommitted naive T lymphocytes into Th2 lymphocytes and regulates the secretion of Th2 cytokines (Ho *et al.,* 2007). Clinical studies have revealed that there is an increase in the number of $GATA3^+$ T lymphocytes in the airways of stable asthmatic subjects (Caramori *et al.,* 2001). Stimulation of T lymphocytes following ligation of the T cell receptor (TCR) and CD28 co-receptor by antigen-presenting cells (APCs), GATA3 is phosphorylated and activated by p38 mitogen-activated protein kinase (MAPK), and subsequently translocate to the nucleus, where it binding to the promoter region of Th2 cytokine genes (Maneechotesuwan *et al.,* 2007). Nuclear factor of activated T cells (NFAT) is a T lymphocyte–specific transcription factor that enhances the transcriptional activation of the Il4 promoter by GATA3 (Ho *et al.,* 2007). Similarly, applying the concept that a specific cytokine exerts its effect on a transcription factor to mediate lymphocyte differentiation has led to the identification of other Th subsets, such as the regulatory T lymphocytes (Tregs) and Th17 lymphocytes (Zhou *et al.*, 2009).

Th9 lymphocytes are a discrete population of IL-9-secreting $CD4^+$ T lymphocytes that depend on transforming growth factor-β (TGF-β) for their development (Veldhoen et al., 2008). Th9 lymphocytes were first identified as a subpopulation of Th2 lymphocytes, but recent experimental analysis revealed that Th9 lymphocytes had divergent regulatory capabilities (Xing *et al.*, 2011). Generation of these Th9 lymphocytes involves expression of PU.1, which is a transcription factor is expressed specifically in subpopulations of Th2 lymphocytes with low IL-4 expression, and secretion of IL-9 from Th9 lymphocytes is

upregulated by IL-25 (Angkasekwinai et al., 2010; Chang et al., 2010). These discoveries strongly establish Th9 lymphocytes as a distinctive Th lymphocytes subtypes.

Th17 lymphocytes are a distinct lineage of $CD4^+$ effector T lymphocytes that express IL-17 and the key transcription factors retinoic acid receptor-related orphan receptor-γt (RORγt) and RORα (Harrington *et al.*, 2005; Park *et al.*, 2005). Tissue-infiltrating $CD4^+$IL-17^+ T lymphocytes and the up regulation IL-17 have been documented in the lungs of patients with asthma (Pene *et al.*, 2008) and the levels of IL-17 correlate with disease severity (Chang *et al.*, 2012; Wang *et al.*, 2011). Furthermore, adoptive transfer of Th17 cells induces neutrophilia and imparts resistance to steroid therapy, suggesting that IL-17 may function to promote neutrophil recruitment (Kolls *et al.*, 2004) and could therefore be particularly important in severe asthma which is often characterized by neutrophilic inflammation and steroid resistance (McKinley *et al.*, 2008). Besides IL-17, Th17 lymphocytes also produce a small amount of IFNγ and no IL-4 (Harrington *et al.*, 2005).

$CD8^+$ T lymphocytes, which play a pivotal role in tumour cell killing and protection during viral infection through the secretion of IFN-γ and cytolytic factors, were considered to be less essential or even as a negative regulator for the development of allergic inflammation. Indeed, studies reported protective effects of CD8+ T lymphocytes in allergic airway disease because of production of IFN-γ and the ability to suppress Th2 responses (Huang *et al.*, 1999; Suzuki *et al.*, 1999). However, cell transfer studies using $CD8^+$ αβ T lymphocytes has demonstrated that not only $CD4^+$ T lymphocytes but also $CD8^+$ T lymphocytes were essential to the development of AHR and allergic inflammation, either directly (Sawicka *et al.*, 2004) or in concert with sensitized $CD4^+$ T lymphocytes (Isogai *et al.*, 2004; Koya *et al.*, 2007; Miyahara *et al.*, 2004). CD8-deficient mice are less susceptible to allergic airway inflammation, but the transfer of *in vitro*-generated antigen-primed effector memory $CD8^+$ αβ T lymphocytes into sensitized CD8-deficient mice increased AHR, eosinophilic inflammation

and the levels of IL-13 in bronchoalveolar lavage (BAL) fluid (Miyahara *et al.*, 2004). While $CD8^+$ αβ T-lymphocytes have been demonstrated to have pro-inflammatory effects, $CD8^+$ γδ T-lymphocytes are believed to have inflammation inhibitory effects (Isogai *et al.*, 2003). Antibody based depletion studies has also shown that depletion of residential $CD8^+$ T lymphocytes enhance the late airway response and airway inflammation in a rat model of asthma (Isogai *et al.*, 2005), and enhance the effects on airway remodelling in a rat model of asthma (Tsuchiya *et al.*, 2009). Cytotoxic T lymphocytes type 2 (Tc2) is a subsets of $CD8^+$ T lymphocytes that produce IL-4, IL-5 and IL-13 but not IFN-γ. Recent studies have demonstrated in the absence of IFN-γ, CD8 T lymphocytes assume a Tc2-biased phenotype and potentiate inflammation (Tang *et al.*, 2012). The presence of $CD8^+$ T lymphocytes in asthmatic airways is well documented. Tc2 are increased in BAL fluid of atopic asthma patients (Cho *et al.*, 2005). Additionally, bronchial biopsies from patients with atopic and non-atopic asthma contain $CD8^+$ T cells that produce IL-4 and IL-5 (Ying *et al.*, 1997). However, despite these correlations, the role play by $CD8^+$ lymphocyte remains to be further explored.

Regulatory T lymphocytes (Tregs) are T lymphocytes that suppress potentially harmful immune responses. The inappropriate immune response to allergens observed in asthmatic patients may be explain by the reduced or altered function of Treg lymphocytes populations in these patients. Several T_{Reg} lymphocyte subsets have been described, including naturally occurring forkhead box P3 $(FOXP3)^+CD4^+CD25^+$ lymphocytes (nTreg) and inducible Treg (iTreg) lymphocytes, which develop *in vitro* or *in vivo* following antigen stimulation (Robinson, 2009). The majority of nTregs express the transcriptional factor FOXP3, and their development and function are dependent upon it. In contrast, iTregs are usually FOXP3 negative, but they can express this transcription factor in certain antigen presentation situations (Langier *et al.*, 2012). Beside the $CD4^+CD25^+$ Tregs that function via

cell-cell contact, other types of regulatory suppressive T lymphocytes that act via modulatory cytokines such as regulatory T helper lymphocytes type 3 (Th3) and regulatory T lymphocytes type 1 (Tr1) have been identified. Th3 are dependent on TGF-β and are observable in mice models in which low doses of the antigen exposure induces oral tolerization (Kumar *et al.*, 2011a). Tr1 lymphocytes are defined by their specific cytokine production profile, which includes the secretion of high levels of IL-10 and TGF-β, and their ability to suppress antigen-specific effector T lymphocytes responses via cytokine-dependent mechanisms (Langier *et al.*, 2012). Transfer of $CD4^+CD25^+$ Treg lymphocytes attenuates airway inflammation and AHR as well as prevents the allergen-induced activation of DCs in the airways (Joetham *et al.*, 2007; Leech *et al.*, 2007; Strickland *et al.*, 2006). Treg lymphocytes exert their inhibitory action through both direct and indirect mechanisms. In the direct mechanism, Treg produce anti-inflammatory cytokines, such as IL-10 and TGF-β, express inhibitory molecules, such as cytotoxic T lymphocyte antigen 4 (CTLA4) (Kearley *et al.*, 2005; Ostroukhova *et al.*, 2004) and induce the down regulation of MHC class II and the co-stimulatory molecules CD80 and CD86 by antigen-presenting cells (APC) (Cederbom *et al.*, 2000). Intriguingly, Treg lymphocytes do not necessarily produce IL-10 themselves but, instead, the Treg lymphocytes can induce IL-10 production from bystander $CD4^+$T lymphocytes to mediate the suppressive effect (Kearley *et al.*, 2005). In asthmatic patients, the presence of $CD4^+CD25^+$ T lymphocytes in the airways correlated positively with the patient's FEV1 and corticosteroid treatment increases the number of $CD4^+CD25^+$ T lymphocytes in the airways of patients with asthma (Hartl *et al.*, 2007). The characterizations for cytokine dependent subset of regulatory T lymphocytes (Th3 and Tr1) have been impeded by the lack of a specific surface marker (Langier *et al.*, 2012). Finally, T lymphocytes play a critical role in the pathophysiology of asthma. Identification of distinct subset of would further advance

our understanding about the disease and may lead to the development of a more specific treatment for asthma.

Eosinophils

Eosinophil in the sputum is a hall-mark of asthma and it has been used as marker for effective corticosteroid therapy or treatment compliance (Hargreave, 2007). In uncontrolled asthma, eosinophils are present in the sputum and also in BAL fluid (Holgate, 2008). Eosinophils differentiate and undergo trafficking under the regulation of the transcription factors GATA binding protein (GATA)-1&2, and CCAAT-enhancer-binding proteins (c/EBP), the chemokine eotaxin, as well as the $CD4^+$ Th2 lymphocyte-derived survival cytokines such as IL-3, IL-5 and granulocyte macrophage colony-stimulating factor (GM-CSF). Eosinophils express receptors for the pro-inflammatory mediators complement component 5a (C5a) and platelet-activating factor (PAF), the cytokines IL-2, IL-3, IL-5, IFN-γ, and GM-CSF, as well as the immunoglobulins IgG and IgA, and C-C chemokine receptor type 3 (CCR3) (Minai-Fleminger *et al.*, 2009). Eosinophils are a rich source of basic granule proteins, such as major basic protein (MBP), eosinophil peroxidase (EPO), and eosinophil cationic protein (ECP) which altogether have been shown to cause significant tissue destruction and involved in the development of AHR (Minai-Fleminger *et al.*, 2009). MBP, which accounts for more than 50% of the eosinophil granule protein mass, was found to activate human lung mast cell and cause histamine release. EPO, which is a cationic enzyme that is capable of catalysing the formation of highly toxic oxidants, has significant potential to induce cellular injury as well (McElhinney *et al.*, 2003). ECP, a ribonuclease which has been attributed with cytotoxic and fibrosis promoting functions, has been developed as a marker for asthma as elevated ECP levels are observed in asthmatic patients (Bystrom *et al.*, 2011). Eosinophils also release and up to 28 cytokines, chemokines and growth factors such as IL-4, IL-6, IL-8, IL-10, IL-13, GM-CSF, stem cell factor (SCF), nerve growth factor (NGF) and

TGF-β as well as eosinophil-derived lipid mediators, such as leukotriene C_4 (LTC_4) and PAF, which potently act as bronchoconstrictors and secretagogues (Minai-Fleminger *et al.*, 2009; Wegmann, 2011) and prostaglandin E_2 (PGE_2) has been shown to enhance eosinophil survival (Profita *et al.*, 2003). Additionally, because eosinophils secrete considerable amounts of TGF-β, eosinophils contribute to processes leading to airway remodelling. As a result, eosinophils have been identified as valuable targets for therapeutic intervention in asthma (Wegmann, 2011).

Mast cell

Mast cells play a key role in asthma. Mast cells, along with dendritic cells are among one of the first immune cells to interact with allergens (Galli *et al.*, 2008a). Mast cells are potentially long-lived cells and mast cell number, distribution, function and phenotype can be regulated by various factors whose local concentrations can change at the sites of innate or adaptive immune responses (Galli *et al.*, 2005). In humans there are two main kinds of mast cell phenotypes, the first contains tryptase and the second stores tryptase, chymase carboxypeptidase A and cathepsin-G (Matsumoto *et al.*, 2007). Mast cells are highly granulated high-affinity IgE receptor (FcεRI) bearing tissue-dwelling cells, which develop from myeloid progenitors expressing CD34, proto-oncogene c-Kit (CD117/c-kit) and alanine aminopeptidase (CD13). Mast cells are widely distributed in connective tissues and mucosal surfaces (Minai-Fleminger *et al.*, 2009). Mast cells are recruited to the airways by stem-cell factor (SCF) released from epithelial cells. Additionally, mast cell are also recruited by interleukin 8 (IL-8/CXCL8**)** and interferon-γ-inducible protein 10 (IP10/CXCL10) produced by airway smooth muscle cells (Brightling *et al.*, 2005; John *et al.*, 2009). These chemokines also prime mast cells for heightened mediator secretion. In return, mast cells secrete CCL19 which, through its CCR7, stimulates airway smooth muscle cell migration and contributes to smooth muscle hyperplasia (Holgate, 2008; Kaur *et al.*, 2006). The cross-linking of IgE-

bound FcεRI complexes in response to activation by specific allergens on mast cell surface leads to the "early phase" of the allergic reaction within minutes. The "early phase" of allergy is characterized by vasodilation, increased vascular permeability, vascular leakage and oedema in which mast cells degranulate and secrete of various granular preformed mediators such as tryptase, chymase, histamine and proteoglycans as well as of newly-synthesized mediators, such as arachidonic acid metabolites, such as prostaglandin D_2 (PGD_2), leukotriene B_4 (LTB_4) and LTC_4 (Galli *et al.*, 2008a; Li-Weber *et al.*, 2003), as well as the later production of many cytokines, chemokines and growth factors such as IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-11, IL-13, GM-CSF, TNF-α, TGF-β, basic fibroblast growth factor (b-FGF), vascular endothelial growth factor (VEGF) and NGF (Minai-Fleminger *et al.*, 2009).

These mediators are responsible for the increase in microvascular permeability and contraction of smooth muscle, which may account for the bronchoconstriction seen in asthma. Concomitantly, the recruitment of macrophages, T lymphocytes and eosinophils takes place (Galli *et al.*, 2008b). These cytokines and chemokines liberated by the inflammatory cells in the early phase initiate the "late phase" that usually peaks a few hours later (Bloemen *et al.*, 2007; Gould *et al.*, 2008). The release of these mediators is tightly controlled by signalling pathways that are propagated through the cell by specific phosphorylation and dephosphorylation events. IgE-dependent activation is mediated through a complex cascade of signal transduction initiated by tyrosine-protein kinase Lyn (Lyn), spleen tyrosine kinase (Syk) and Fyn kinases from the Src family (Roth *et al.*, 2008). Mast cells homeostasis is controlled by IgE, IL-4, IL-10 and TGF-β1, which down regulate important effector proteins such as c-Kit and FcεRI in long-term mast-cells cultures (Ryan *et al.*, 2007). Among the mediators released by mast cells, the lipid mediators as well as histamine have an essential role in the recruitment of Th2 lymphocytes and of eosinophils to the inflammatory site and in sustaining the allergic process (Minai-Fleminger *et al.*, 2009).

Macrophages

Macrophages are the most abundant immune cell population in normal lung tissue and serve critical roles in innate and adaptive immune responses. Macrophages exert its prominent defensive effects of the respiratory tract from airborne pathogens. Macrophages are specialized to recognize, phagocytose, and destroy these infectious agents and then promote appropriate tissue repair after successful pathogen clearance (Byers *et al.*, 2011). Macrophages also play an important role in the pathogenesis of asthma. Studies have suggested that macrophages could be directly involved in the development of AHR as macrophages modulate the contractility of ASM by inducing histamine release from mast cells and basophils, by augmenting cholinergic neurotransmission via the generation of thromboxane A2, and by impairing airway β-adrenergic function through the production of oxygen radicals (Yang *et al.*, 2012). Activated macrophages may also be an important effector cells in airway inflammation as experimental model of an acute exacerbation of chronic asthma have demonstrated that macrophages can stimulate Th2 cytokine secretion by primed $CD4^+$ T lymphocytes via mechanisms involving the expression of CD80/86 costimulatory molecules (Herbert *et al.*, 2010).

Alveolar macrophages (AMs) and interstitial macrophages (IMs) represent the 2 major populations of macrophages that occupy different compartments in the lung (Moreira *et al.*, 2011). AMs are found in alveoli lumen, presiding a nonspecific innate defence mechanism while IM inhabit the interstitium where they interact with interstitial lymphocytes and help in mounting a specific immune response. In general, IMs are considered as antigen-presenting macrophages, but when AMs are primed with allergen *ex-vivo*, they can also help in expanding allergen-specific T-lymphocytes response (Balhara *et al.*, 2012). AM can be derived from circulating blood monocytes, which colonize the tissues under inflammatory and non-inflammatory states, or from a stable, self-sustaining population of resident lung

macrophages, the IMs, which serve as obligate intermediate between blood monocytes and AMs (Yang *et al.*, 2012). Local proliferation in human and murine airways is also sustained by colony-stimulating factor (CSF) (Balhara *et al.*, 2012). The monocytes migrate to the lungs in response to chemoattractants such as monocyte chemotactic protein-1 (MCP-1/CCL2), acting on C-C chemokine receptor type 2 (CCR2) (Deshmane *et al.*, 2009).

Generally, macrophages (including AMs and IMs), are exhibit unique activation patterns upon exposure to cytokines and/or toll-like receptor (TLR) agonists. Two such subpopulations have been extensively studied and these are the classical (M1) and alternatively activated (M2) macrophages (Mantovani *et al.*, 2009). M1 macrophages are differentiated by IFN-γ and lipopolysaccharide (LPS) in both mice and humans (Joshi *et al.*, 2010; Moreira *et al.*, 2010). M1 macrophages release inflammatory cytokines and chemokines such as IL-6, IL-12, TNF-α, interferon-γ-inducible protein 10 (IP-10/CXCL10), and Macrophage inflammatory protein-1α (MIP-1α/CCL3). These cells also produce high levels of nitric oxide (NO) and are known to drive inflammation in response to intracellular pathogens (Cassol *et al.*, 2009). The Th2 cytokines IL-4 and IL-13 are involved in the polarization of alternatively activated (M2) macrophages (Moreira *et al.*, 2011). Both mouse and human M2 macrophages have enhanced expression of scavenger and mannose receptors (Varin *et al.*, 2010), and increased phagocytic activity of foreign pathogens and apoptotic cells (Mills *et al.*, 2000), but are inept at the clearance of intracellular pathogens (Schuh *et al.*, 2003). Besides the classical M1 and M2 macrophages stimuli, other factors such as IL-10 and TGF-β or immune-complexes are also involved in polarizing the macrophage phenotype (Liu *et al.*, 2011). The polarization of M2 macrophages by IL-4 and IL-13 is dependent on the induction of signal transducer and activator of transcription 6 (STAT6), which leads to the activation of pro-fibrotic cytokines, chitinase-like molecules (Moreira *et al.*, 2010), and "found in inflammatory zone-1" (FIZZ1) (Nair *et al.*, 2005). Both M1 and M2 macrophages

are shown to be involved in the developments of asthma although M2 are intuitively present given the Th2-driven lung environment in asthma (Moreira *et al.*, 2011). The pro-inflammatory cytokines IL-1β and IL-6 produce by the M1 macrophages have direct effects on Th2 lymphocytes proliferation (Doganci *et al.*, 2005), and promote fibroblast activation (Gallelli *et al.*, 2008). The recruitment and the survival of eosinophils in the lung is increase by IL-1β and IL-6 as both of this cytokine increase the production of GM-CSF by macrophages and fibroblast (Ilmarinen-Salo *et al.*, 2010). M1 polarized macrophages are consider as a component of asthma exacerbation (Moreira *et al.*, 2011). M2 polarized macrophage on the other hand are potent inflammatory cells release pro-fibrotic and pro-inflammatory factors, that can cause increased inflammatory cell recruitment, mucus secretion, and AHR in asthma (Moreira *et al.*, 2011).

Epithelial Cells

Airway epithelium is an important controller of inflammatory, immune and regenerative responses to allergens, viruses and environmental pollutants that contribute to the pathogenesis of asthma. Airway epithelial cells (ECs) represent the first line of defence against microorganisms, gases and allergens as they lie at the interface between the host and the environment (Xiao *et al.*, 2011). As a barrier to the external environment, ECs express many pattern recognition receptors (PRRs) such as TLRs, NOD-like receptor (NLR), C-type lectins and protease-activated receptors (PAR) that rapidly detect and react to pathogen-associated molecular patterns (PAMPs) originate from microbes or to damage-associated molecular patterns (DAMPs) released upon tissue damage, cell death or cellular stress (Lambrecht *et al.*, 2012). Epithelial PRRs activation leads to the release of cytokines, chemokines and antimicrobial peptides that attract and activate innate and adaptive immune cells. Although the epithelium was initially considered to function solely as a physical barrier,

recent studies have demonstrated that EC activation is a play a central role in influencing the recognition of inhaled allergens that activates the local network of dendritic cells (DCs), which coordinate the ensuing immune response, and thereby bridging innate and adaptive immunity (Hammad *et al.*, 2008). Human respiratory EC express huge number of TLRs and have been shown to react functionally to a number of TLR ligands. Upon ligand engagement, TLRs signal via a series of adaptor proteins, leading to the activation of transcription factor nuclear factor-κB (NF-κB) and the subsequent production of the expression of an array of inflammatory cytokine genes (Sha *et al.*, 2004). Intriguingly, studies have demonstrated that the recruitment, activation and intraepithelial migration of DCs in response to inhaled endotoxin require only epithelial Toll-like receptor 4 (TLR4) triggering (Hammad *et al.*, 2009). The crucial role of NF-κB in regulating of airway inflammation was elucidated in mice deficient in the NF-κB subunits p50 or p65, which demonstrated reduced responses to endotoxin and allergens (Yang *et al.*, 1998). Recent studies have also shown that constitutive activation of NF-κB in airway ECs was sufficient to activate DCs, breach inhalational tolerance and promote sensitization to OVA (Ather *et al.*, 2011; Sheller *et al.*, 2009), while inhibition of epithelial NF-κB in the contrary reduced Th2 lymphocytes recruitment and airway remodelling (Broide *et al.*, 2005). Th2 effector cytokines may subsequently sustained the epithelial responses to allergens and provides an important feedback loop that can perpetuate disease (Lambrecht *et al.*, 2012). IL-4 and IL-13 induce airway ECs to produce GM-CSF and the chemokines IL-8 (CXCL8), which attract neutrophils, eotaxin-1 (CCL11) which attract eosihophils and thymus and activation regulated chemokine (TARC/CCL17) which attract Th2 lymphocytes (Lordan *et al.*, 2002). Hence it is evidence that airway ECs orchestrates various aspects of allergic sensitization and is a critical player in allergic inflammation.

Mediators of Asthma

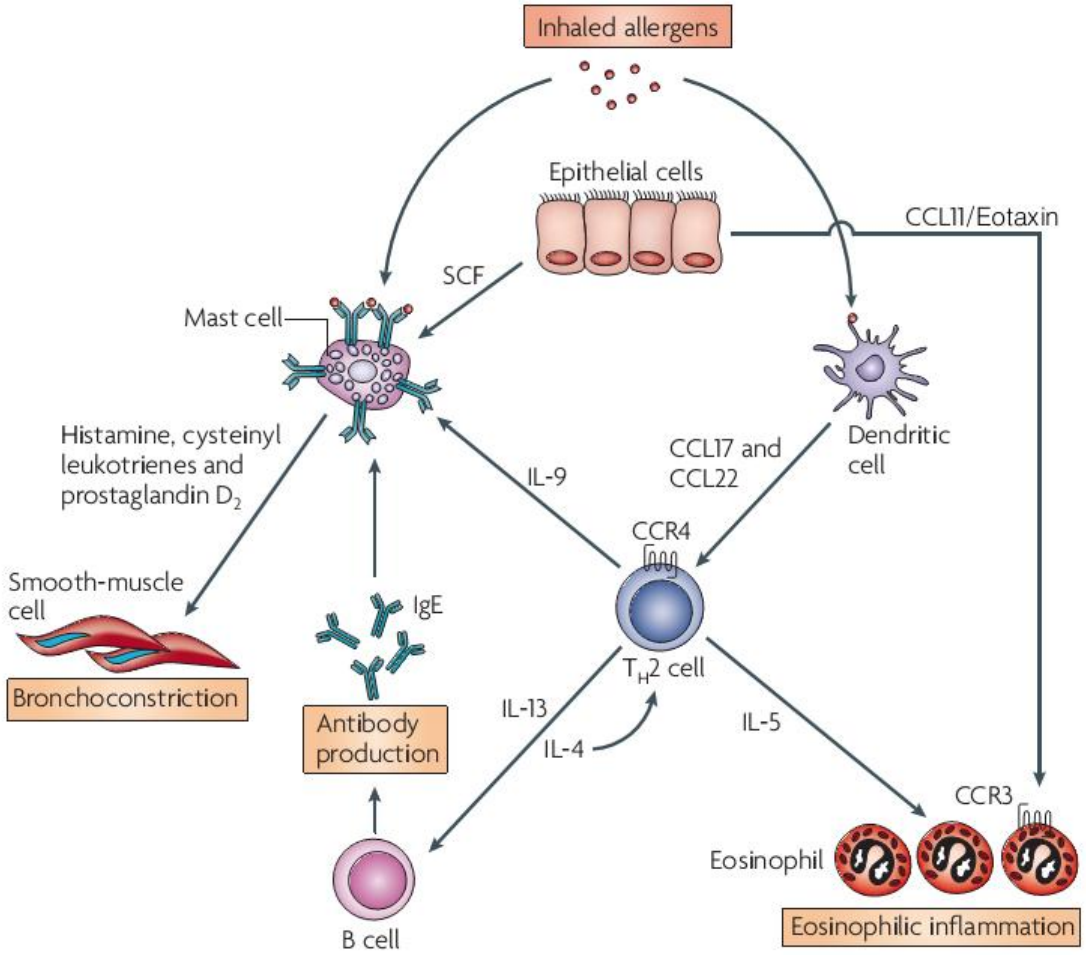


Figure 1.3 Cytokines involved in asthma. Myeloid dendritic cells processed the allergens are by release the chemokines CC-chemokine ligand 17 (CCL17) and CCL22, which act on CC-chemokine receptor 4 (CCR4) to attract T helper 2 (T_H2) cells. Th2 cells have a central role in orchestrating the inflammatory response in allergic asthma through the release of IL-4 and IL-13 (which stimulate B cells to synthesize IgE), IL-5 (which is necessary for eosinophilic inflammation), and IL-9 (which stimulates mast cell proliferation). Epithelial cells play an important role in orchestrating the inflammation of asthma through the release of various cytokines. Epithelial cells release stem-cell factor (SCF), which is important for maintaining mucosal mast cells at the airway surface. Epithelial cells may also release CCL11, which recruits eosinophils via CCR3. Inhaled allergens activate sensitized mast cells by crosslinking surface-bound IgE molecules to release several bronchoconstrictor mediators, including histamine, cysteinyl leukotrienes (CysLTs) and prostaglandin D_2 (PGD_2). Adapted from Barnes (Barnes, 2008c)

1.2.2.2 Mediators of Asthma

Th2 Cytokine

Th2 cytokine play a vital role in the development of atopic asthma as the expression of Th2 cytokines could be related to activity of disease, symptom scores, airway eosinophilia, and airway hyperresponsiveness (Georas *et al.*, 2005). In addition, the expression of Th2 cytokines are increased after allergen inhalation challenge in patients with atopic asthma and decreased with corticosteroid treatment (Lloyd *et al.*, 2010). The main Th2 cytokines involved are those encoded by the IL-4 gene cluster, which contains the genes encoding IL-4, IL-5, IL-9, IL-13, and GM-CSF on chromosome 5q31-q33. This cluster of cytokine genes is co-regulated, providing coordination of the allergic cascade (Robinson, 2010). Studies in mouse models in which Th2 cytokines such as IL-4, IL-5, IL-9 or IL-13 have been individually genetically knocked out or immunologically block antibodies have provided important evidence that the Th2 axis can drive eosinophilic airway inflammation and AHR (Lloyd *et al.*, 2010). When Th2 lymphocytes failed to develop in response to antigen, such as in IL-4Rα$^{-/-}$ or Stat6$^{-/-}$ mice, allergic airway inflammation is not induced (Cohn *et al.*, 2004). Both IL-4 and IL-13 have a crucial role to promote isotype switching of B cells to IgE and goblet cell metaplasia by acting on epithelia, whereas IL-4, but not IL-13, is crucial for maintaining the Th2-lymphocytes phenotype (Oettgen, 2000).

IL-5 is a cytokine that play a key role in eosinophil differentiation, recruitment, activation, and survival at sites of allergic inflammation. Systemic and local (inhaled) administration of IL-5 to asthmatic patients results in an increase in circulating eosinophils (Stirling et al., 2001). Additionally, IL-5, which is mainly produced by Th2 lymphocytes and to a lesser degree by activated mast cells, is also involved in B-cell differentiation (Wegmann, 2011). The expression of IL-5 is elevated in BAL fluid in patients with asthma and the level of IL-5 in BAL fluid and the bronchial mucosa correlates with disease activity (Flood-Page *et*

al., 2007). The roles of IL-5 underlying the pathogenesis of asthma have been demonstrated by a number of in vivo studies. Mice deficient for IL-5 do not develop the eosinophilia, lung damage, and airways hyperreactivity normally resulting from ovalbumin (OVA) sensitization and aeroallergen challenge (Foster *et al.*, 1996). Similarly, the IL-5 receptor α-chain (IL-5R alpha)-deficient mice demonstrated an impaired IL-5 induce eosinophilia in response to parasitic infections (Yoshida *et al.*, 1996). Furthermore, mice that constitutively overexpressed IL-5 in the lung spontaneously developed prominent airway eosinophilia associated with AHR (Lee *et al.*, 1997). When IL-5 was targeted by antisense molecules or by an anti–IL-5 monoclonal antibody (mAb), significant reduction of airway eosinophilia and consequently the pathologic features of experimental asthma, including AHR was observed (Hamelmann *et al.*, 1997; Lach-Trifilieff *et al.*, 2001). In spite of these encouraging in vivo results, clinical trials with the anti–IL-5 monoclonal (mAb), mepolizumab reported a reduction in blood and sputum eosinophils in patients with mild-to-moderate asthma but lack of improvement in asthmatic symptoms, AHR, and allergic late-phase response (Flood-Page *et al.*, 2007; Leckie *et al.*, 2000). Although the outcomes of these clinical trial prompted discussion about the role of eosinophils in the pathogenesis of asthma, more recent clinical trials that investigated the therapeutic effect of mepolizumab in patients with severe corticosteroid-refractory and highly eosinophilic form of asthma, have shed new light on the role of eosinophils in asthma and especially in distinctive asthma phenotypes (Haldar *et al.*, 2009; Nair *et al.*, 2009). Both of these clinical studies have reported a significantly reduced exacerbation rate and a reduction in blood and sputum eosinophils. As such, these studies also indicate the need of distinguishing distinct asthmatic phenotypes that might require different therapeutic approaches.

IL-9 is involved in the onset and progression of asthma and IL-9 is detected in biopsies from patients with asthma and localizes to $CD4^+$ T lymphocytes, although there are

several other cellular sources of IL-9, including mast cells and eosinophils (Soroosh et al., 2009). Biological targets for IL-9 include mast cells, B lymphocytes, T lymphocytes, T lymphocytes clones, hemopoietic progenitors, and immature neuronal cell lines (Steenwinckel *et al.*, 2007). Overexpression of IL-9 in mice induces inflammation mediated by eosinophils, mucus hyperplasia, mastocytosis, AHR, and increased expression of other Th2 cytokines and IgE (Zhou *et al.*, 2001). Targeting IL-9 with antibody inhibits pulmonary eosinophilia, mucus hypersecretion, and AHR after allergen challenge of sensitized mice. The effects of IL-9 in mice such as pulmonary eosinophilia and mucus hypersecretion are mediated via the release of IL-13, whereas its effects on mastocytosis and B lymphocytes lung infiltration seem to be direct (Steenwinckel *et al.*, 2007). IL-9 plays an key role in differentiation and proliferation of mast cells and interacts synergistically with SCF (Barnes, 2008a). The presence of IL-9 in mouse and human is highly associated with mast cell accumulation in the airways, as well as AHR and globlet cell metaplasia (Lambrecht *et al.*, 2009).

IL-13 induces AHR and mucus hypersecretion by activating STAT6 in the airway epithelium (Kuperman *et al.*, 2002) and produces several of the structural changes seen in chronic asthma, including goblet cell hyperplasia, airway smooth muscle proliferation and subepithelial fibrosis (Wills-Karp, 2004). IL-13 also increases the expression of acid mammalian chitinase (AMCase) and neutralization of AMCase inhibits IL-13 mediated AHR and Th2-driven inflammation (Zhu *et al.*, 2004). The airways of asthmatic patients have an increased expression of IL-13. Additionally, there is a transient increase in IL-4 in BAL fluid after allergen challenge, whereas the secretion of IL-13 is sustained and correlates with the increase in the number of eosinophils in the airways (Barnes, 2008a). In addition to Th2 cells, IL-13 is produced by several cell types including mast cells, basophils, and eosinophils (Barnes, 2008a).

IL-17

The IL-17 family consists of six members in mammals including IL-17 (now synonymous with IL-17A), IL-17B, IL-17C, IL-17D, IL-17E (also called IL-25), and IL-17F. Among the IL-17 family members, the most investigated cytokine is IL-17 (Park *et al.*, 2010). IL-17 and IL-17F share the greatest similarity showing 50% identity at the amino acid level. Genes encoding IL-17 and IL-17F are located on chromosome 1-A4 region in mice and on chromosome 6p12 location in humans. IL-17 and IL-17F may have derived via a gene duplication event owing to their sequence similarity and proximity in the genome (Pappu *et al.*, 2008). Other than Th17 lymphocyte, other cell types such as $CD8^+$ T cells, γδ T cells, and natural killer T cells also produce IL-17 while eosinophils, neutrophils, macrophages, and monocytes can also be sources of IL-17 in some cases (Park *et al.*, 2010).

Similar to the IL-17 cytokine family, IL-17 receptors form a unique family composed of five members which are IL-17RA, IL-17RB, IL-17RC, IL-17RD, and IL-17RE (Gaffen, 2009). The IL-17 receptor (IL-17R, renamed IL17-RA) is ubiquitously expressed and ligand binding causes secretion of pro-inflammatory cytokines and chemokines such as IL-8, IL-6, growth-related oncogene α (GROα/CXCL1), macrophage inflammatory protein-2-beta (MIP-2β/CXCL3), CXCL5 and granulocyte chemotactic protein 2 (GCP-2/CXCL6) (Nograles *et al.*, 2008). Signaling through the IL-17R is critically required to develop allergic asthma since IL-17R-deficient mice fail to develop allergic asthma in response to intranasal ovalbumin (OVA) after intraperitoneal sensitization (Souwer *et al.*, 2010).

IL-17 exerts a wide variety of biological activities due to ubiquitous distribution of its receptor (Pappu *et al.*, 2008). IL-17 plays an important role in driving allergic inflammation. IL-17 has been reported in the lungs, sputum, and BAL fluid of asthma patients (Park *et al.*, 2010). The up-regulation of IL-17 mRNA expression in the airways of an asthma mouse model further substantiated the evidence for the involvement of IL-17 in the pathogenesis of

asthma (Hellings *et al.*, 2003). Recent Study have reveal that inhibition of IL-17 by administration of IL-17-specific antibody resulted in significantly less AHR and fewer neutrophils in the BAL fluid and administration of recombinant IL-17 to mice increased their susceptibility to AHR (Lajoie *et al.*, 2010). Activation of the IL-17RA by the binding of IL-17, activates NF-κB and MAPK pathways (Park *et al.*, 2010). IL-17 orchestrate local inflammation by inducing the release of proinflammatory cytokines such as TNF-α, IL-1β, granulocyte colony-stimulating factor (G-CSF) production by bronchial fibroblasts and epithelial and airway smooth muscle cells. Additionally, IL-17 can act in synergy with IL-6 to induce mucus proteins (Muc5B and Muc5AC) or with IL-1β and TNF-α to enhance Vascular endothelial growth factor (VEGF) (Wang *et al.*, 2008). Moreover, IL-17 induces the expression of not only eosinophil-guiding chemokines like regulated and normal T cell expressed and secreted (RANTES/CCL5) and eotaxin (CCL11) but also other inflammatory mediators like intercellular adhesion molecule 1 (ICAM-1) and cyclooxygenase-2 (COX-2). Taken together, IL-17 acts as an orchestrating cytokine in immune and inflammatory responses.

Blockade of IL-17 activity with anti-IL-17 monoclonal antibody that is injected before allergen inhalation bronchial neutrophilic influx are significantly reduced. Because neutrophils may be important in airway remodeling in chronic severe asthma, targeting IL-17 may hold therapeutic potential in human asthma (Hellings *et al.*, 2003). Receptors for IL-17 such as IL-17RA and IL-17RC may be the other possible targets for inhibition of IL-17 activities as human bronchial epithelial cells pretreated with anti-IL-17R antibody demonstrated a decrease in IL-17 activity (McAllister *et al.*, 2005). A soluble form of IL-17RC, which binds to IL-17 and IL-17F with high affinity, has been shown to inhibit the signalling of IL-17 in fibroblast (Kuestner *et al.*, 2007). Based on these data, the therapeutic potential of inhibiting IL-17 activities is anticipated.

IL-33

IL-33, also called IL-1F11 or nuclear factor from high endothelial venules (NF-HEV), is a member of the IL-1 family of cytokine (Joshi *et al.*, 2010; Prefontaine *et al.*, 2010). IL-33 is an inducer of the Th2 branch of adaptive immunity and signals through a complex membrane bound interleukin 1 receptor-like 1 (IL1RL1/ST2) protein (Schmitz *et al.*, 2005). The IL-33/ST2 pathway also contributes to allergen-induced airway inflammation and AHR (Kearley *et al.*, 2009), both important features of asthma. The IL-33/ST2 axis triggers the release of several proinflammatory mediators, such as chemokines and cytokines, and induces systemic Th2-type inflammation in vivo (Schmitz *et al.*, 2005). In the presence of antigen, IL-33 binds the surface receptor ST2 and polarized naïve CD4+ T lymphocytes into a population of T lymphocytes which produce IL-5 and IL-13, but not IL-4. This polarization are modulated by ST2 receptor and myeloid differentiation primary response gene (88) (MyD88), but not IL-4 or signal transducer and activator of transcription 6 (STAT6). The IL-33-induced T-lymphocytes differentiation is also dependent on the phosphorylation of mitogen-activated protein kinase and NF-κB, but not the induction of GATA-binding protein 3 (GATA3) or T lymphocytes–specific T-box transcription factor (T-BET) (Kurowska-Stolarska *et al.*, 2008). Additionally, IL-33 also acts as a selective chemoattractant of Th2 lymhocytes (Komai-Koma *et al.*, 2007). Aside from Th2 lymphocytes, ST2 is present on mast cells, basophils, eosinophils, macrophages, and ECs (Prefontaine *et al.*, 2010). Human macrophages constitutively expressed the membrane-associated (ST2L) and the soluble (sST2) ST2 receptors. Studies has demonstrated that bone marrow derived human macrophages primary responses to IL-33 by skewing in favours of M1 chemokine generation while IL-33 addition to polarized human macrophages promotes or amplifies M2 chemokine expression, thus signifying that IL-33 might amplify allergic responses by promoting an overall activation of both M1 and M2 macrophages (Joshi *et al.*, 2010). In response to allergen exposure, airway

ECs released IL-33 (Chustz *et al.*, 2011). IL-33 is typically sequestered in the nucleus of ECs, but it can be secreted upon epithelial activation. IL-33 activates lung DCs during antigen presentation and thereby drives a Th2-type response in allergic lung inflammation by acting on the ST2 receptor (Besnard *et al.*, 2011). By blocking IL-33, the development of Th2 response due to sensitization to inhaled antigens by DCs was abolished (Lambrecht *et al.*, 2000). The expression of IL-33 is higher in airway ECs of subjects with asthma (Prefontaine *et al.*, 2010). Taken together, these data support a role for IL-33 in the pathogenesis of allergic asthma.

Tumour necrosis factor-alpha (TNF-α)

TNF-α is a pro-inflammatory cytokine that has been implicated in many aspects of the airway pathology in asthma. Induced sputum and BAL fluid from asthmatic patients have elevated levels of TNF-α (Obase *et al.*, 2001). An up-regulated expression of TNF-α has also been detected in alveolar macrophages, mast cells, leukocytes and bronchial epithelial cells (Ackerman *et al.*, 1994; Cembrzynska-Nowak *et al.*, 1993; Gosset *et al.*, 1991; Ying *et al.*, 1991). Expression of TNF-α in the asthmatic airway play an important role in amplifying inflammation by the activation of NF-κB and the expression of numerous immune and inflammatory response genes (Berry *et al.*, 2007). TNF-α induces the expression of multiple airway epithelial genes, including cytokines (IL-5, GM-CSF), chemokines (eotaxin, MCP-1, RANTES), adhesion molecules (ICAM-1) and mucins (Muc5AC) (Matera *et al.*, 2010). Other than increasing the epithelial expression of adhesion molecules, TNF-α is also a chemoattractant for neutrophils and eosinophils (Lukacs *et al.*, 1995). Furthermore, TNF-α directly induces histamine release from human mast cells (van Overveld *et al.*, 1991) and induce transient AHR (Thomas *et al.*, 1995). Additionally, TNF-α stimulate T lymphocytes activation and cytokine release (Chen *et al.*, 2011). TNF-α also reduce glucocorticoid

responsiveness in monocytes and up-regulates the pathways involved in chronic airway remodelling and subepithelial fibrosis (Hansbro *et al.*, 2011). Several humanized anti-TNF-α neutralizing antibodies (infliximab, adalimumab and golimumab) are available (Desai *et al.*, 2009). Nonetheless, the research article which demonstrated that infliximab could suppress exacerbations in patients with moderate asthma has recently been retracted (2011; Erin *et al.*, 2006). Blocking of TNF-α with etanercept, which is a human soluble TNF-α receptor (TNFR), attenuated AHR and improved lung function in a small study of patients with refractory asthma. However, serious concerns remain over the safety of TNF-α blockade, which may increase susceptibility to respiratory infection (Berry *et al.*, 2006).

Chemokines and Adhesion Molecules

During inflammation, leukocytes are recruited into tissues by adhesion molecules and chemokines. At sites of inflammation, cytokines produced in the tissue induce endothelial cell adhesion molecule expression. The vascular recruitment of leukocytes is a three-step process involving low affinity rolling of leukocytes on the endothelium followed by arrest of the leukocyte on the endothelium through high affinity adhesion, and the subsequent transmigration of the leukocyte through the endothelium. The rolling of leukocytes on the vessel wall is mediated by selectins. Selectins are a family of calcium-dependent, type I transmembrane glycoprotein family of adhesion molecules (McEver, 2002). The low-affinity nature of selectins allows the characteristic "rolling" action of leukocytes. Currently known selectins include E-selectin, P-selectin, and L-selectin, named for the cell type in which they were originally identified: endothelium, platelet, and leukocyte respectively. IL-1, LPS and TNF-α mediate the expression of E-selectin by endothelium (Kelly *et al.*, 2007). Additionally, rolling can also be mediated by very late antigen 4 (VLA4) (which is also known as leukocyte α4β1-integrin) in its low affinity state interacting with vascular cell adhesion molecule-1

(VCAM-1) on the endothelium (Alon et al., 1995). Binding of selectins on leukocytes increases the affinity of the integrin family of receptors that then bind to the endothelial cell adhesion molecules such as ICAM-1 or VCAM-1 which are up regulated by Th2 cytokines (Cook-Mills et al., 2011). The high affinity integrin binding of leukocytes mediates the arrest of the leukocytes on the endothelium, and the subsequent migrate on chemokine gradients into the tissue. While both neutrophils and eosinophils bind to E-selectin, the chemokine provided the specificity for leukocyte cell type base on the chemokine receptors it has. Eotaxin (CCL11) selectively recruits eosinophils by binding to CCR3 which is not expressed by neutrophil and monocytes (Baggiolini *et al.*, 1997).

Regulated upon activation in normal T cells, expressed, and secreted (RANTES/CCL5) are crucial to the delivery of eosinophils and T lymphocytes into the airways during asthma and during OVA-induced allergic airway disease (Hogan *et al.*, 2008). RANTES which is produced by endothelial cells, fibroblasts, T lymphocytes, eosinophils, platelets and other cells (Zhang *et al.*, 2010), may activate cells by binding to CC chemokine receptor-1 (CCR1), CCR3, or CCR5, which it shares with other chemokines (Schuh *et al.*, 2003). Although RANTES was constitutively expressed in the airways, it expression was elevated in patients with asthma (Zhang *et al.*, 2010).

Monocyte chemotactic protein-1 (MCP-1/CCL2) recruits monocytes and T lymphocytes to the sites of inflammation and it is produced by bronchial epithelial cells, macrophages, and smooth muscle cells. Levels of MCP-1 are elevated in the airways of asthmatics (Sutcliffe *et al.*, 2009). Moreover, IL-13-induced inflammation in mice is associated with an increase in MCP-1 release and that the disease process is attenuated in MCP-1 hematopoietic cell receptor CC chemokine receptor 2 (CCR2) knockout mice (Zhu *et al.*, 2002). Additionally, MCP-1 stimulate the release of histamine or leukotriene from mast

cells or basophils, induce production of transforming growth factor-β (TGF-β) and procollagen by fibroblast and enhance Th2 lymphocytes polarization (Melgarejo *et al.*, 2009).

Chitinases

Chitinases have been suggested to play a pivotal role in Th2-mediated allergic diseases such as asthma (Guan *et al.*, 2009; Sutherland *et al.*, 2009). Chitinases such as chitotriosidase and acidic mammalian Chitinase (AMCase) are enzymes that degrade chitin, the second most abundant biopolymer that is an essential structural component of the fungal cell wall and is present in the exoskeleton of arthropods and the microfilarial sheath of nematodes (Tharanathan *et al.*, 2003). There are also chitinase-like proteins such as YKL-40, Ym1 and Ym2 that lack chitinolytic activity but retain chitin-binding ability. Although chitinases were originally believed to function in host defense against parasitic infections, there is currently ample evidence to support an association of acidic mammalian chitinase and YKL-40 with allergic asthma. Zhu and coworkers reported the first clinically relevant finding regarding the role of chitinase in asthma (Zhu *et al.*, 2004) where exaggerated quantities of AMCase were detected in the epithelial cells and macrophages of lung biopsies taken from patients with asthma. Subsequently, utilizing a 2D gel electrophoresis proteomics approach, a concurrent elevation of the BAL fluid protein level of AMCase, Ym1 and Ym2 was also observed in a OVA-induced mouse asthma model (Zhao *et al.*, 2005). On the contrary, there is no change observed in chitotriosidase protein level in BAL fluids. In addition to AMCase, serum and lung tissue levels of a chitinase-like protein YKL-40 have been found to be increased in patients with asthma. Moreover, circulating YKL-40 levels correlated positively with asthma severity, thickening of the lung subepithelial basement membrane, frequency of rescue inhaler use, and deterioration in pulmonary function in asthmatic subjects studied (Chupp *et al.*, 2007). Thus, YKL-40 and AMCase levels may be useful biomarkers for Th2-

dependent allergic airway inflammation, and the AMCase level may be useful for monitoring the effectiveness of anti-inflammatory therapy in asthma.

1.2.2.3 Airway Hyperresponsiveness (AHR)

Physiologically, airway responsiveness describes the ability of the airways to narrow after exposure to constrictor agonists. AHR consists of an increased sensitivity of the airways to constrictor agonists, which is presented by a smaller concentration of a constrictor agonist needed to initiate the bronchoconstrictor response, a steeper slope of the dose-response and a higher maximal response to the agonist (Jiang *et al.*, 2012; O'Byrne *et al.*, 2003). AHR is therefore an exaggerated constriction of the airways in response to bronchoconstrictor stimuli. Viral-induced exacerbations, allergen exposure and occupational exposures can also briefly enhance the underlying AHR in individual patients. AHR has been identified as an important feature in patients with current, symptomatic asthma and AHR is regarded as a key diagnostic criterion of asthma (Bousquet *et al.*, 2007; Busse, 2010). Clinically, AHR is documented by a decreased bronchial airflow after inhalation challenge with methacholine or histamine as the severity of AHR correlates with the severity of asthma and with the amount of treatment needed to control symptoms; The patients with more severe airway disease often have a greater degree of AHR (Galli *et al.*, 2008b). Conversely, an improvement in AHR is associated with better control of asthma (Busse, 2010). Thus, the methods for measuring airway responsiveness have been standardized and are widely accepted.

To understand the components that comprise AHR, the factors that contribute to AHR has been suggested to be divided into two categories, the persistent and the variable AHR (Busse, 2010; Covar, 2007; O'Byrne *et al.*, 2009). The persistent aspects of AHR have been largely attributed to structural changes in the airway, particularly in those patients with more severe disease; these structural changes alter the architecture of the airways to make them

thicker, less compliant, and more narrowed, all features associated with a greater degree of constriction and closure when stimulated by contractile substances. The variable component of AHR is believed to relate to inflammatory events in the airway, which are variable and influenced by numerous environmental events such as exposure to allergens, respiratory infections and treatment. Nonetheless, it is understood that these processes are interrelated and likely to be interdependent. Hence, the level of AHR represents both collective and synergistic events in the airway and is the result of multiple processes (Busse, 2010).

1.2.3 The mouse model of asthma

Studies in animal models form the basis for much of our current understanding of the pathophysiology of asthma, and are central to the development of drug therapies. While there are comparative dissimilarities in genetics, immunology, anatomy and physiology, the use of laboratory animals clearly allows investigators to test hypothesis about the causal mechanisms underlying disease in ways that cannot be done in man.

We use mouse asthma model in our study as it has the benefit of being easy to handle, together with its obvious practical advantages related to cost and gestation period (Corry *et al.*, 2006). The mouse has also become the species of choice for our studies because of the availability of immunological and molecular tools available to study them as the entire mouse genome has been sequenced, and the variety of in-bred strains with different phenotypic traits. Our research focused primarily on generating allergic inflammation by sensitizing and challenging animals with OVA, leading to an acute inflammatory response and airways hyperresponsiveness. The protocol starts by systemically sensitising the animals via intraperitoneal injections separated by one week to ovalbumin OVA absorbed onto an adjuvant. Aluminium hydroxide ($Al(OH)_3$) was used as the adjuvant as it primes the immune response towards a Th2 phenotype (Stevenson *et al.*, 2011). This is followed by allergen

exposure to the airways via aerosol administration one week after the last sensitisation. These models have provided important insights into the nature of the allergic airway inflammation, particularly the significance of the Th2 phenotype in this disease and have been useful in the identification of potential drug targets for interventions involving allergic pathways.

Nonetheless, mouse models are not exact replicas of human asthma. There are a number of issues with existing animal models of asthma that must be recognized including the requirement for adjuvant during senitisation in most models, the acute nature of the allergic response that is induced and the use of adult animals as the primary disease model. It has also been proposed that chronic OVA exposure leads to tolerance and poorly maintained inflammation, possibly through the induction of Treg lymphocytes, which limits the ability to mimic the chronic aspect of asthma and the associated airway remodelling (Stevenson *et al.*, 2011).

That being said, the OVA-induced allergic lung inflammation mouse model can replicate many of the central features of allergic asthma including increased specific IgE production, mucus hypersecretion, bronchoconstriction responses, a Th2-biased inflammation rich in eosinophils and nonspecific AHR. Observations from mouse models of allergic asthma support many existing paradigms, and therefore it is utilized to examine our hypotheses.

1.2.4 Current Treatment

The treatment for current asthmatic patients includes the use of medication for short-term relief, the daily use of preventive medication to avert attacks, monitoring of early symptoms, avoiding factors that trigger attacks and removing risks such as tobacco smoke or mould from the environments (Akinbami *et al.*, 2011). As the airway obstruction of asthma is due to inflammation and AHR, bronchodilating and anti-inflammatory therapy is usually applied (Hoshino *et al.*, 2009). Inhaled corticosteroids and short-acting and long-acting β-2-

adrenoceptor agonists are now the mainstay of asthma treatment. Inhaled glucocorticoids are the priority for asthma treatment as airway inflammation in asthma responds well to steroid. On the other hand, if an asthma patients only have a mild intermittent asthma, the patient will only be treated with short-acting inhaled β-2-selective agonist such as salbutamol (Cazzola *et al.*, 2011). Asthma is a disease that can be managed with the use of anti-inflammatory therapy as a huge portion of asthmatic patients are reasonably well controlled with inhaled corticosteroids, cysteinyl leukotriene receptor antagonists and/or injections of anti-IgE antibody, omalizumab. Theophylline has also been used as a bronchodilator, but the cardiac and central nervous system side effects and relative low efficacy have led to a marked reduction in its use (Hansel *et al.*, 2004).

The cysteinyl leukotrienes (CysLTs), leukotrienes (LT) C_4, D_4 and E_4 are one of the most potent contractile agonists of airway smooth muscle. By interacting with the Cysteinyl leukotrienes receptor 1 (CysLT1), they increasing vascular permeability and stimulate mucus secretion, all of which would induce an acute asthmatic attack. Corticosteroids treatments are not effective against the biosynthesis and the actions of cysteinyl leukotrienes (Gyllfors *et al.*, 2006). The currently available oral cysteinyl leukotrienes receptor 1 antagonists such as montelukast are not only beneficial in treatment of chronic asthma (Reiss *et al.*, 1998), it has also recently found to be effective in acute asthma exacerbation (Ramsay *et al.*, 2011). Although CysLTs antagonists can be used as a monotherapy in mild to moderate asthma, their main use is as an adjunct therapy to inhaled corticosteroids (Polosa, 2007) as these drugs are generally less effective than inhaled glucocorticoids (Montuschi *et al.*, 2010).

Although inhaled corticosteroids are effective at suppressing airway inflammation, they do not influence the natural history of the disease, even when the corticosteroids treatment is started early in childhood (Bisgaard *et al.*, 2006; Guilbert *et al.*, 2006). Moreover, the use of higher dose of corticosteroids has associated morbidities (Blakey *et al.*, 2012).

Inhaled corticosteroids are also not effective in virus-induced exacerbations (Harrison *et al.*, 2004) and in those asthmatics who smoke (Chaudhuri *et al.*, 2003). The failure of corticosteroids to decrease the level of expression of TNF-α in asthmatic airways might explain why corticosteroids have limited effects in more severe forms of the disease (Truyen *et al.*, 2006).

Despite intensive use of these anti-inflammatory interventions, 5–10% of asthmatics have severe asthma, which does not respond to treatment, and continue to have significant disabling and/or life-threatening symptoms (Moore *et al.*, 2007). Patients with severe asthma account for 50% of asthma-related healthcare costs (Yang *et al.*, 2012). Severe corticosteroid-refractory asthma patients face the greatest morbidity and mortality. There are currently limited therapeutic option for patients whom only have incomplete control and thus, it is imperative to derive novel treatment for this group of patients.

1.3 Chronic obstructive pulmonary disease (COPD)

COPD is a disease state characterized by slow and progressive development of airflow limitation that is not fully reversible, in sharp contrast to asthma where there is variable airflow obstruction that is usually reversible spontaneously or with treatment. The airflow limitation is associated with dysregulated inflammatory response of the lungs to noxious particles or gases (Naeije, 2005). Physiologically, the chronic airflow limitation is reflected by reducing expiratory flow, such that a slow forced expiration is required to empty the lungs.

The severity of COPD is defined by the most popular classification of COPD, the Global Initiative for Chronic Obstructive Lung Disease (GOLD) classification (Rabe et al., 2007) which is based on the forced expiratory volume in 1 second (FEV_1) as the most important criterion, categorize patients into 4 stages, described as mild (I), moderate (II), severe (III), or very severe (IV). A COPD patient may start seeking medical attention at stage

II due to the chronic respitory symptoms or an exacerbation of their disease but are usually unaware of lung function abnormality during stage I. Stage III is characterized by further worsening of airflow limitation, greater shortness of breath, reduce exercise capacity, fatigue, and repeated exacerbations that almost always have an impact on patients' quality of life. At the most severe stage of COPD, stage IV, respiratory failure may occur and quality of life is very appreciably impaired and exacerbations may be life threatening (GOLD, 2011). In current clinical practice, COPD is diagnosed and monitored via symptoms, lung function and the assessment of responses to inhaled pharmacological agents (Rabe *et al.*, 2007).

The deterioration of lung function in COPD is associated with chronic obstructive bronchitis and emphysema that commonly co-exist. Bronchitis, which is an inflammation of the bronchus, is resulted from structural remodelling of the lung that includes the narrowing of the small airways due to peribronchiolar fibrosis and luminal obstruction by inflammatory mucus exudates (Dournes et al., 2012). Airway obstruction occurs long before the patient is aware of symptoms because of its slowly progressive nature. Emphysema is a disease with enlargement of airspaces, destruction of lung parenchyma which reduce the gas-exchange surface area of the lung and loss of lung elasticity as a consequence of skewed activation of proteases (Brusselle et al., 2011; Cosio et al., 2009; Mocchegiani et al., 2011). The pathologic changes which resulted from repeated injury and repair, correlate with disease severity and persist on after smoking cessation (Rabe et al., 2007). In general, inflammatory cell infiltration, the imbalance of proteinase-antiproteinase system, oxidative stress and airway remodelling contributes to the decline in pulmonary function associated with the development of COPD (Cosio *et al.*, 2009; Larsson, 2007; Shapiro, 2002).

1.3.1 Epidemiology and Burden of COPD

COPD is a global health problem that is estimated to cause the death of at least 2.9 million people annually (Lopez et al., 1998). As 210 million people worldwide are estimated to have COPD, it is predicted to become the third most common cause of death after ischaemic heart disease and cerebrovascular disease and the fifth most prevalent disease worldwide by 2020 (Lopez et al., 1998) . It affects over 5% adults (age 18-74), is currently the fourth leading cause of death worldwide and is the only major cause of death that is still rising (Pauwels *et al.*, 2004; Sin *et al.*, 2005). Subsequently, COPD will be the direct underlying cause of 7.8% of all deaths by 2030 (Mathers et al., 2006). The economic burden of COPD and the health care costs has exceeded those of asthma by more than 3 folds (Barnes, 2000). Exacerbations of COPD account for the greatest burden on the health care system in developed countries. Indeed, the total direct costs of respiratory disease in the European Union are estimated to be about 6% of the total health care budget, where COPD alone accounts for 56% of this cost of respiratory disease or aproximately €38.6 billion (Rabe et al., 2007). In the United State, the direct costs of COPD were $18 billion and the indirect costs totalled $14.1 billion (Skrepnek et al., 2004). Because of its persistently increasing prevalence, mortality and disease burden, COPD is a major public health concern and the focus of intense research.

1.3.2 Etiology

Cigarette smoking is the single most important identifiable major risk factor for the development of COPD (Huvenne et al., 2011). Although COPD also occurs in non-smokers that is exposures to a range of both environmental fumes and dusts, such as coal dusts, or person with α1-antitrypsin deficiency and history of childhood respiratory infections, cigarette smoking has been accounted for 80–90% of all COPD cases (El-Zein et al., 2012; Gaschler et

al., 2010). Smoking directly correlates with the early onset of chronic obstructive pulmonary disease (COPD). Nonetheless, individuals vary greatly in their susceptibility to the effects of tobacco smoke (Castaldi et al., 2011). In spite of this, according to the World Health Organization (WHO), over 1.3 billion people worldwide continue to smoke (World Health Organization., 2005; World Health Organization., 2003). Cigarette smoke is a rich source of potent oxidants both in gaseous and water-soluble phases (Pryor, 1992). The cigarette smoke-derived oxidants damage airway epithelium and alveolar wall, leading to infiltration of macrophages and neutrophils (Cantin, 2010; Foronjy *et al.*, 2008; Yao *et al.*, 2011). In additionally, cigarette smoke is not just a direct exogenous source of reactive oxygen species (ROS), it also induces endogenous production of ROS from activated inflammatory cells, such as alveolar macrophages and neutrophils (MacNee et al., 2001) in the lung, resulting in an imbalance between oxidants and antioxidants (Rahman *et al.*, 2006a). This is likely due to the high oxidative burden in the lungs of smokers which has been estimated to be on the order of 10^{15} free radicals per puff (Church et al., 1985). Furthermore, the degree of oxidative stress increases greatly with the severe exacerbations in COPD (Drost et al., 2005). Hence, reduction of oxidative stress in COPD patients is clinically beneficial as it could reduce inflammation and reverse corticosteroid resistance. Nonetheless, N-acetyl cysteine, the current and the only approved antioxidant is inadequate in reducing progression and exacerbation of COPD (Decramer et al., 2005). Thus, novel anti-oxidant activator is very much needed.

1.3.3 Inflammatory cells

COPD is a complex disease that involves several types of inflammatory cells (Barnes, 2003). Although both asthma and COPD involve chronic inflammation in the respiratory tract, there is a discernible difference in the type of inflammatory cells involved and in the site of inflammation (Barnes, 2004). Increased numbers of macrophages and neutrophils are

observed in sputum and BAL fluid of COPD patients (Keatings *et al.*, 1996; Pesci *et al.*, 1998a). Similarly, BAL fluid and sputum also demonstrate increased inflammatory biomarkers such as cytokines, proteases, and soluble cytokine receptors (Barnes *et al.*, 2006). However, in contrast to asthma, eosinophils are not prominent in COPD patients' lung except during exacerbation or when patients have concomitant asthma (Fabbri et al., 2003). Inflammation in COPD is not a separate entity by itself but is integrally related to oxidative stress and protease–antiprotease imbalance. Chronic inflammation plays a critical role in the pathogenesis of COPD through their potential release of ROS, cytokines, chemokines, elastase, and metalloproteinase in response to cigarette smoke. Although cigarette smoke is the major cause of chronic inflammation of the lungs, the inflammation in COPD patient does not stop after cessation of smoking (Lapperre et al., 2006). Moreover, the inflammatory cell infiltration correlates well with the severity of airflow obstruction (Hogg et al., 2004). In contrast, chronic bronchitis, which is define by a productive cough for more than 3 months for more than 2 successive years is not necessarily associated with airflow limitation (Barnes, 2003). Thus, inflammation is highly implicated in the disease process.

Neutrophils

Neutrophil accumulation in the lung is a prominent feature of COPD. BAL of smokers has an increase in neutrophils compared with nonsmokers as cigarette smoke is known to induce macrophages to release neutrophilic chemotactic factors. Higher number of neutrophils have been found in bronchial epithelium and lamina propria in smokers compared with controls (Pesci *et al.*, 1998b), and the number of neutrophil is higher in the small airways than large airways (Battaglia *et al.*, 2007). When compared to healthy nonsmokers and healthy smokers, bronchial biopsies from GOLD stage III and IV COPD patients (severe/very severe) demonstrate a corresponding increase in neutrophils in the airway submucosa (Di Stefano *et*

al., 2009). Furthermore, acute COPD exacerbations are often associated with microbial infections and neutrophilic inflammation (Sethi *et al.*, 2009).

Neutrophils are a rich source of inflammatory mediators, reactive oxygen species and tissue proteases (Barnes et al., 2003; Stockley, 1999). Activation of lung neutrophils leads to the release of granule proteins such as neutrophil elastase (NE) and myeloperoxidase (MPO) (Borregaard et al., 2007) that could contribute to the bronchial inflammation and to structural changes such as peribronchiolar fibrosis and emphysema (Baraldo et al., 2004; Woolhouse et al., 2005). In emphysema, neutrophil degranulation leads to alveolar wall destruction which resulted to mucociliary dysfunction and reduced mucociliary clearance. In chronic bronchitis, inflammation which is characterized by mucosal infiltration with neutrophils, macrophages, and lymphocytes, resulted in epithelial disruption, smooth muscle hypertrophy and fibrosis (Quint et al., 2007).

Neutrophils are responsible for significant damage when they accumulate at sites of inflammation and are harmful to healthy tissue. The neutrophils role in the cellular host defence against microorganisms relies in part on their ability to generate large amounts of superoxide anion (O2–) and related ROS through activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase on the plasma membrane, an ability which is known as respiratory bursting. Oxidant such as O2– and hydrogen peroxide (H_2O_2) serve as intracellular signaling molecules for a variety of chemokines, including LTB4, TNF-α, IL-1, and TGF-β1, as well as various other growth factors (Circu et al., 2010).

The accumulation of neutrophils is a dynamic process that consists of recruitment from the bloodstream and clearance from the lungs as a result of phagocytosis of apoptotic cells. Recently, cigarette smoke has been discovered to impair the ingestion of neutrophils by macrophages and decreases the ability of macrophages to phagocytose apoptotic cells through inhibition of actin rearrangement (Minematsu et al., 2011). The inability to clear neutrophils

would lead to a greater release of destructive neutrophil products. This leads to a cascade of events that damages central and peripheral airways along with terminal airspaces, causing the physiologic and clinical abnormalities in COPD.

Macrophages

Macrophages play an important role in the pathophysiology of COPD. Macrophages account for the majority of inflammatory cells recovered by airway lavage, regardless of whether or not the subjects are non-smokers, healthy smokers or smokers with airways disease(O'Donnell et al., 2006). There is however a significant increase of macrophages in airways, lung parenchyma, BAL fluid and sputum in patients with COPD. Moreover, macrophage numbers in the airways correlate well with the severity of COPD (Di Stefano et al., 1998). The increase of macrophages numbers in COPD patients and smoker are partly due to the increased recruitment of monocytes from circulation in response to monocyte-selective chemokines and partly due to the increase proliferation and prolonged macrophages survival in the lungs. Macrophages can be directly activated by cigarette smoke to release inflammatory mediators including TNF-α, MCP-1, reactive oxygen species (ROS), and neutrophil chemotactic factors such as keratinocyte-derived chemokine (KC) (Keatings et al., 1996). Macrophages also secrete a wide array of tissue proteases, including matrix metalloproteinase (MMP) which mediate the lung parenchymal damage (Barnes et al., 2003).

Epithelial Cell

Epithelial cells are key regulators of neutrophil trafficking into the airway lumen (Quint *et al.*, 2007). Cigarette smoke exposure induced the expression of IL-1β, IL-8 and GM-CSF in normal human bronchial epithelial cells (NHBE) via the activation of both the NF-κB and MAPK pathways (Hellermann *et al.*, 2002). The airway epithelium is one of the first targets of cigarette smoke. Studies have demonstrated that alveolar epithelial cell apoptosis

increased disproportionately to alveolar cell proliferation in emphysema (Calabrese *et al.*, 2005). The damage to epithelial cells of the lower respiratory tract is partly due to cigarette smoke-derived oxidants which cause direct injury to membrane lipids, proteins, carbohydrates and DNA, resulting in loss and destruction of lung tissue (Demedts *et al.*, 2006; Morissette *et al.*, 2009). The epithelial barrier breakdown reduce the ability of lung to exclude foreign and reactive materials and increase the likelihoods of respiratory infections (Taylor, 2010).

1.3.4 Mediators of COPD

1.3.4.1 Chemokines

Neutrophil Chemoattractant

Neutrophil recruitment to the airways is mediated by various mediators. IL-8 (CXCL8) is one of the most notable chemoattractant of neutrophils (Gernez *et al.*, 2010). IL-8 level correlate with the increased proportion of neutrophils and are markedly increased in induced sputum of patients with COPD (Keatings et al., 1996). Additionally, the level of IL-8 in BAL fluid can be used as a marker to determine the susceptibility of current smokers to pulmonary emphysema (Tanino *et al.*, 2002).

Cigarette smoke also induces macrophages to release IL-8 (Quint *et al.*, 2007). Additionally, IL-8 are also released by neutrophils themselves to attract more neutrophils and therefore, a self-perpetuating inflammatory state may be established (Scapini et al., 2000). When anti-IL-8 antibodies are use therapeutically, the neutrophils chemotactic activity is only partially reduced, of the order of approximately 30%, signifying blocking IL-8 alone may not be sufficient as a therapeutic strategy to attenuate neutrophil inflammation in the respiratory tract (Beeh et al., 2003).

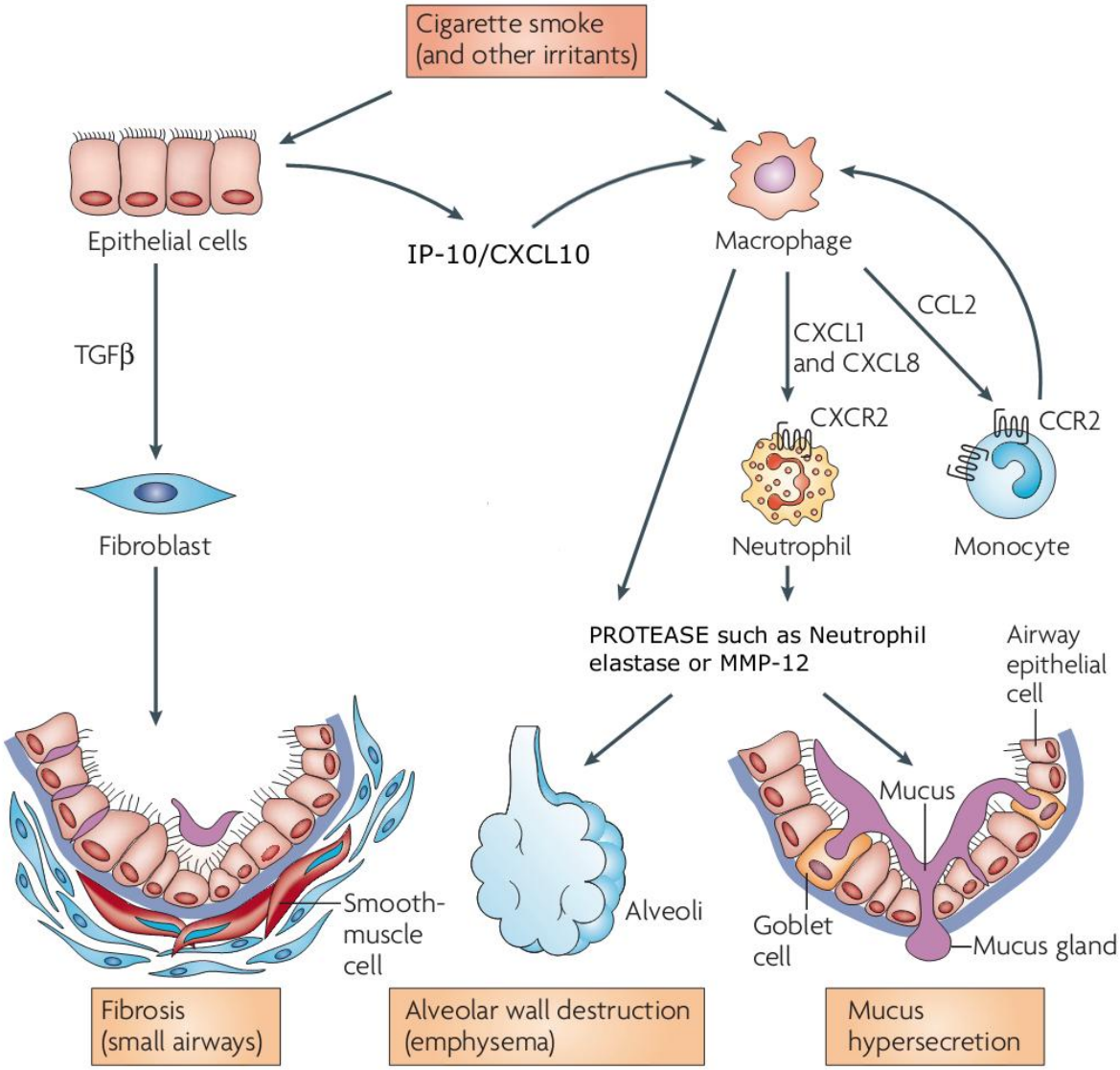


Figure 1.4 Cytokines involved in COPD. Inhaled irritants, such as cigarette smoke, activate epithelial cells and macrophages to release multiple cytokines, including proinflammatory cytokines TNF-α, and IL-1β which amplify inflammation, and several chemokines that attract circulating cells into the lungs. MCP-1/CCL2 acts via CCR2 to attract monocytes (which differentiate into macrophages in the lungs); KC/CXCL1 and IL-8/CXCL8 act via CXCR2 to attract neutrophils and monocytes; and IP-10/CXCL10 act via CXCR3 to attract T lymphocytes. Adapted from Barnes (Barnes, 2008c).

There are two receptors for IL-8, one of which is CXCR1, which is a low-affinity receptor that is specific for IL-8, and CXCR2, which has high affinity and is share by others CXC chemokines including CXCL1. IL-8 also induces neutrophils to release of myeloperoxidase (MPO) which contributes to further recruitment of inflammatory cells and sustain inflammation (Quint *et al.*, 2007).

CXCL1 is a small cytokine belonging to the CXC chemokine family that is also known as Growth-related oncogene-α (GRO-α) or keratinocyte chemoattractant (KC). CXCL1/KC is secreted by alveolar macrophages and airway epithelial cells in response to stimulation with TNF-α (Schulz et al., 2004). CXCL1 activates both neutrophil and monocytes via CXCR2 (Geiser et al., 1993). The concentrations of CXCL1/KC were significantly elevated in induced sputum and BAL of patients with COPD compared with non-smokers or normal smokers (Traves et al., 2002). In animal models of inflammation, blockade of CXCR1 and CXCR2 by specific inhibitors significantly attenuated neutrophilic airway inflammation (Chapman *et al.*, 2007). In endobronchial biopsies from patients with severe COPD, both CXCR1 and CXCR2 are expressed in high levels and play important roles in severe exacerbations (Qiu *et al.*, 2003).

In the airways of COPD patients, E-selectin, which is necessary for slowing down circulating neutrophils, is upregulated on endothelial cells, while ICAM-1, which regulates neutrophil adhesion and transcellular migration, is upregulated in on basal epithelial cells. (Di Stefano *et al.*, 1994; Scott *et al.*, 2002). Neutrophil influx is also dependent on the presence of leukotriene B_4 (LTB_4), TNF-α, GM-CSF and macrophage-derived matrix metalloproteinase (MMP)–12 (Gernez *et al.*, 2010). LTB_4 stimulates neutrophil chemotaxis, degranulation, release of lysosomal enzymes, and generates reactive oxygen species (ROS). TNF-α also plays a role in cellular migration by stimulating the secretion of GM-CSF, IL-6 and IL-8.

(Quint *et al.*, 2007). MMP-12 induced an auto-feedback loop that causes macrophages to release TNF-α, further enhancing the neutrophil influx.

Monocyte Chemoattractant

Monocyte chemotactic protein-1 (MCP-1/CCL2) which is a potent chemoattractant for monocytes, is express by alveolar macrophages, T lymphocytes and epithelial cells. MCP-1 activates CCR2 on monocytes and T lymphocytes (Rose et al., 2003). CCR2 play a key role in COPD as sputum, BAL and lung of patients with COPD has an increase of MCP-1 level (Traves et al., 2002). Macrophage inflammatory protein-1α (MIP-1α/CCL3) is a chemoattractant for monocytes and neutrophils via CCR1. MIP-1α is released by macrophages. Interferon-γ-inducible protein 10 (IP-10, CXCL10) is a member of the non-ELR CXC chemokine family that is produced by several cell types, particularly epithelial cells (Sauty et al., 1999). IP-10 has been attributed to several roles, including chemoattraction for monocytes (Dufour et al., 2002) and inducing the production of MMP-12 in human alveolar macrophages (Grumelli et al., 2004).

1.3.4.2 Cytokine

TNF-α

TNF-α is a pleiotropic inflammatory cytokine that has a broad spectrum of inflammatory effects relevant to COPD. TNF-α is produced by alveolar macrophages, neutrophils, T lymphocytes, mast cells and epithelial cells following contact with different pollutants including cigarette smoke (Chung, 2006). In animal models, TNF-α has been shown to induce an inflammatory cell infiltrate into the lungs, pulmonary fibrosis and emphysema, all of which are pathological features associated with COPD (Lundblad *et al.*, 2005). TNF-α concentrations are increased in peripheral blood, bronchial biopsies, induced

sputum and BALF of patients with stable COPD compared with control subjects. Furthermore, TNF-α level in sputum was shown to increase significantly during acute COPD exacerbations (Matera *et al.*, 2010). The activation of NF-κB by TNF-α will leads to the transcription of inflammatory genes, including cytokines, chemokines and proteases. TNF-α is synthesized as a precursor before being converted to the biologically active TNF-α by metalloproteinase (MMP). It has been demonstrated that the release of active TNF-α from the macrophages after acute smoke exposure is dependent on matrix metalloproteinase-12 (MMP-12) (Churg et al., 2003). Unexplained weight loss occurs in about 50% of patients with severe COPD and chronic respiratory failure. TNF-α release from circulating cells was increased in COPD patients with weight loss (Agusti *et al.*, 2008). Infliximab is a monoclonal antibody against TNF-α. Infliximab was used to treat autoimmune diseases such as psoriasis, Crohn's disease, ankylosing spondylitis, psoriatic arthritis, rheumatoid arthritis, and ulcerative colitis (Matera *et al.*, 2010). Nonetheless, infliximab failed to alleviate symptoms, lung function, and exercise performance of COPD patients, as assessed by effects on when administrated at the doses that are effective in individuals with rheumatoid arthritis (Rennard *et al.*, 2007). Infliximab was also no effective in patients with severe COPD who have cachexia (Dentener *et al.*, 2008). These surprising findings may indicate that the drug does not adequately neutralize local concentrations of TNF-α or that other cytokines are more important. Moreover, when TNF-α are block in COPD patients, there was an increase in the incidence of chest infections and cancers, raising concerns that suppression of TNF-α may be detrimental in patients with COPD who are already predisposed to bacterial lung infections and cancer.

IL-1β

Similar to TNF-α, IL-1β is a potent activator of alveolar macrophages from COPD patients (Russell et al., 2002b). IL-1β activates macrophages to secrete various inflammatory cytokines and chemokines as well as stimulating the expression of elastolytic MMPs from multiple cell types (Culpitt *et al.*, 2003). In addition, IL-1β also stimulate the bronchial epithelial cells to produce extracellular matrix glycoproteins (Chung, 2006). The increases in sputum and lavage fluid IL-1β have been documented in smokers compared with non-smokers (Ryder et al., 2002). Furthermore, IL-1β together with TNF-α has been identified as key cytokine that is able to initiate inflammatory cascades during exacerbations of COPD (Hackett *et al.*, 2008). As such, studies investigating the efficacy of canakinumab, an fully human mAb that neutralizes the bioactivity of human IL-1β in patients with COPD are currently in progress (Church *et al.*, 2009).

Granulocyte-macrophage colony stimulating factor (GM-CSF)

GM-CSF play an important role in cigarette smoking-related lung diseases because it governs the growth, activation, and survival of leukocytes directly implicated in the pathogenesis of COPD (Vlahos *et al.*, 2006). GM-CSF, which is released from macrophages and epithelial cells, exerts its effect by increasing cytokine expression and priming inflammatory cells to amplify their responses to other stimuli (Hamilton, 2002). Moreover, GM-CSF which is a direct neutrophil chemotactic factor (Gomez-Cambronero et al., 2003) increases neutrophil survival in the respiratory tract (Barnes et al., 2003). GM-CSF is also directly implicated in the development of small airway fibrosis (Xing *et al.*, 1996). The concentrations of GM-CSF in BAL fluid are increased in stable COPD and are significantly elevated during exacerbations (Balbi *et al.*, 1997). Similarly, studies have also reveal that the levels of GM-CSF in sputum are increased in stable COPD (Saha *et al.*, 2009), and during exacerbations of COPD (Tsoumakidou *et al.*, 2005). Recent studies have shown that

neutralization of GM-CSF by intranasal administration of anti–GM-CSF antibody to mice exposed to cigarette smoke significantly reduce cigarette smoke-induced airways inflammation and suggested potential therapeutic implication in COPD (Vlahos *et al.*, 2010).

1.3.4.3 Proteases and antiproteases

The protease-antiprotease imbalance has been proposed to play a major pathogenic role in the development of emphysema. The observation that the instillation of papain, an plant elastolytic proteinase, into the lungs of hamsters led to pronounced emphysema (Gross et al., 1965) was essential to the formation of the proteinase-antiproteinase imbalance hypothesis. These observations led to the notion that emphysema develops due to the release of uncheck proteases by the inflammatory cells which lead to the destruction of the parenchymal matrix (Abboud *et al.*, 2008). Hence, early studies of COPD focus on the effect of protease induce lung inflammation and the subsequent lung injuries.

Neutrophil elastase (NE) and α1-antitrypsin (α_1-AT)

NE is a potent serine protease that that hydrolyze many proteins in addition to elastin. NE which is stored in azurophilic granules in neutrophils play an important role in host defence as mice lacking neutrophil elastase has been demonstrated to have an impaired response to gram negative bacterial induce sepsis (Belaaouaj, 2002). However, as a result of NE capacity to efficiently degrade extracellular matrix, overproduction of NE has been implicated in a variety of destructive diseases including COPD. Administration of elastase, either by intratracheal instillation or aerosol inhalation (Wright et al., 2008) not only lead to emphysema, it also cause infiltration of neutrophils and lymphomononuclear cells, mucus cell metaplasia, pulmonary edema, hemorrhage and rupture of the respiratory epithelium (Birrell *et al.*, 2005c). These changes lead to alterations in lung function that are consistent with those

observed in COPD patients (Birrell *et al.*, 2005b). The alpha-1 antitrypsin (α_1-AT) is a serine protease inhibitor that protects the pulmonary tissue from deleterious effects of proteolytic enzymes (Kohnlein *et al.*, 2008). As such, α_1-AT deficiency is known to predispose to COPD and is symptomatically treated as COPD (Rabe *et al.*, 2007). However, α_1-AT deficiency only occur in a small portion of COPD patients and COPD patients with or without α_1-AT deficiency shows different molecular and cellular characteristics due to the specific deficiency present in α_1-AT deficiency (Hattesohl *et al.*, 2011). As elastase can be rapidly induced emphysema, many early animal COPD models used elastase as the sole agent to study the effect potential drugs in treating COPD. However, as the inflammation of the elastase model is transient and resolves within a week of elastase administration, it does not reflect the progressive, slowly resolving inflammation associated with COPD and the model has gradually fallen out of favours (Stevenson *et al.*, 2011).

MMPs and Tissue inhibitor of MMPs (TIMP)

MMPs constitute a large family of zinc-dependent endopeptidases that are capable of degrading extracellular matrix components. Alveolar macrophages may play a pathogenic role in emphysema as they express MMPs when induced by cigarette smoking (Lemaitre et al., 2006). Among the MMPs, MMP-12 is the prominent MMP in the mouse. MMP-12 knock-out mice prevented emphysema induced by chronic cigarette smoke exposure (Hautamaki et al., 1997). Similarly, MMP-12 knock-out mice has attenuated emphysema (Wang et al., 2000; Zheng et al., 2000). MMP-9 knockout mice however are not protected against cigarette smoke induced emphysema (Lanone et al., 2002). Moreover, as aforementioned, MMP-12 also cleaves membrane-bound 26kDa pro-TNF-α to the 17kDa mature TNF-α protein, acting as a "TNF-α converting enzyme" (Black et al., 1997; Moss et al., 1997). The MMPs are counteracted by four tissue inhibitor of MMPs (TIMP1-4). TIMPs may be either secreted as

soluble proteins (TIMP-1, TIMP-2, and TIMP-4) or bound to extracellular matrix (ECM) components (TIMP-3) (Melendez-Zajgla *et al.*, 2008). The TIMPs are synthesized by connective tissue cells and leukocytes and form non-covalent complexes with MMPs. TIMPs bind to the zinc binding catalytic site of the MMPs with a 1:1 molar ratio (Mocchegiani et al., 2011). Among the TIMP, only TIMP-1 is highly implicated in COPD. The Secretion of TIMP-1 from alveolar macrophages is increased in response to inflammatory stimuli but the cells derived from COPD patients has a reduce TIMP-1 and therefore favouring increased in elastolysis (Russell et al., 2002a).

NEs and MMPs "support" each other by inhibiting their counterpart endogenous inhibitors. NEs inhibits TIMPs, and MMPs degrade α_1-AT (Shapiro, 2002). These proteinases cleave components of the ECM to generate elastin fragments or collagen-derived peptides such as N-acetyl proline-glycine-proline (PGP), which have been shown to be chemotactic for monocytes and neutrophils (Weathington *et al.*, 2006). Although unchecked proteinase has deleterious effects on the lungs, the imbalance of protease and anti-protease are largely due to oxidative stress in the overall pathogenesis of COPD as oxidant not only induce proteases secretion, cigarettes or biomass smoke exposure also can inactivate endogenous antiproteases activities (Cavarra *et al.*, 2001).

1.3.4.4 Oxidant and antioxidant

Oxidative stress is a cardinal feature of COPD. While oxidant generation is part of the normal cellular metabolism and is critical for cell homeostasis, oxidative stress can occurs when the burden of oxidants is not well counterbalanced by the antioxidant defence system and results in harmful effects (Lin *et al.*, 2010). Inhaled reactive oxygen species (ROS) and reactive nitrogen species (RNS) and reactive chemicals (e.g. aldehydes) from cigarette smoke, together with endogenous oxidants arise from inflammatory cells, such as activated

neutrophils and macrophages, constitute a major oxidative burden to the lungs (Mak, 2008). Oxidants in cigarette smoke may also activate resident cells in the lung such as epithelial cells and alveolar macrophages to release chemokine which recruit additional inflammatory cells into the lung (Louhelainen *et al.*, 2008). Chronic exposure to cigarette smoke perpetuates this response, leading to the increased production of inflammatory cytokines and degradative enzymes as well as defects in homeostatic mechanisms such as inactivation of anti-proteases, anti-oxidants and repair mechanisms (van der Toorn *et al.*, 2007). The down regulation of antioxidant pathways has also been associated with acute exacerbations of COPD (Lin *et al.*, 2010). Lung injury due to oxidants is linked to oxidation of proteins, DNA, and lipids (Biswas et al., 2009). Pulmonary levels of oxidant biomarkers such as 3-nitrotyrosine (3-NT), 8-isoprostane and 8-hydroxydeoxyguanosine (8-OHdG) have been shown to correlate positively with COPD severity (Inonu *et al.*, 2012; Louhelainen *et al.*, 2008; Yao *et al.*, 2011). The degree of pulmonary oxidant-antioxidant imbalance correlates well with the severity of COPD (Drost et al., 2005). Thus, the ability of the lungs to respond to oxidative stress is an important determinant of their relative resistance or susceptibility to COPD.

Antioxidants are agents that decrease steady-state ROS and protect cellular macromolecules from oxidative modification. An antioxidant rapidly reacts with oxidant to produces less reactive species. The lung has a large reserve of endogenous enzymatic and non-enzymatic antioxidants. Antioxidant enzymes include superoxide dismutase (SOD), catalase, glutathione peroxidases (GPx), glutathione S-transferase (GST) and the heme oxygenase-1 (HO-1) system. Non-enzymatic antioxidants include glutathione (GSH), bilirubin, carbon monoxide and ferritin.

GSH is the most abundant free thiol antioxidant that reacts with peroxides to form glutathione disulfide (GSSG) and is recycled by glutathione reductases (GR). GPx also catalyze a variety of GSH reactions including the breakdown of H_2O_2. GSH is more

concentrated in the epithelial lining fluids and plays an important role in maintaining optimal redox balance in the lungs. In addition, under catalysis of GST, GSH is capable of rapidly detoxifying harmful xenobiotic components of tobacco smoke (Harju et al., 2008). GSH is sensitive to oxidative stress. In response to acute cigarette smoke exposure, there is a transient upregulation of GSH in the epithelial lining fluids to counteract the increased oxidants, but it is eventually overwhelmed by ongoing oxidative stress. Cigarette smoke also affects GPx, GR, and GST, all of which are detoxifying enzymes involved in the GSH redox system in the lungs. These observations suggest that smokers are predisposed to oxidative injuries (Lin et al., 2010). Similar to GPx and GSH, catalase catalyse the decomposition of H_2O_2 to water and oxygen. Polymorphisms of the catalase gene have been shown to be related to an imbalanced of oxidant and antioxidant system in COPD patients (Mak et al., 2007). SODs are antioxidant enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. There are three forms of superoxide dismutase, SOD1 (Cu/Zn SOD), SOD2 (MnSOD) and SOD3 (extracellular SOD, ecSOD). SOD1 and SOD2 are primarily localized in lung cells and SOD3 is mainly in the extracellular space of the lung (Oberley-Deegan et al., 2009).

Heme oxygenase (HO) is an enzyme that catalyzes the degradation of heme to generates biliverdin, iron, and carbon monoxide (CO). The products of heme catabolism mediate the antioxidant properties of HO. In the airways, HO-1 is expressed in the epithelium, smooth muscle, type II pneumocytes, and alveolar macrophages. HO-1 expression which can be induced by heme, hypoxia, hyperoxia, NO, endotoxin and pro-inflammatory cytokines (An et al., 2012), is extremely sensitive to agents that cause oxidative stress. HO-1 exhibits powerful antioxidant and anti-inflammatory properties. HO-1 expression is upregulated in several pulmonary diseases, including COPD and asthma. The over-expression of HO-1 in animal models is beneficial in several lung diseases including COPD (Shinohara et al., 2005).

The biliverdin that is generated by HO-1 is subsequently converted to bilirubin, a potent endogenous antioxidant (Fredenburgh et al., 2007). In vitro and in vivo studies have shown carbon monoxide (CO) has both anti-inflammatory and anti-oxidant capacities (Montuschi et al., 2001). In fact, a recent clinical trial which have been carried out to determine the effect of CO in the treatment of stable COPD have found that CO treatment led to trends in reduction of sputum eosinophils and improvement of responsiveness to methacholine (Bathoorn et al., 2007). The generation of iron-catalysed ROS has been minimized as free iron produced by HO-1 is taken up by ferritin, a major intracellular iron storage protein that removes excess free iron (Lin et al., 2010).

The association of COPD with genetic variations of antioxidant genes has also been reported. There is a decrease of HO-1expression in COPD patients. Studies have shown that the polymorphism of the HO-1 gene is associated with increased susceptibility to emphysema in smokers (Hirai et al., 2003). Furthermore, in response to oxidative stress, this polymorphism resulted in reduced transcriptional activity of the HO-1 gene and decrease HO-1 antioxidant capacity. Similarly, single-nucleotide polymorphisms (SNPs) in SOD3 have been reported to either protect (Young et al., 2006) or promote (Dahl et al., 2008) development of COPD.

Nuclear factor erythroid-2-related factor 2 (Nrf2) is a redox-sensitive basic leucine zipper transcription factor and is critical in protecting the lung against oxidative stress. Nrf2 regulates expression of antioxidants including GPx, GR and HO-1 by binding to the Antioxidant Response Element (ARE) present in the promoters of these genes (Adair-Kirk et al., 2008; Hubner et al., 2009). The selective inactivating mutations in Kelch-like ECH-associated protein 1 (KEAP1), an Nrf2 inhibitor, enhances Nrf2-directed constitutive expression of multiple antioxidants genes (Singh *et al.*, 2006a). Impaired Nrf2 activities have been implicated in several pulmonary diseases including acute respiratory distress syndrome,

pulmonary fibrosis, asthma and COPD (Boutten et al., 2011; Cho et al., 2006). Studies have revealed a significant decline in NRF2-regulated antioxidant defenses such as HO-1 and GCLM as well as a higher degree of oxidative damage in peripheral lung tissues of patients with COPD as compared with lungs of patients without COPD (Malhotra et al., 2008). In animal models, Nrf2 gene disruption resulted in ablated expression of antioxidants gene and enhanced susceptibility to emphysema after cigarette smoke exposure (Rangasamy et al., 2004). Activation of Nrf2 by triterpenoids such as CDDO-imidazolide has demonstrated beneficial effects in a cigarette smoke-induced emphysema animal model (Sussan et al., 2009). Drug discovery of novel compounds with property of strengthening antioxidant defense is a vital strategy for COPD drug development.

1.3.5 Mouse Models of COPD

Animal models have played an instrumental role in broadening our mechanistic understanding of COPD. In our studies, cigarette smoke model were utilized to investigate our hypothesis as the model is induced by same insult as in humans. Cigarette smoking model are advantages over other models for COPD as it uses the predominant disease-causing agent to model several key features of the disease in small animals. Moreover, as there are over 4000 hazardous compounds, of which 200 are highly toxic (Brunnemann et al., 1991) and it has more than 10^{15} free radicals in every puff of smoke (Church et al., 1985), single stimuli such as elastase and LPS are not likely to replicate the complex response to smoke. Chronic cigarette smoke exposure to animals produces emphysema, airway inflammation and airway remodelling. Cigarette smoke also causes lung inflammation where macrophages and neutrophils are prominent (Pauwels et al., 2001; Rabe et al., 2007). Moreover, cigarette smoke induces inflammation exposure that is not as pronounced as the response to either LPS or elastase and it is insensitive to glucocorticoids treatments (Marwick et al., 2009). Similar to

physiological alterations in humans, cigarette smoking appear to effectively mimic the progressive, low-grade, slow resolving and steroid-insensitive inflammation associated with COPD. As acute exacerbations in COPD patients, which contribute to the accelerated lung function decline and disease progression, are a major cause of hospitalization and death (Makris *et al.*, 2007), several groups have begun to attempt to model the pathologies and physiological changes associated with late-stage COPD by challenging animals exposed to smoke with agents known to cause exacerbations such as bacteria and viruses. Studies combining virus and cigarette smoke exposure demonstrated that virus infection may reduce or enhance the inflammatory response depending depended on the type of virus (Gualano *et al.*, 2008; Meshi *et al.*, 2002) and the dose of virus infection (Robbins *et al.*, 2006). Additionally, infecting small animals with bacteria has also been used to model COPD acute exacerbation-like changes. The effects of bacterial infection on cigarette smoke exposed mice are also dependent on the species of bacteria involved. Bacterial infection generally enhanced inflammation but the clearance of bacteria may be enhanced (Gaschler *et al.*, 2009) or reduced (Drannik *et al.*, 2004) dependent on the species of bacteria involved. These studies underscore the complexity of combining cigarette smoke exposure with human pathogens to model these acute events. As the entire mouse genome has been sequenced and mouse can readily be manipulated to generate genetically modified mouse models, mouse has become the species of choice for investigators to test hypothesis about the causal mechanisms underlying disease in ways that cannot be done in human. Mice with Nrf2 (Rangasamy et al., 2004) and MMP-12 knockout (Hautamaki et al., 1997) have shed light on the molecular mechanisms involved in mediating the inflammation and lung pathologies induced by cigarette smoke exposures. In our studies, BALB/C mice were preferred over other strain of mice as mouse strains comparison studies have found that although the types of changes in response to cigarette smoke exposure were similar among the strains studied, the degree of the

changes did vary and BALB/C mice consistently had the greatest response to cigarette smoke exposure in term of the proportion and numbers of neutrophils and levels of KC in the BAL fluid, whereas C57BL/6 mice consistently had the smallest response (Morris *et al.*, 2008). Female mice were used in the present study as there is evident that women are more susceptible to the effects of smoke than men (Gan et al., 2006; Langhammer et al., 2003; Martinez et al., 2007; van Durme et al., 2009). Even in mouse models, female A/J mice develop emphysema earlier than male A/J mice (March et al., 2006). However, cigarette smoke induced mouse COPD model requires months of smoke exposure (around 4 to 6 months) to generate pathologies and functional changes consistent to those observed in COPD patients. This approach is thus an expensive and time-consuming proposition. Hence, many laboratories use an acute (<1 week) model of cigarette smoke induced lung inflammation as primary model to examine the efficacy of prospective therapies. Although emphysema is not obvious in the acute model, it does provide preliminary effect on cigarette smoke induce oxidative stress and inflammatory response that is useful in guiding further studies as the changes are very mild compared to those observed in human. The pathologies and lung function changes associated with these models would most likely constitute mild COPD (GOLD 1 and 2) (Churg et al., 2007; Rabe et al., 2007). Regrettably, most cases of COPD are not diagnosed until the disease has become severe (GOLD 3 and 4) (Rabe et al., 2007). Moreover, cigarette smoking models do not produce the severe disabling disease seen in humans and the lesions due to cigarette smoking do not appear to progress after cessation of smoke exposure (Wright et al., 2008). Thus, we must bear these limitations in mind when we design and interpret the results from animal models.

1.3.6 Current Drug

Similar to asthma, current guidelines for COPD management advocate the use of inhaled β_2-agonists, inhaled anticholinergics and inhaled corticosteroids for symptomatic management (GOLD, 2011). Long acting bronchodilators such as the anti-cholinergic tiotropium, and the β_2-agonists salmeterol and formoterol have all been shown to improve lung function, quality of life and reduce the time to first exacerbation when compared to placebo (Donohue *et al.*, 2003; Tashkin *et al.*, 2004). Similarly, inhaled steroids have been demonstrated to significantly reduce the rate of exacerbations in COPD in several clinical trials (Alsaeedi *et al.*, 2002; Calverley *et al.*, 2007). In spite of these beneficial effects and in sharp contrast to asthma, neither bronchodilators nor inhaled steroids are able to alter the rate of decline of lung function (Vestbo *et al.*, 1999) or improve survival (Calverley *et al.*, 2007) in COPD. The inability of current drug therapy to halt the relentless progression of lung function deterioration in COPD patients may be due in part to the inability of these drugs to fully reverse the inflammatory changes that occur in COPD (Loppow *et al.*, 2001). For example, although corticosteroids are effective in attenuating the release of IL-8, TNF-α and MMP-9 from macrophages of normal subject and normal smokers in vitro, corticosteroids are not effective in macrophages from patients with COPD (Culpitt et al., 2003). Although bronchodilators such as phosphodiesterase 4 inhibitor roflumilast has been newly approved for the treatment of COPD, it suffers from dose-limiting major side effects such as diarrhoea, headache, nausea and weight loss (Page *et al.*, 2012). In addition, current drug do not alter the oxidative or apoptotic processes that are critical to the pathogenesis of COPD (Imai *et al.*, 2005; Zheng *et al.*, 2005). Given the importance of antioxidant in COPD, researchers have sought to determine whether antioxidant administration would improve disease outcomes in COPD. The most commonly used antioxidant for these studies is ***n***-acetylcysteine (NAC), which is a known precursor of glutathione, as it has established safety profile and is relatively

easy to tolerate in its oral form (Dekhuijzen *et al.*, 2006). Nonetheless, the therapeutic use of NAC in COPD has yield conflicting results, and while some studies have shown beneficial effects of NAC with regard to lung function (Stav *et al.*, 2009) and COPD exacerbations (Hansen *et al.*, 1994), some clinical trials concluded that NAC was ineffective at preventing deterioration of lung function or preventing exacerbations (Schermer *et al.*, 2009). Although smoking cessation, supplemented oxygen therapy for hypoxemic patients, lung reduction surgery for selected patients with emphysema alters the course of COPD, there is currently no specific treatments halting the relentless progression of COPD, which leads to increasing symptoms and disability and increases the risk of premature death in COPD patients (Barnes, 2008b; Barnes, 2010; Morjaria *et al.*, 2010). As a result, the lack of effective treatments and the escalating healthcare burden has created an impetus for researchers to seek novel and more effective compounds aimed at reducing the progression of COPD and control the underlying disease process.

1.4 Andrographolide and DDAG

1.4.1 *Andrographis paniculata*

Andrographis paniculata (Burm. F.) Nees, commonly known as 'king of bitters' due to its bitterness, is an herbaceous plant belonging to the Family Acanthaceae. Similar to weeds, *A. paniculata* is easily grown and propagate over a broad eco-geographical range (Aromdee, 2012). *A. paniculata* is a plant indigenous to Southeast Asian countries and it is highly cultivated for mass production in many Asian countries due to the high demand for its active constituents (Aromdee, 2012). *A. paniculata* has a long history of therapeutic usage in Indian and Chinese medicine as it is traditionally was used for the prevention and treatment of upper respiratory tract infection in Asian countries and also in Scandinavia (Chang *et al.*, 2008a; Coon *et al.*, 2004; Poolsup *et al.*, 2004). With the long history of uses, *A. paniculata* is

known by various vernacular names in various places where it is traditionally use. It is known as *Kalmegh* in India, Chuan-Xin-Lian in China, Fah Tha Lai in Thailand, and Hempedu bumi in Malaysia.

1.4.2 Andrographolide

A number of active constituents are described from the plant, which mainly include diterpene lactones, flavonoids and polyphenols (Koteswara Rao *et al.*, 2004). However, andrographolide (Fig. 4), 14-deoxy-11,12-didehydroandrographolide (DDAG) (Fig. 5) and neoandrographolide are the three prime constituents isolated from *A. paniculata* (Lim *et al.*, 2012; Pholphana *et al.*, 2004). Among the isolated compound, andrographolide ($C_{20}H_{30}O_5$) is the most abundant diterpenoid in *A. paniculata*, making up about 4% of dried whole plant, 0.8~1.2% of dried stem and 0.5~6% of dried leaf extracts (Chao *et al.*, 2010b).

Andrographolide has been demonstrated to have multiple pharmacological properties and it has been proposed to be a potential chemotherapeutic agent (Varma *et al.*, 2009). As such, the toxicity profile has been extensively studied. Intraperitoneal injection affording doses of 4-50 mg/kg of of andrographolide over body weight in mice (Iruretagoyena *et al.*, 2005; Mishra *et al.*, 2011) has been investigated. At the doses used, andrographolide was well tolerated by mice, and no evidence of toxicity was observed. Indeed, the LD50 for intraperitoneal administration of andrographolide was 11.6 g/kg in rats. (Handa *et al.*, 1990). Even in human subjects, no side effects were observed when participants were monitored for changes in liver and kidney functions, blood counts, and other laboratory measures when treated with 1,200 mg/day of andrographolide for 5 days (Hancke *et al.*, 1995).

Figure 3 Andrographis paniculata. Adapted from Valdiani (Valdiani *et al.*, 2012)

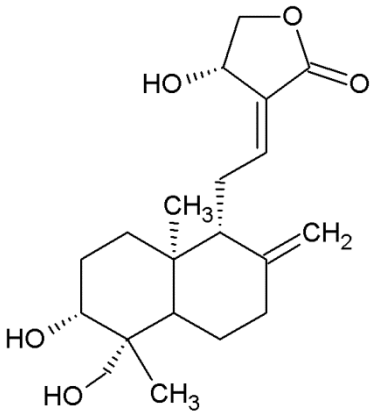


Figure 4. Andrographolide

Andrographolide has also been reported to have diverse pharmacological potential including hepatoprotective (Negi *et al.*, 2008), antiviral (Aromdee *et al.*, 2011b; Calabrese *et al.*, 2000; Ko *et al.*, 2006; Lin *et al.*, 2008; Wiart *et al.*, 2005), anti-cancer (Kumar *et al.*, 2004b; Lim *et al.*, 2012; Varma *et al.*, 2009), antipyretic (Suebsasana *et al.*, 2009), antibacterial (Aromdee *et al.*, 2011a; Jiang *et al.*, 2009; Wang *et al.*, 2010) and anti-inflammatory (Abu-Ghefreh *et al.*, 2009; Bao *et al.*, 2009) activities.

Recently, andrographolide has also been shown to have antioxidant activities by inhibiting oxygen radical production in neutrophils and inhibition in phorbol 12-myristate 13-acetate (PMA) induced superoxide and nitric oxide formation in BALB/c mice (Sheeja *et al.*, 2006). The exact molecular mechanism that mediates these anti-inflammatory effects of andrographolide has not been unequivocally determined. However, andrographolide has been reported to have the ability to activate antioxidant system and probably acting thorough Nrf2 activation pathway.

It has been demonstrated that intraperitoneal treatment of andrographolide in mice significantly increase the glutathione (GSH) concentration and glutathione peroxidase (GPx) activities (Neogy *et al.*, 2008). Besides, oral andrographolide administration significantly induced the activities of GSH, glutathione reductase (GR) and GPx in the liver of hexachlorocyclohexane (BHC)-treated mice. The studies shown that andrographolide effectively activate antioxidant in severe liver damage (Trivedi *et al.*, 2007).

It has been demonstrated that incubation of andrographolide in rat primary hepatocyte also increase the expression of glutathione S transferase (GST) protein, mRNA level and activities (Chang *et al.*, 2008b). The study showed that GST-P enhancer-1 (GPE1) (a critical promoter of GST) luciferase activities was up regulated by andrographolide treatment. This finding further supports the notion that Nrf2 activities was up-regulated by andrographolide as Nrf2 is a transcriptional factor that bind to GPE1.

Studies using a hypoxia/reoxygenation model has showed that treatment with andrographolide protected the stressed cardiomyocytes by up-regulating the cellular-reduced glutathione (GSH) level and antioxidant enzyme activities. After treating the cell with andrographolide alone, the enzymatic activities of GPX, GR and the cellular GSH level were all increased when compare to vehicle controls (Woo *et al.*, 2008). Subsequently, to determine the molecular mechanism of andrographolide in activating the endogenous antioxidant system, it was discover that the glutamate-cysteine ligase, catalytic subunit (GCLC) and glutamate-cysteine ligase, modifier subunit (GCLM) mRNA and protein level were up-regulated upon andrographolide treatment (Woo *et al.*, 2008). The GSH synthesis is regulated by the induction of the GCL transcript which is controlled by antioxidant response elements (ARE) (Rahman *et al.*, 2000). These findings have shown for the first time the direct effect of andrographolide on Nrf2 activities as Nrf2 is the essential positive regulator of ARE-mediated gene expression.

In addition, to better understand the specific effect andrographolide has on gene expression, an mRNA microarray analysis on andrographolide treated cells is conducted to screen for andrographolide's effect in mouse gene transcription. Out of the 28,853 genes analysed, the study found that 25μM of andrographolide treatment of primary mouse hepatocytes up-regulated 18 genes and almost all of them were related to metabolism, and oxidation/reduction. Among the genes that were up-regulated, genes such as Gst, Srxn1, Gclm, Nqo1, Gpx2 and Gsr (Glutathione reductase gene) are Nrf2-dependent genes (Rangasamy *et al.*, 2004). That study further support the notion that andrographolide is a Nrf2 activator.

Andrographolide has been shown to promote Nrf2 nuclear translocation in a human endothelial cell line. By probing Nrf2 protein in the cell nuclear extracts, and by measuring Nrf2-induced antioxidant response element (ARE) activation in cells transfected with ARE-luciferase construct, it has been demonstrated that andrographolide treatment induced a time-

dependent Nrf2 nuclear translocation and increase in transcriptional activities (Yu *et al.*, 2010).

Given that the known anti-oxidative effects of andrographolide and the protective effect against oxidative stress, it is therefore proposed that andrographolide protects the lung against cigarette smoke-induced lung inflammation.

1.4.3 14-deoxy-11,12-didehydroandrographolide

DDAG is another major naturally occurring analogue isolated from *A. paniculata*, and DDAG has also been identified as a metabolite of andrographolide when given systemically to rats (He, 2003). Both andrographolide and DDAG are structurally similar, although andrographolide contains a hydroxyl group and two additional hydrogen atoms (Tzeng *et al.*, 2012).

At low doses, both andrographolide and DDAG compounds were not cytotoxic, and both of the compounds are effective in inhibiting pro-inflammatory cytokine secretion induced by TNF-α activation in astrocyte cultures (Tzeng *et al.*, 2012)

In some cases, there are difference in activities between andrographolide and DDAG. For instance, DDAG is known to inhibit α-glucosidase more potently than andrographolide (Dai *et al.*, 2006). Conversely, DDAG has been reported to be less cytotoxic to a variety of tumour cell lines as compared to andrographolide (Kumar *et al.*, 2004b; Nanduri *et al.*, 2004; Tan *et al.*, 2005). DDAG has been shown to have lower cytotoxicity as the 50% of cytotoxic concentrations (CC50) to Vero cells for DDAG was 243µM, but CC50 of Andrographolide and 14-deoxyandrographolide to Vero cells were 76µM and 80µM, respectively. These findings indicate that DDAG is safer to be used for activities other than anticancer purpose (Aromdee *et al.*, 2011b).

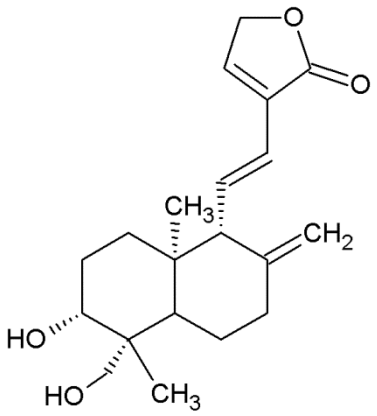


Figure 5. 14-deoxy-11,12-didehydroandrographolide (DDAG)

In addition, DDAG which demonstrated a potent hypotensive effect (Zhang *et al.*, 1998), has been shown to effectively inhibit cytokine and nitric oxide production from Raw 264.7 macrophage cell line stimulated with LPS and IFN-γ. DDAG has also been shown to attenuate high glucose-induced fibrosis and apoptosis in a renal mesangial cell line (Chao *et al.*, 2010a; Lee *et al.*, 2010). Nonetheless, while andrographolide prevented PC12 toxicity due to the synergistic effects of H_2O_2 and astrocyte-conditioned medium (ACM), DDAG on the other hand was not able to rescue PC12 cell death that occurs due to the same treatment (H_2O_2 and ACM).

The differences in pharmacological activities of these compounds are related to their structures. The molecular mechanism that mediates the actions of DDAG has not been unequivocally determined. Nevertheless, as both andrographolide and DDAG significantly attenuated TNF-α, IL-6, macrophage inflammatory protein-2 (MIP-2), and nitric oxide (NO) secretions from LPS/IFN-γ stimulated RAW 264.7 cells, the inhibition of NF-κB transcriptional activity by these compounds are strongly supported (Chao *et al.*, 2010a; Xia *et al.*, 2004). Since we have recently reported a broad-spectrum of anti-inflammatory effects for andrographolide in an OVA-induced allergic lung inflammation model (Bao *et al.*, 2009), it is necessary to elucidate the effects of DDAG, as an analogue of andrographolide, in allergic asthma.

2. RATIONALE AND OBJECTIVES

COPD and asthma are global pandemics that are major causes of morbidity and mortality. COPD is currently the fourth leading cause of death worldwide (Pauwels et al., 2004) while asthma is an airway disease which affects around 300 million people of all ages and ethnic backgrounds (Masoli *et al.*, 2004). Despite the fact that cigarette smoke is a major cause for COPD, efforts sought to restrict tobacco consumption have thus far ineffective, leading to an increase in prevalence of COPD especially in less develop countries (Huvenne et al., 2011). The increase incidence of COPD will lead to a significant increase in economic and social burden as currently there is no effective drug for the treatment of COPD as most current drugs only reduce the symptoms but fail to attenuate the underlying inflammation and the progressive deterioration of lung function (Barnes, 2010). On the other hand, current mainstay therapies for asthma are β2 agonists and inhaled corticosteroids which although are effective in controlling asthma, 5-10% of patients who suffer from uncontrollable asthmatic symptoms are refractory to corticosteroids treatment (Moore *et al.*, 2007). There is an imperative need to discover and develop novel, safe and potent drugs for both asthma and COPD.

Andrographolide and DDAG are both bioactive compounds isolated from the plant *Andrographis paniculata* (Lim *et al.*, 2012). Andrographolide has been shown to activate nuclear factor erythroid-2-related factor 2 (Nrf2), a redox-sensitive antioxidant transcription factor (Yu *et al.*, 2010). As Nrf2 activity is reduced in COPD, we hypothesize that andrographolide may have therapeutic value for COPD. On the other hand, although novel anti-inflammatory effects of andrographolide in a mouse asthma model have been reported (Abu-Ghefreh *et al.*, 2009; Bao *et al.*, 2009), andrographolide has been shown to possess cytotoxic activity, especially in tumour cell lines (Nanduri *et al.*, 2004; Tan *et al.*, 2005). We hypothesized that DDAG, as an analogue of andrographolide, may retain the anti-inflammatory effects for asthma but is devoid of cytotoxicity.

The main aim of these studies was to explore the potential therapeutic effects of andrographolide as a novel anti-oxidative stress compound on COPD in a cigarette smoke induce lung injury model and to examine the potential anti-inflammatory DDAG in allergic asthma mouse model and elucidate their molecular mechanisms. The findings in this study may shed light on novel molecular drug target for both asthma and COPD and broaden our understanding in rational drug design. The studies could also lead to the development of efficacious therapies that have minimal side effects.

3. MATERIAL AND METHODS

3.1. Materials and reagents

Drugs and chemicals used in this PhD project are as follows:

- 3-[(E)-2-[(1R,4aβ)-Decahydro-6α-hydroxy-5α-(hydroxymethyl)-5,8aα-dimethyl-2-methylenenaphthalen-1-yl]vinyl]furan-2(5H)-one (14-Deoxy-11,12-didehydroandrographolide) (>98% purity, TCM Institute of Chinese Materia Medica, Nanjing, People's Republic of China);

- 3-[2-[decahydro-6-hydroxy-5-(hydroxymethyl)-5,8α-dimethyl-2-methylene-1-napthalenyl]ethylidene]dihydro-4-hydroxy-2(3H)-furanone (andrographolide),
- anti-β-actin monoclonal antibody, 10% neutral buffered formalin, acetyl-β-methylcholine chloride, aluminium hydroxide ($Al(OH)_3$), bovine serum albumin (BSA), chicken ovalbumin (OVA), dimethyl sulfoxide (DMSO), eosin Y, Harris hematoxylin solution, hematoxylin solution Gill no. 3, heparin, periodic acid solution, Schiff's reagent, skim milk powder (Fluka) and Tween 20 (Sigma-Aldrich, St Louis, MO, USA);

- 3R4F research cigarettes (Tobacco and Health Research Institute, University of Kentucky, Lexington, KY, USA);

- Fast SYBR Green Master Mix, RNAlater (Applied Biosystems, Foster City, CA, USA);

- Alkaline phosphatase (AP) conjugate substrate kit, agarose, blotting paper, polyvinylidene difluoride (PVDF) membrane, tetramethylethylenediamine (TEMED),

3,3´,5,5´-tetramethylbenzidine (TMB) substrate kit, Triton X-100 (Bio-Rad Laboratories, Hercules, CA, USA);

- Ammonium chloride (NH_4Cl) (BDH Laboratory Supplies, Poole, England);

- Agarose, phosphate buffered saline (PBS), sodium dodecyl sulfate (SDS), and tris-acetate-EDTA (TAE) (1st BASE, Singapore);

- Diethylpyrocarbonate (DEPC)-treated water, penicillin, streptomycin, random primer, Roswell Park Memorial Institute (RPMI) medium, Trizol, and trypan blue (Invitrogen, Carlsbad, CA, USA);

- Mouse keratinocyte chemoattractant (KC/CXCL1) Duoset, mouse interferon-γ-inducible protein 10 (IP-10/CXCL10/CRG-2) DuoSet, mouse monocyte chemoattractant protein-1 (MCP-1/CCL2/JE) DuoSet and Mouse IL-1 beta DuoSet, Mouse IL-13 DuoSet, mouse CCL11/Eotaxin DuoSet (R&D Systems, Minneapolis, MN, USA);

- Avidin-horseradish peroxidase (HRP), biotinylated anti-mouse IgE, biotinylated anti-mouse IgG1, biotinylated anti-mouse IgG2a, OptEIA™ mouse total IgE set, OptEIA™ mouse IL-4 set, and OptEIA™ mouse IL-5 set, (BD Biosciences Pharmingen, San Diego, CA, USA);

- Bicinchonic acid (BCA) protein assay kit, calf serum, fetal bovine serum (FBS), Restore™ PLUS western blot stripping buffer (Thermo Fisher Scientific Inc, Waltham, MA ,USA);

- M-PER Mammalian Protein Extraction Reagent containing HALT protease inhibitor cocktail (Pierce, Rockford, IL, USA);

- Cotinine ELISA (Bio-Quant, San Diego, CA, USA);

- 8-isoprostane (8-iso-$PGF_{2\alpha}$) and 8-OHdG EIA kit (Cayman Chemicals, Ann Arbor, MI, USA);

- 3-nitrotyrosine (3-NT) ELISA kit (Upstate/Millipore, Billerica, MA, USA);

- Sodium carbonate (Na_2CO_3) (Kanto Chemical, Tokoyo, Japan);

- Absolute ethanol, isopropanol (Merck, Darmstadt, Germany);

- HistoClear and Histomount (National Diagnostics, Atlanta, GA, USA);

- Avian myeloblastosis virus (AMV) reverse transcriptase, dNTP mix, polymerase chain reaction (PCR) master mix, and ribonuclease inhibitor (Promega, Madison, WI, USA);

- Ethidium bromide (Research Organics, Cleveland, OH, USA);

- Anti-β actin and anti-p65 polyclonal antibody (Cell Signaling Technology, Beverly, MA, USA);

- Anti-TATA binding protein (TBP, Abcam, Cambridge, UK);

- Enhanced chemiluminescent (ECL), western blotting detection reagents, and hyperfilm (GE Healthcare, Piscataway, NJ, USA);

- Protease inhibitor cocktail (complete) (Roche Diagnostics, Indianapolis, IN, USA);

- HRP-conjugated anti-mouse Ig, HRP-conjugated anti-rabbit Ig, AP-conjugated anti-mouse Ig, and AP-conjugated anti-rabbit Ig (Dako, Glostrup, Denmark);

- TNF-α (BioSource, Camarillo, CA, USA); and

- Nuclear extract kit, TransAM™ NF-κB p65 Kit, TransAM™ Nrf2 Kit (ActiveMotif, Carlsbad, CA, USA).

3.2. Mouse Model

Female BALB/c mice of 6 to 8 weeks old (Animal Resources Centre, Canning Vale, Western Australia, Australia) were maintained in the animal housing unit on a 12-hours light-dark cycle with food and water available *ad libitum*. The temperature in the animal room ranged from 18°C to 26°C with an average temperature of 22°C. Maintenance diets generally contain 4-5% fat and 14 % protein. Generally a minimum of 3 days of acclimatization is required for mice to adapt to their new surroundings. Animal experiments were performed

according to the Institutional guidelines for Animal Care and Use Committee of the National University of Singapore.

3.2.1. Asthma mouse model and DDAG treatment protocol

BALB/c mice, were sensitized and challenged with ovalbumin (OVA) as described previously (Bao *et al.*, 2009). Briefly, mice were sensitized by intraperitoneal (i.p.) injections of 20 μg OVA and 4 mg $Al(OH)_3$ as adjuvant suspended in 0.1 ml saline on day 0 and 14. Aluminium hydroxide is the most commonly used adjuvant as it primes the mice immune response towards a Th2 phenotype. Following this, the mice were challenged with 1% OVA aerosol for 30 minutes administer by DeVilbiss Ultra-Neb Large-Volume Ultrasonic Nebulizer (Sunrise Medical Respiratory Products, Somerset, PA) as shown in figure 3.1 on day 22, 23 and 24. DDAG (0.1, 0.5, and 1 mg/kg) or vehicle (1% DMSO) in 0.1 ml saline was given by i.p. injections 2 hours before and 10 hours after each OVA aerosol challenge. Saline aerosol was used as a negative control. To ensure the strict hygiene standard of the cages' environment, we changed the cage beddings three times a week.

Figure 3.1. Allergen aerosol delivery system. The ultrasonic nebulizer was used to aerosolize 1% of OVA saline solution and pump the aerosol mist into an adjacent chamber where the aerosolized solution was inhaled into the airways of the mice. Up to 8 mice can be put into the chamber during OVA or saline challenge.

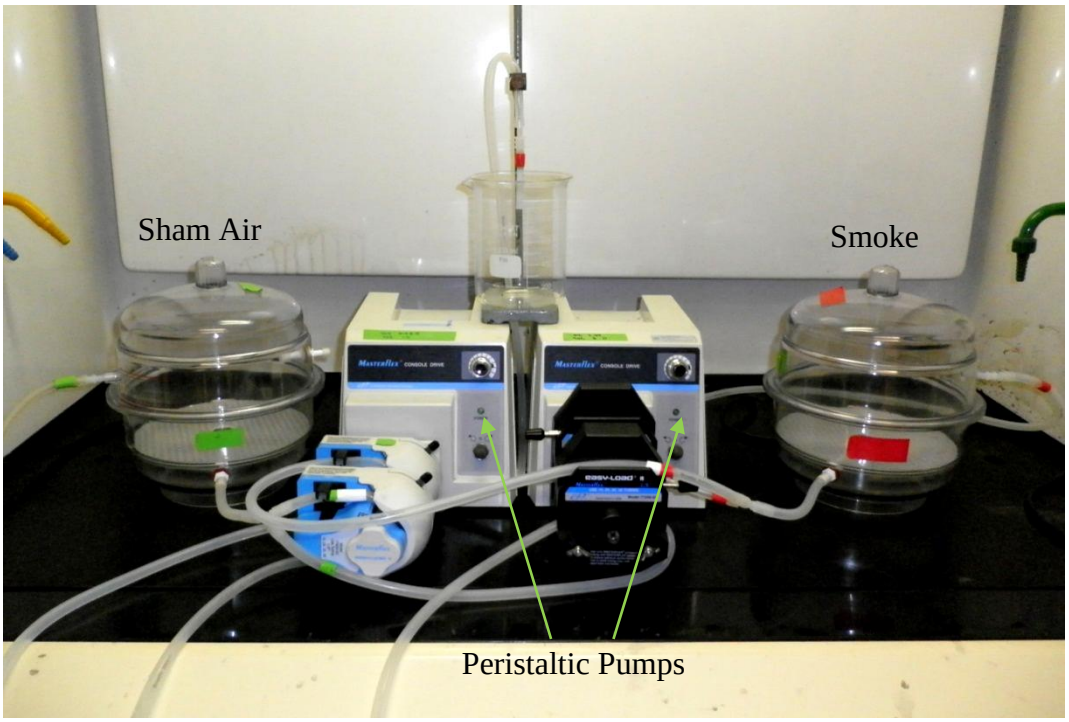


Figure 3.2. Cigarette smoke and Sham Air delivery system. Peristaltic pump are used to deliver room air or cigarette smoke to the chamber. Both sham air and cigarette smoke chamber receive a constant flow of fresh air and have an exhaust tubing to keep the chamber ventilated. Additionally, the smoke chamber receives a constant flow of cigarette smoke while the sham air chamber receives the similar flow of room air.

3.2.2. Cigarette smoke-induced lung injury and andrographolide treatment protocol

Mice were placed in a ventilated chamber filled with 4% cigarette smoke delivered by a peristaltic pump (Masterflex, Cole-Parmer Instrument Co., Niles, IL, USA) at a constant rate of 1 L/min as shown in figure 3.2 according to methods described by Chan et al. (Chan *et al.*, 2009). Total suspended particulate of 4% cigarette smoke was 493.5±49.6 mg/m^3 (n=4) recorded using the MicroDust Pro-aerosol monitor (Casella CEL, Bedford, UK). To develop cigarette smoke-induced lung injury, mice were exposed to 10 sticks of 3R4F research cigarettes (Tobacco and Health Research Institute, University of Kentucky, Lexington, KY, USA) over a period of 60 minutes a day for 5 consecutive days (Braber *et al.*, 2011). Mice in the sham air group were simultaneously placed in another ventilated chamber but exposed to fresh air. Andrographolide (0.1, 0.5, and 1 mg/kg; Sigma, St. Louis, MO, USA) or vehicle (1% DMSO) in 0.1 mL saline was given by intraperitoneal injection 2 hours before each cigarette smoke exposure as described by Bao *et al*. (Bao *et al.*, 2009). Mice were sacrificed 24 hours after the last cigarette smoke or sham air exposure, and lung samples were collected for various analyses.

3.3. Collection of bronchoalveolar lavage (BAL) fluid from mice

BAL fluid was collected 24 hours after the last saline or OVA challenge as previously described (Bao *et al.*, 2009) for the asthma mouse model or 24 hours after the last cigarette smoke exposure for the cigarette smoke induce lung injury model. Mice were anaesthetized by an i.p. injection of 300 µl of an anaesthetic mixture (ketamine: medetomidine: H2O = 3: 4: 33, Parnell, Alexandria NSW, Australia & Pfizer, Auckland, New Zealand). Tracheotomy was performed by inserting a blunt needle (20G) into the trachea. Ice-cold PBS (0.5 ml × 3) was instilled into the lungs and a final volume of 1.2~1.3 ml of BAL fluid was retrieved from the lungs. BAL fluid total and differential cell counts were determined immediately after BAL

fluid extraction. BAL fluid supernatants were stored at −80 °C for cytokine, chemokine levels and oxidative lung damage markers analysis.

3.4. Total and differential BAL fluid cell counts

The BAL fluid was centrifuged at 3000 rpm for 5 minutes at 4°C. Supernatant was collected and stored at -80°C until further experiments. The pellet was resuspended in 200 µl of 8.5 mg/ml NH_4Cl for 5 min at room temperature to remove red blood cells. The cell suspension was centrifuged at 3000 rpm for 5 min at 4°C and the supernatant was discarded. Subsequently, the cell pellet was resuspended in 200 µl of RPMI supplemented with 10 mg/ml BSA. A total number of viable cells was enumerated using a haemocytometer (10 µl cell suspension: 10 µl 0.4 % trypan blue) under a microscope (magnification ×200). Following the total cell count, aliquots (10^5 cells/150 µl) of the cell suspension were cytospinned onto a slide in a Shandon Cytospin 3 (Thermo Electron Corporation, Pittsburgh, PA) at 600 rpm for 10 min at room temperature. The BAL fluid cells were stained with a modified Wright staining (Bao *et al.*, 2009). Briefly, cytospin slides were fixed and stained with 800 µl of Liu A for 30 seconds followed by 1600 µl of Liu B for 90 seconds. Differential cell count was then performed on a minimum of 500 leukocytes (magnification ×1000). Four types of inflammatory cells, namely eosinophils, macrophages, neutrophils, and lymphocytes were identified and their respective percentage in the total inflammatory cells was enumerated, based on standard morphological criteria and staining (Figure 3.3 and Figure 3.4). The absolute number of four types of inflammatory cells was calculated by their percentages and total inflammatory cell count. The epithelial cells were excluded in the differential cell count.

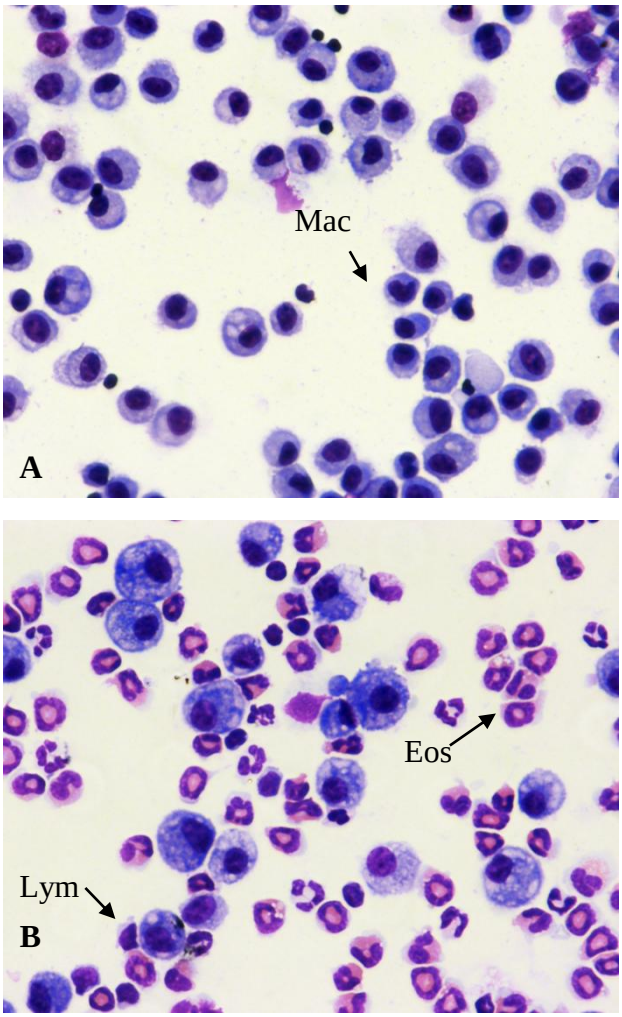


Figure 3.3. Type of cells found in BAL fluid of mice. (A) Control BAL fluid collected from the mouse sensitized with OVA and challenged with saline; (B) BAL fluid collected from the mouse sensitized with OVA and challenged with OVA. Mac, macrophages; Eos, Eosinophil; Lym, lymphocyte; Neu, neutrophil.

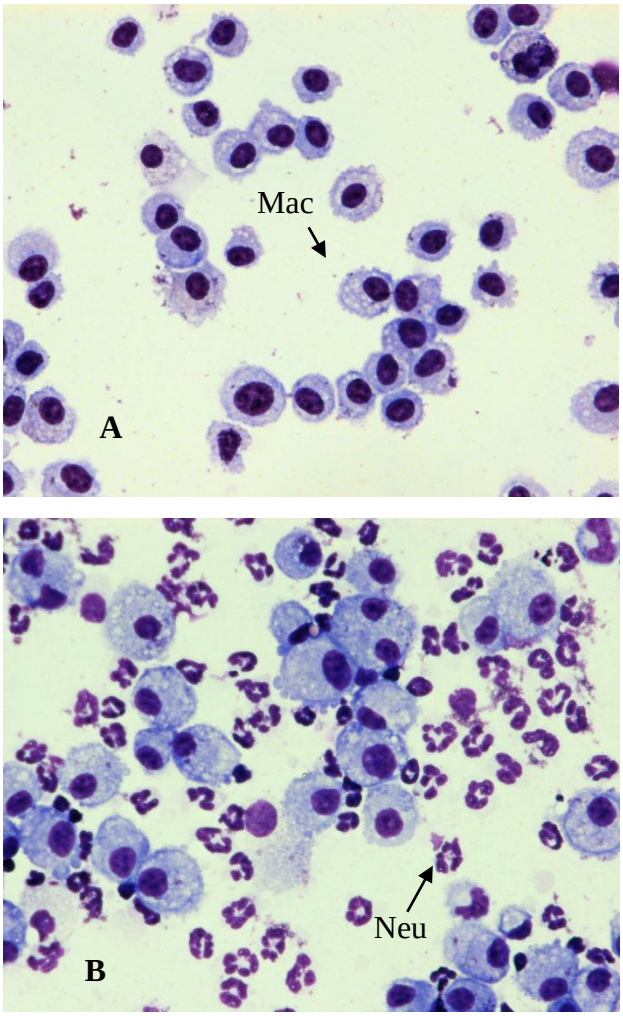


Figure 3.4. Type of cells found in BAL fluid of mice. (A) Control BAL fluid collected from mouse exposed to Sham Air; (B) BAL fluid collected from mouse exposed to cigarette smoke. Mac, macrophages; Eos, Eosinophil; Lym, lymphocyte; Neu, neutrophil.

3.5. Lung total protein extraction

Lungs were isolated from the thoracic cavity 24 hours after the last OVA or saline challenge and stored in -80°C until study. Before the lungs were remove, cardiac puncture carried out after the mice were anaesthetized by an i.p. injection of 300µl of an anesthetic mixture (ketamine: medetomidine: H2O = 3: 4: 33, Parnell, Alexandria NSW, Australia & Pfizer, Auckland, New Zealand). Lung lobes were then cut into small pieces using scissors and homogenized in ice-cold M-PER Mammalian Protein Extraction Reagent containing HALT protease inhibitor cocktail (Pierce, Rockford, IL, USA) with a homogenizer (SilentCrusher M, Heidolph Elektro GmbH & Co, Kelheim, Genman). Lysates were then incubated on ice for 30 minutes before centrifugation (18,000g for 5 minutes); supernatants were collected, and protein concentrations were determined using a BCA protein assay kit (Thermo Fisher Scientific Inc, Waltham, MA ,USA).

3.6. ELISA

3.6.1. Cytokines and chemokine levels in BAL fluid

For the asthma mouse model, levels of IL-4, IL-5, IL-13 and eotaxin in the supernatant of the BAL fluid were determined by ELISA according to the manufacturer's instructions. IL-4 and IL-5 ELISA were obtained from BD PharMingen (San Diego, CA). Eotaxin and IL-13 were purchased from R&D Systems (Minneapolis, MN). For the cigarette smoke model, BAL fluid levels of keratinocyte chemoattractant (KC/CXCL1), interferon-γ-inducible protein 10 (IP-10/CXCL10), monocyte chemoattractant protein-1 (MCP-1/CCL2) and IL-1β were measured using ELISA R&D Systems (Minneapolis, MN) according to the manufacturer's instructions. Briefly, 50 µl of diluted capture antibody (diluted to the appropriated concentration in relevant coating buffer) was coated to each well of an ELISA

plate (NUNC, Denmark). The plate was sealed with parafilm and incubated overnight at 4°C. Next day, the coating buffer was aspirated and the plate was washed with wash buffer (PBS with 0.05% Tween-20). Following the last wash, any remaining wash buffer was removed by inverting the plate and blotting it against clean paper towels. Ensuing washing, the plate was blocked with 300 µl PBS with 10% FBS for 2 hours. Following blocking, 50 µl of standards or BAL fluid samples were added into each well accordingly and incubated for 2 hours at room temperature. Next, after washing away the unbound samples and standards, the plate was incubated with biotinylated-detection antibody plus HRP for 1 hour (BD OptEIA™ Kit) or with biotinylated-detection antibody for 1 hour followed by HRP for 45 minutes (R&D). Subsequently, after washing, 50 µl of a substrate solution was added to each well, and incubated for 30 min in the dark, followed by addition of 50 µl stopping solution (1M H_2SO_4). Finally, the optical density of each well in the plate was read at 450 nm with λ correction at 570 nm within 30 min. The detection limits for the cytokines and chemokine used are as follows: 4 pg/ml for IL-4; 4 pg/ml for IL-5; 15.6 pg/ml for IL-13; and 2 pg/ml for eotaxin. 9 pg/ml for IL-1β; 15 pg/ml for IP-10; and 16 pg/ml for both MCP-1 and KC.

3.6.2. Oxidative damage marker level in BAL fluid

BAL fluid levels of oxidative damage markers were measured using competitive ELISA kit for 3-nitrotyrosine (3-NT) (Upstate/Millipore, Billerica, MA, USA), in addition to 8-isoprostane (8-iso-$PGF_{2\alpha}$) and 8-hydroxy-2-deoxy Guanosine (8-OHdG) (Cayman Chemicals, Ann Arbor, MI, USA) according to the manufacturers' instructions (Ho *et al.*, 2012). This assay was based on competition between the samples' oxidative damage markers, the antigen (8-isoprostane, 8-OHdG and 3-NT) and the supplied competing antigen (8-isoprostane-acetylcholinesterase conjugate/8-isoprotane tracer, 8-OHdG-acetylcholinesterase conjugate/8-OHdG tracer and nitrated BSA) for a limited number of specific primary

antibodies. Both 8-isoprostane and 8-OHdG kits come with secondary antibody pre-coated plate and enzyme link antigen (8-isoprostane Tracer, 8-OH-dG Tracer respectively) while the 3-NT kits comes with the enzyme link secondary antibody and antigen (nitrated BSA) for well coating. Briefly, for the 3-NT assay, 96-well plates were coated with 5 μg/mL nitrated bovine serum albumin (BSA) overnight, and blocked with blocking buffer for 2 hours. 50 μl of BALF sample or standard samples and 50 μl of 2×anti-nitrotyrosine were added to each well. Subsequently, the plates were incubated at 37 °C for 60 min. After un-bound antibody were remove by washing, 100 μl per well of enzyme link secondary antibody (1× anti-rabbit IgG, HRP-conjugate) was added and incubated at 37 °C for 60 min. For the 8-isoprostane and 8-OHdG assay, a 50 μl BALF sample or standard, 50 μl competing enzyme link antigen and the primary antibody (8-OHdG monoclonal antibody and 8-Isoprostane antiserum) were added to each pre-coated well, incubating for 14 hours at 4°C. For the entire assays, the plates were washed between each step. Following competitive binding, substrate solution (Ellman's reagent for 8-isoprostane and 8-OH-dG, LumiGlo Chemiluminescent® Substrate for Nitrotyrosine) were added into each well in the dark. The product of the enzymatic reaction for 8-isoprostane and 8-OH-dG assay produces a distinct yellow colour which was determined spectrophotometrically at wavelength between 405-420 nm. While the products for the enzymatic reaction for 3-NT was measured with the luminescence as relative light units (RLU). Luminescence was measured using a microplate reader and the background luminescence subtracted from the values. The levels were calculated with the standard curve and expressed as picograms per milliliter of BAL fluid (8-isoprostane, 8-OH-dG) or nanomolar per milliliter of BAL fluid (Nitrotyrosine).

3.6.3. Immunoglobulin levels in serum

For the asthma mouse model, blood was collected from the mouse through cardiac puncture, and allowed to clot by leaving at room temperature for 3 hours. The clear supernatant was collected as the serum, and stored at -80°C until further experiments. Serum levels of total IgE, OVA-specific IgE, OVA-specific IgG1 and OVA-specific IgG2a were assayed by ELISA. Briefly, ELISA plate was incubated at 4°C overnight with coating buffer containing either 20 µg/ml OVA (for OVA-specific IgE, OVA-specific IgG1 and OVA-specific IgG2a) or IgE capture antibody (for total IgE measurement). The following day, the plate was blocked with 10% FBS in 300 µl PBS for 2 hours at room temperature. Following blocking, total IgE standards or diluted serum samples were added into the respective wells and incubated for 2 hours. Next, the respective detection antibodies were added and the mixtures were incubated for 1 hour. After that, HRP-conjugated antibody was added and incubated for 45 min, followed by substrate solution for 30 minutes in the dark. Finally, the optical density of each well was read at 450 nm with λ correction at 570 nm within 30 minutes of adding stopping solution. The detection limit for total IgE is 2 ng/ml.

3.6.4. Cotinine Measurement

For the cigarette smoke model, cotinine is the major metabolite of nicotine (Benowitz, 2009). Plasma cotinine levels was measured as a marker of cigarette smoking. Immediately after sham air or cigarette smoke exposure, blood was drawn and the blood samples were centrifuged for 15 minutes at 5,000 g at 4° C. The resultant plasma cotinine levels were measured by ELISA (Bio-Quant, San Diego, CA, USA) according to the manufacturer's instructions. Absorbance was measured at 450 nm in a microplate reader.

3.7. Measurements of airway hyperresponsiveness (AHR)

Mice were anesthetized and tracheotomy was performed as described (Bao *et al.*, 2009). The animal was intubated with a cannula that is connected to a multiport that leads to the pneumotach, ventilator and nebulizer within the FinePointe Series RC Sites (Buxco Research System, Wilmington, NC). The mouse was ventilated at a fixed breathing rate of 140 breaths/min and the lung resistance (Rl) and dynamic compliance (Cdyn) in response to

Figure 3.5. The FinePointe™ system (Buxco Research Systems, Wilmington, NC, United States). The system measures the resistance and compliance of mouse in response to aerosolized methacholine. The system comes with in-line aerosol, ventilator, and heated bed.

increasing concentrations of nebulized methacholine (0.5– 8.0 mg/ml) were recorded using FinePointe data acquisition and analysis software (Buxco Research System). The methacholine used was dissolved in PBS and administered through the system nebulizer at a volume of 10µl per aerosol. PBS is nebulised before and after methacholine administration to serve as a baseline and to clean up the nebulizer. A different nebulizer was used for different animal and the system was air dried before proceeding to the next animal. Results are expressed as a percentage of the respective basal values in response to PBS.

3.8. Histology

Histological studies were performed for the allergic mouse model studies. Essentially, lungs were isolated from the thoracic cavity 24 hours after the last OVA or saline challenge after the injection with a lethal dose of anaesthesia (300µl of an anaesthesia mixture) and after cardiac puncture was performed, fixed in 10% neutral buffered formalin solution for at least 48 hours, and processed in a tissue processor (Leica Microsystems, Wetzler, Germany).

Briefly, lungs were dehydrated in a series of ethanol mixtures (70% to 80% to 90% to 100%, 30 minutes each and 2 hours for 100%), and immersed in xylene for 10.5 hours. Lungs were infiltrated with hot paraffin for 3 hours and embedded in paraffin wax. The specimens were then cut into 5 µm sections using a microtome (Leica Microsystems, Wetzler, Germany) and fixed on slides. For H&E staining, slides were deparaffinized with HistoClear for 10 min and rehydrated in a series of ethanol to water mixtures (100% to 90% to 70% to water and each for 2 minutes). The sections were then stained with Harris haematoxylin for 5 minutes, washed in the distilled water, and differentiated in 0.1% acid alcohol solution for 30 seconds. The sections were washed with tap water for 5 minutes and counter stained with Eosin for 1 minute. Subsequently, the sections were dehydrated in a series of ethanol solutions (70 % to 90% to 100% and each for 30 seconds) and immersed in HistoClear for 10 minutes.

Evaluation of inflammation around peribronchial and perivascular areas was semi-quantitatively performed in a blind manner as previously described (Myou *et al.*, 2003). A subjective scale (0 - 4) was assigned as follows: 0: no inflammatory cells; 1: occasional cuffing with few inflammatory cells; 2: most bronchi or vessels surrounded by a thin layer of inflammatory cells; 3: most bronchi or vessels surrounded by a thick layer (2 - 4 cells layer deep) of inflammatory cells; 4: most of bronchi or vessels surrounded by a thicker layer (more than 4 cells layer deep) of inflammatory cells. Likewise, for periodic acid Schiff (PAS) staining, slides were deparaffinized with HistoClear for 10 minutes and rehydrated in a series of ethanol to water mixtures (100% to 90% to 70% to water and each for 2 minutes). Subsequently, the sections were sequentially immersed in the periodic acid (5 minutes), water (5 minutes), and Schiff`s reagent (15 minutes). The sections were washed with tap water for 5 minutes and stained in Gill Haematoxylin for 90 seconds. Next, the sections were dehydrated in a series of ethanol mixtures (70% to 90% to 100% and each for 2 minutes) and cleared with HistoClear for 10 minutes. Evaluation of mucus production or goblet cell hyperplasia was semi-quantitatively performed in a blind manner as previously described (Grunig *et al.*, 1998). According to the percentage of PAS-positive mucin-producing cells in the epithelium, scores (0 - 4) were assigned as follows: 0, no goblet cells; 1, < 25%; 2, 25–50%; 3, 50–75%; 4, >75%. Lung tissue mast cells were stained using toluidine-blue, and the number of intact and degranulating mast cells was counted in the entire lung sections as described (Pushparaj *et al.*, 2009). Briefly, toluidine-blue staining for lung mast cells were perfomed by deparaffinized paraffin sections slides with HistoClear for 10 minutes and rehydrated in a series of ethanol to water mixtures (100% to 90% to 70% to water and each for 2 minutes). Next, the sections were rinsed in water and immersed in the toluidine-blue stains for 2 minutes. The sections were washed with tap water for 10 minutes and dehydrated in a series of ethanol mixtures (70% to 90% to 100% and each for 2 minutes) and cleared with

HistoClear for 10 minutes. Mast cells were identified as those cells that contained toluidine blue–positive granules. For both H&E and PAS staining, bronchioles with the maximum internal diameter two times greater than the minimum internal diameter were not used for analysis. The scoring of inflammatory, goblet cells as well as intact and degranulating mast cells was performed in 2 - 4 preparations of each mouse and mean scores were calculated from 4 - 5 mice.

3.9. Cell cultures

A549 human lung epithelial cells, BEAS-2B human transformed lung epithelial cells, and RBL-2H3 rat basophilic leukemia cells were obtained from the American Type Tissue Collection (American Type Tissue Collection, Rockville, MD, USA). A549 and BEAS-2B cells were cultured in RPMI 1640, while RBL-2H3 cells were grown in DMEM. Both media were supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100μg/mL), and cells were grown at 37°C in a humidified 5% CO_2 incubator.

3.9.1. Cell viability assay

A549 cells (3×10^4/well), BEAS-2B cells (5×10^4/well) and RBL-2H3 cells (3×10^4/well) were seeded on flat-bottomed 96-well plates for overnight, and then incubated with increasing concentrations (3–120 μM) of DDAG or andrographolide for 24 hours and 48 hours at 37°C. Both DDAG and andrographolide was dissolved in DMSO. Cell viability was analyzed using the CellTiter 96® AQ_{ueous} non-radioactive cell proliferation assay (Promega, Madison, WI) according to the manufacturer's protocol. This MTS assay is based on the ability of viable cells to convert a soluble tetrazolium salt to a colour formazan product. Absorbance was

recorded at 490 nm (Tecan microplate reader infinite M200; Mannedorf, Switzerland) and equal volume of DMSO treated cells were used as control.

3.9.2. In Vitro Inflammation model

To explore the anti-inflammatory mechanism of action of DDAG, A549 cells (1×10^6 cells) were seeded in a 100 mm Petri dish, and at 90% confluence, cells were treated with 30μM DDAG or vehicle (0.1% DMSO) for 4 h before stimulation with 10 ng/mL TNF-α for 30 min and protein lysates were prepared for analysis.

3.9.3. In vitro cigarette smoke exposure model

Cigarette Smoke Extract (CSE) was freshly prepared immediately before each experiment by bubbling smoke from one cigarette to 10 mL of RPMI 1640 medium supplemented with 1% FBS at a rate of one cigarette every 10 min as described (Kode *et al.*, 2008). CSE was adjusted to pH 7.4 and sterile-filtered through a 0.45-μm Acrodisc filter (Pall, Ann Arbor, MI, USA). CSE preparation was standardized by setting the optical density at 0.8 ±0.05 with absorbance wavelength at 340 mm. Control medium was prepared in the same way as CSE except for bubbling fresh air into 10 mL of RPMI medium. BEAS-2B were cultured in RPMI 1640 supplemented with 1% FBS. Cells were pre-incubated with a non-toxic concentration of 30 μM andrographolide (Bao *et al.*, 2009) or vehicle (0.05% DMSO) for 1 h before exposure to 2% CSE for indicated times to explore the anti-oxidants mechanism of action of andrographolide in cigarette smoke extract exposed medium. Using the MTS assay, 2% CSE had no adverse effect on the viability of BEAS-2B cells.

3.10. Nuclear Protein extraction

For the allergic inflammation model, nuclear proteins were extracted from lung tissues and A549 cells using a Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA). Using the same kit, for the cigarette smoke exposure model, cytoplasmic and nuclear extractions were performed according to manufacturers' instructions. Briefly, the cells are firstly collected in ice-cold PBS in the presence of phosphatase inhibitors to limit further protein modifications such as proteolysis, and dephosphorylation. Subsequently, the cells are resuspended in hypotonic buffer to swell the cell membrane and make it fragile. Detergent was then added to cause the leakage of the cytoplasmic proteins into the supernatant. After collection of the cytoplasmic fraction, the nuclei are lysed and the nuclear proteins are solubilized in the lysis buffer in the presence of the protease inhibitor cocktail. The extracted proteins were used for immunoblotting studies or transcription factor transactivation assay.

3.10.1. NF-κB and Nrf2 DNA-transactivation Assay

For the allergic inflammation model, nuclear proteins were also analyzed for NF-κB DNA-binding activity using the TransAM™ NF-κB p65 transcription factor assay kit (Active Motif). For the cigarette smoke exposure model, nuclear extracts were used for measuring Nrf2 binding activity to immobilized antioxidant response elements (ARE) using a TransAM™ Nrf2 kit (Active Motif). Briefly, both of this kit contains a 96-well plate with immobilized oligonucleotide containing either the NF-κB consensus binding sequence (5'-GGGACTTTCC-3') or ARE consensus binding site (5'-GTCACAGTGACTCAGCAGAATCTG-3') which the active form of Nrf2 contained in the nuclear extract will specifically bind to. Nuclear extracts (5 µg) were incubated in the wells for 1 hour and anti-p65 primary antibodies or anti-Nrf2 antibodies were then added into the

wells following washing. A HRP-conjugated secondary antibody was subsequently added to detect the bound primary antibody. Developing solution and stopping solution were used to develop a colorimetric reaction. The absorbance was measured at 450 nm with a reference wavelength of 655 nm by a microplate reader (Tecan microplate reader infinite M200; Mannedorf, Switzerland). In addition, the wild-type or mutated NF-κB or Nrf2 consensus oligonucleotide was added into the reactions as the competitive or mutated competitive control to monitor the specificity of the assay.

3.11. Immunoblotting

For the NF-κB p65 nuclear translocation studies, lung and cell culture nuclear proteins were utilized while for Nrf2 nuclear translocation studies, both cell culture cytoplasmic and nuclear proteins were used for immunoblotting studies. A similar amount of protein (30 µg) mixed with sample buffer were separated by 10% SDS-PAGE in a Trans-Blot tank (Bio-Rad Laboratories, Hercules, CA, USA) and transferred to a PVDF membrane using a semi-try transblotter (ATTO Corp, Tokyo, Japan). The PVDF membrane was blocked with 5% skim milk powder (Fluka, Sigma) in Tween-20 Tris buffered saline (TTBS) for 2 hours and probed overnight at 4°C with either with anti-p65 (Cell Signaling Technology, Beverly, MA) for NF-κB p65 nuclear translocation studies or with rabbit anti-Nrf2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies for Nrf2 nuclear translocation studies. The PVDF was subsequently washed with TTBS for 10 times (2 - 3 min each), and incubated in HRP-conjugated anti-rabbit or anti-mouse antibodies for two hours before being developed on hyperfilms using an ECL reagent. After washing several times in TTBS, ECL reagent was removed with western blot stripping buffer. Subsequently, the PVDF was blocked again, reprobed with anti-TATA box binding protein (TBP) (Abcam, Cambridge, UK) or mouse anti-β-actin (Cell Signaling Technology, Beverly, MA) and developed using an AP substrate

kit. Band intensity was quantitated using ImageJ software (NIH) as described previously (Goh *et al.*, 2012).

3.12. RNA extraction and Reverse Transcription

Lungs were isolated from the thoracic cavity 24 hours after the last OVA or saline challenge for the allergic mouse model as well as 24 hours after the last cigarette smoke exposure for the cigarette smoke lung injury model. The isolated lung are immediately stored in RNAlater at -80°C. Before RNA isolation, lung tissues were removed from the RNAlater with sterile forceps, immersed in 1 ml Trizol (Invitrogen, Carlsbad, CA, USA), and homogenized on ice using the homogenizer. Next, RNA isolation was performed using Trizol according to the instructions from Invitrogen. Similarly, for Nrf2 transactivation in vitro studies, mRNA is extracted at the mentioned time point using Trizol following manufacturer instructions. After dissolving the isolated RNA in DEPC water, spectrophotometric measurements was perform using NanoDrop ND-1000 (Thermo Fisher Scientific Inc, Waltham, MA, USA) to quantify the amount and purity of the RNA present in the sample. Both A260/A280 (DNA/protein) and A260/A230 (DNA/organic contaminants) ratio were recorded as an indicator of purity of RNA. An acceptable level of purity for RNA extracts should be around 1.9 to 2.1 for both A260/A280 and A260/A230 ratio. Subsequently, cDNA was synthesized from 1 µg of isolated RNA by a random primer and AMV reverse transcriptase using a multi-well thermal cycler (GeneAmp PCR system 2700, Applied Biosystems, Foster City, CA, USA).

3.13. Polymerase Chain Reaction (PCR)

For the allergic mouse model studies, PCR amplifications were then performed on 1 µl of cDNA template in a 25 µl reaction volume containing 12.5 µl of 2×PCR master mix (50 units/ml TaqDNA polymerase, 400 µM dATP, 400 µM dGTP, 400 µMdCTP, 400 µM dTTP, and 3 mM MgCl2), 9.5 µl nuclease-free water, 1 µl forward primer (10 µM) and 1 µl reverse primer (10 µM) using the multiwell thermal cycler. Primers used in the PCR reactions are listed in Table 3.1. PCR products were separated by electrophoresis on 2% agarose gels stained with ethidium bromide (100 V, 30 min) and were visualized under an ultraviolet transilluminator. Furthermore, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene to normalize variations between sample loadings. For the cigarette smoke exposed mouse and Beas-2B cells studies, quantitative real time PCR were perform to quantify the gene expression profile. Template cDNA (100ng) in PCR mixture containing Fast SYBR Green Master Mix (Applied Biosystems, Foster City, California, USA) with a total reaction mixture volume of 20 µl were added in MicroAmp optical 96-well reaction plates. Plates were sealed, centrifuged, and then subjected to amplification. Quantitative real time PCR was performed using a sequence detection system (ABI 7500 Cycler; Applied Biosystems, Foster City, CA). The PCR protocol consisted of 2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. All measurements were done in triplicate. The primers for inflammatory and antioxidant genes are listed in Table 3.2. The mRNA expression levels for all samples were normalized to the level of the housekeeping gene 18S. Following amplification, melting curve analysis was performed to determine the specificity of the amplified products. Data acquisition and analysis were performed using the ABI 7500 software, version 2.0.5 (Applied Biosystems).

Table 3.1. Primer sets for RT-PCR analysis

Target (Accession ID)		Primer Sequence	No. of Cycle	Length (bp)	Reference
AMCase (gi|37999744)	Forward Reverse	5'-TGGGTTCTGGGCCTACTATG-3' 5'-GCTTGACAATGCTGCTGGTA-3'	32	483	(Zhao *et al.*, 2005)
Ym1 (gi|285015)	Forward Reverse	5'-CTGGAATTGGTGCCCCTACA-3' 5'-CAAGCATGGTGGTTTTACAGGA-3'	32	624	(Zhao *et al.*, 2005)
Ym2 (gi|22123907)	Forward Reverse	5'-CAGAACCGTCAGACATTCATTA-3' 5'-ATGGTCCTTCCAGTAGGTAATA-3'	32	429	(Zhao *et al.*, 2005)
YKL-40 (gi|142347793)	Forward Reverse	5'-GTACAAGCTGGTCTGCTACT-3' 5'-GTTGGAGGCAATCTCGGAAA-3'	30	277	(Bao *et al.*, 2009)
E-selectin (gi|118130193)	Forward Reverse	5'-AACGCCAGAACAACAATTCC-3' 5'-TGAATTGCCACCAGATGTGT-3'	35	227	Primer 3
COX-2 (gi|118130137)	Forward Reverse	5'-GGAGAGACTATCAAGATAGT-3' 5'-ATGGTCAGTAGACTTTTACA-3'	40	861	(Jung *et al.*, 2007)
Muc5ac (gi|114431223)	Forward Reverse	5'-GAGTGACATTGCAGGAAGCA-3' 5'-CAGAGGACAGGAAGGTGAGC-3'	38	361	Primer 3
IL-17A (gi|142367609)	Forward Reverse	5'-CCGCAATGAAGACCCTGATAGA-3' 5'-CAGCATCTTCTCGACCCTGAAA-3'	35	187	(Egan *et al.*, 2008)
IL-33 (gi|257900494)	Forward Reverse	5'-TCCTTGCTTGGCAGTATCCA-3' 5'-TGCTCAATGTGTCAACAGACG-3'	35	52	(Verri *et al.*, 2008)
VCAM-1 (gi|31981429)	Forward Reverse	5'-CAAGGGTGACCAGCTCATGAA-3' 5'-TGTGCAGCCACCTGAGATCC-3'	30	518	(Bao *et al.*, 2009)
GADPH (gi|126012538)	Forward Reverse	5'- AGGTCGGTGTGAACGGATTTG-3' 5'-GGGGTCGTTGATGGCAACA-3'	30	95	(Lampton *et al.*, 2008)

Table 3.2. Primer sets for real time-PCR analysis

Target (Accession ID)		Primer Sequence	Length (bp)	Reference
MMP-12 (gi|115392137)	Forward Reverse	5'-TTTCTTCCATATGGCCAAGC-3' 5'-GGTCAAAGACAGCTGCATCA-3'	198	(Deguchi *et al.*, 2005)
TIMP-1 (gi|113205064)	Forward Reverse	5'-GTGGGAAATGCCGCAGAT-3' 5'-GGGCATATCCACAGAGGCTTT-3'	67	(Manoury *et al.*, 2006)
GM-CSF (gi|145301581)	Forward Reverse	5'-GGGCGCCTTGAACATGAC-3' 5'-TTGTGTTTCACAGTCCGTTTCC-3'	76	(Wuthrich *et al.*, 2005)
TNF-α (gi|133892368)	Forward Reverse	5'-TCGAGTGACAAGCCCGTAGC-3' 5'- CTCAGCCACTCCAGCTGCTC-3'	68	(Mashreghi *et al.*, 2008)
MIP-2α (gi|118130527)	Forward Reverse	5'-AGTGAACTGCGCTGTCAATGC-3' 5'-AGGCAAACTTTTTGACCGCC-3'	143	(Hu *et al.*, 2004)
iNOS (gi|146134510)	Forward Reverse	5'-CGGGCAAACATCACATTCAGATCCCG-3' 5'-TATATTGCTGTGGCTCCCATGTT-3'	69	(Boyer *et al.*, 2011)
GCLM (gi|53759142)	Forward Reverse	5'-AATCAACCCAGATTTGGTCAGG-3' 5'-GAGATACAGTGCATTCCAAGACA-3'	56	Primer Bank
GCLC (gi|308199422)	Forward Reverse	5'-GGAGGAAACCAAGCGCCAT-3' 5'-CTTGACGGCGTGGTAGATGT-3'	79	Primer Bank
GR (gi|305410788)	Forward Reverse	5'-CACTTGCGTGAATGTTGGATG-3' 5'-TGGGATCACTCGTGAAGGCT-3'	242	Primer Bank
GPx-2 (gi|375163496)	Forward Reverse	5'-GGTAGATTTCAATACGTTCCGGG-3' 5'-AGCCACATTCTCAATCAGCAC-3'	52	Primer Bank
HO-1 (gi|298676487)	Forward Reverse	5'-GCAGAGGGTGATAGAAGAGGC-3' 5'-GATGTTGAGCAGGAACGCAGT-3'	46	Primer Bank
18S (gi| 225637497)	Forward Reverse	5'-GCCGCTAGAGGTGAAATTCTTG-3' 5'-CATTCTTGGCAAATGCTTTCG-3'	66	(Medicherla *et al.*, 2008)

3.14. Biochemical Assay Antioxidant Activities

3.14.1. Antioxidant Activities in Lung Tissue

Frozen lung tissues were homogenized in PBS and used for assessment of the activities of catalase, superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR) using specific biochemical assays, according to the manufacturer's instruction (Cayman Chemical, Ann Arbor, Michigan, USA). Catalase activity was determined based on the reaction of the enzyme with methanol in presence of an optimal concentration of hydrogen peroxide. The formaldehyde produced is measured spectrophotometrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole as the chromogen. The catalase activity was expressed as nmol/min/mg of protein in the sample. The quantification of SOD activity was determined by using tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. The SOD assay measures all three types of SOD (Cu/Zn, Mn, and FeSOD). One unit of superoxide is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. The glutathione peroxidase (GPx) activity was examined spectrophotometrically in lung through an indirect couple reaction with glutathione reductase. Oxidized glutathione, produced on reduction of hydroperoxide by GPx, is recycled to its reduced state by glutathione reductase and NADP reduced. The oxidation of NADP reduced to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm. Under conditions in which the GPx activity is rate limiting, the rate of decrease in the A_{340} is directly proportional to the GPx activity in the sample. The GR activity was monitor by measuring the rate of NADPH oxidation. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340nm and is directly proportional to the GR activity in the lung sample.

3.14.2. Glutathione Assay

BEAS-2B cells were stimulated with CSE for 24 hours as describe above. Glutathione (GSH) levels were determined using glutathione assay kit (Cayman Chemical, Ann Arbor, MI) according to manufacturer instructions. Briefly, BEAS-2B cells were harvested by gently scraping the culture dish and centrifuged. The cells are subsequently resuspended in PBS buffer before sonication and centrifugation to obtain the cell lysate supernatant. 50 µl of supernatant are used for the quantification of GSH. Principally, the kit used glutathione reductase (GR) for the quantification of GSH. The sulfhydryl group of GSH reacts with DTNB and produces a yellow coloured TNB (5-thios-2-nitrobenzoic acid). The mixed disulfide is reduced by GR to recycle the GSH and produce more TNB. Therefore, the rate of TNB production is directly proportional to this recycling reaction which in turn is directly proportional to the concentration of GSH in the sample. The estimation of GSH in the sample is measured by absorbance at 405 nm.

3.15. Statistical analysis

Data are presented as means ± SEM. One-way *ANOVA* followed by Dunnett's test was used to determine significant differences between treatment groups. Significant levels were set at $p<0.05$.

4. ANTI-INFLAMMATORY EFFECTS OF 14-DEOXY-11,12-DIDEHYDROANDROGRAPHOLIDE IN ALLERGIC ASTHMA MOUSE MODEL

4.1. Results

4.1.1. DDAG is less cytotoxic than andrographolide

To determine the cytotoxicity profile of DDAG and andrographolide, both of these compounds were incubated with 3 different cell-lines at two different time points, 24 hours and 48 hours. MTS assays were carried out to determine the viability profile with DMSO as vehicle control for both andrographolide and DDAG. MTS assay are colorimetric assays generally used to determine cytotoxicity of potential medicinal agents as these agents would stimulate or inhibit cell viability and growth. Our present findings reveal that andrographolide dose-dependently and time-dependently reduced the viability of A549 (Figure 4.1) and BEAS-2B human lung epithelial cells (Figure 4.2) and RBL-2H3 mast cells (Figure 4.3). At low dosage, andrographolide were less cytotoxic toward BEAS-2B cells, which is an epithelial virus transformed normal human bronchial epithelium obtained from autopsy of non-cancerous individuals as compare to the tumour cell lines tested, such as A549 and RBL-2H3 cells. At 30 μM, andrographolide treatment at 48 hours reduced the viability of A549 and RBL-2H3 cells to approximately 60% and 40% of control respectively while maintaining the viability of BEAS-2B cells lines of above approximately 90% compared to control at the same concentration of andrographolide treatment. In contrast, DDAG did not reduce cell viability of these cultured cells at all concentrations tested and at both 24 and 48 hours of exposure. On the other hand, even with 48 hours of high dosage of DDAG (120 μM) treatment, all the cell lines tested show an show a viability above approximately 90% as compare to vehicle control. These data suggest that andrographolide treatments are more cytotoxic compared to DDAG treatment especially towards tumour cell lines.

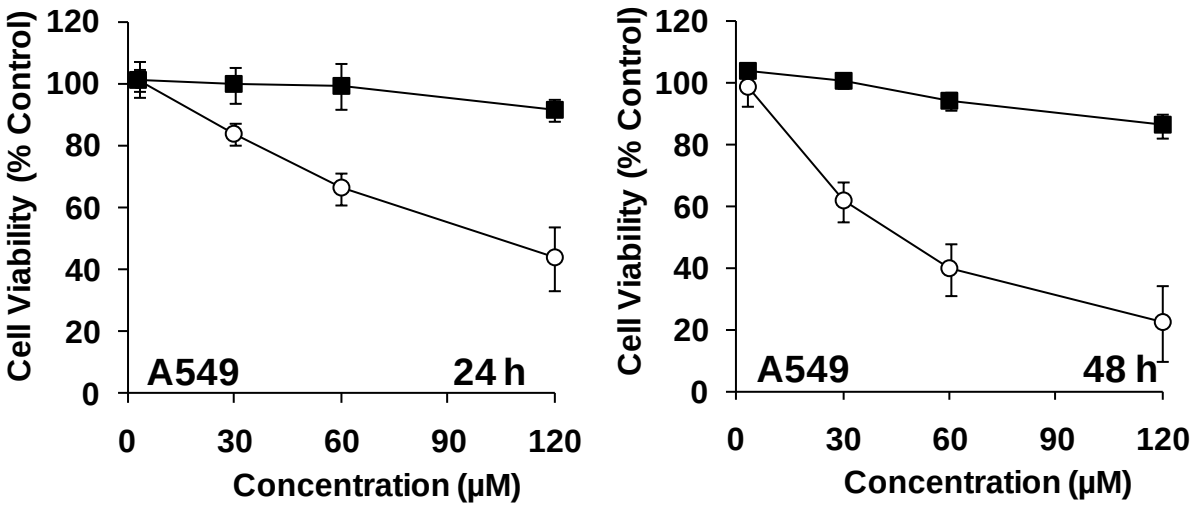


Figure 4.1. Effects of andrographolide (open circles) and 14-deoxy-11,12-didehydroandrographolide (DDAG) (filled squares) on cell viability of A549 cells at 24 and 48 h time intervals. Andrographolide dose-dependently decreased the cell viability of A549 ccells. In contrast, DDAG had no effect on the viability of A549 cells at all concentrations used and both time intervals. Values shown are the means ± SEM of six separate experiments. *Significant difference between andrographolide and DDAG, $p < 0.05$.

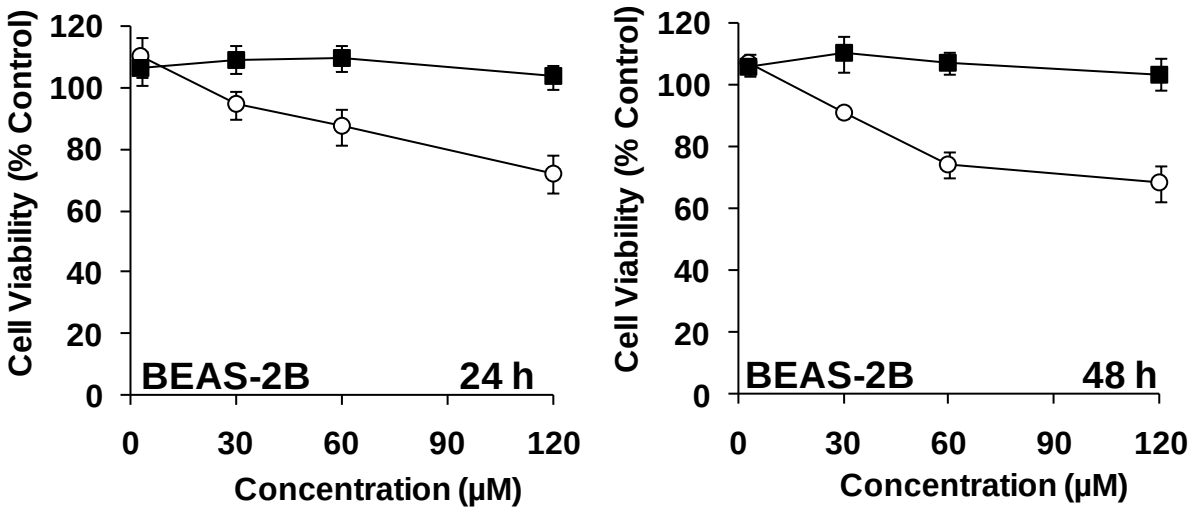


Figure 4.2. Effects of andrographolide (open circles) and 14-deoxy-11,12-didehydroandrographolide (DDAG) (filled squares) on cell viability of BEAS-2B cells at 24 and 48 h time intervals. Andrographolide dose-dependently decreased the cell viability of BEAS-2B cells. In contrast, DDAG had no effect on the viability of BEAS-2B cells at all concentrations used and both time intervals. Values shown are the means ± SEM of six separate experiments. *Significant difference between andrographolide and DDAG, $p < 0.05$.

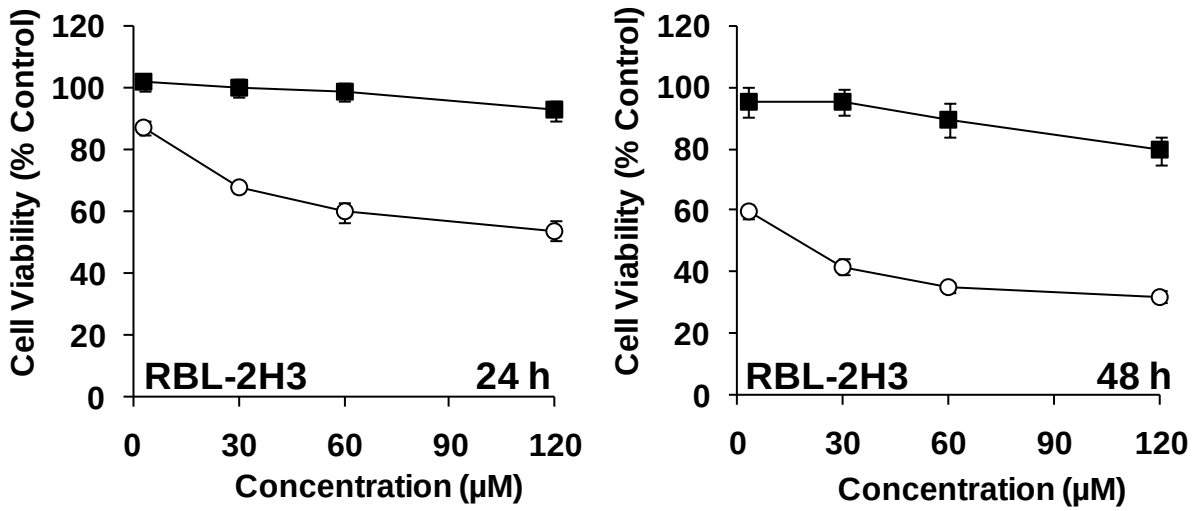


Figure 4.3. Effects of andrographolide (open circles) and 14-deoxy-11,12-didehydroandrographolide (DDAG) (filled squares) on cell viability of RBL-2H3 cells at 24 and 48 h time intervals. Andrographolide dose-dependently decreased the cell viability of RBL-2H3 cells. In contrast, DDAG had no or minor effects on the viability of RBL-2H3 cells at all concentrations used and both time intervals. Values shown are the means ± SEM of six separate experiments. *Significant difference between andrographolide and DDAG, $p < 0.05$.

4.1.2. DDAG reduces bronchoalveolar lavage fluid Th2 cytokines

BAL fluid was collected 24 hours after the last OVA or saline aerosol challenge. After separation of infiltrating cells, the bronchoalveolar lavage (BAL) fluids were quickly stored in -80°C until ELISA. By using ELISA, we observed that OVA challenge caused a notable increase in IL-4, IL-5, IL-13 and eotaxin levels in BAL fluid of OVA sensitize mice as compare to Saline challenge mice. The increase of these cytokines represent an enhance Th2 response typically observe in allergic asthma patients. IL-5 together with eotaxin lead to an increase of eosinophils infiltration while IL-4 and IL-13 are critical in IgE class switching. DDAG significantly ($p < 0.05$) reduced IL-4, IL-5, and IL-13 and, to a lesser extent, eotaxin levels in BAL fluid, in a dose-dependent manner (Figure 4.4).

4.1.3. DDAG reduces serum immunoglobulins

To study the effects of DDAG on the continuing OVA-specific Th2 response in vivo, levels of total IgE, OVA-specific IgE, OVA-specific IgG1 and OVA-specific IgG2a were determined using ELISA. Substantial elevation in serum total IgE, OVA-specific IgE, and OVA-specific IgG1 levels was observed, with no changes in OVA-specific IgG2a levels, in OVA-sensitise and challenged mice (Figure 4.5). DDAG significantly ($p < 0.05$) suppressed OVA-specific IgE levels and, to a lesser extent, the serum levels of total IgE and OVA-specific IgG1 (Figure 4.5). DDAG had no effects on the serum level of OVA-specific IgG2a. The production of subclasses of immunoglobulins is regulated by cytokines derived mainly from T helper cells. The Th2 cytokine IL-4 induces IgG1 and IgE production, whereas the Th1 cytokine IFN-γ induces IgG2a in mice. Therefore, these findings imply that DDAG is able to modify the Th2 immune activity in the OVA mouse asthma model.

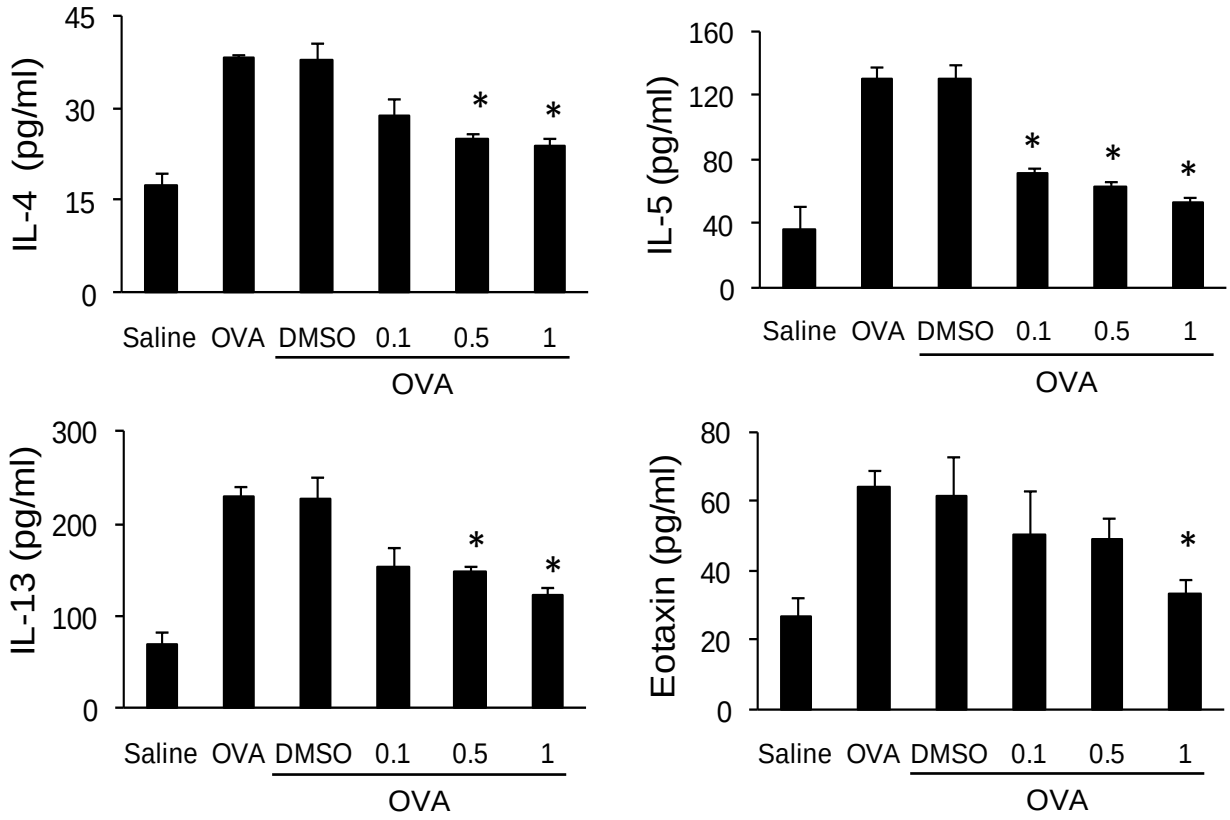


Figure 4.4. Effects of 14-deoxy-11,12-didehydroandrographolide (DDAG) on BAL fluid Th2 cytokines. BAL fluids were collected 24 h after the last OVA aerosol challenge. Levels of IL-4, IL-5, IL-13, and eotaxin were analyzed using ELISA (n = 6–9 mice). Lower limits of detection were as follows: IL-4 and IL-5 at 4 pg/mL, IL-13 at 15.6 pg/mL, and eotaxin at 2 pg/mL. Values shown are the means ± SEM. *Significant difference from DMSO control, $p < 0.05$.

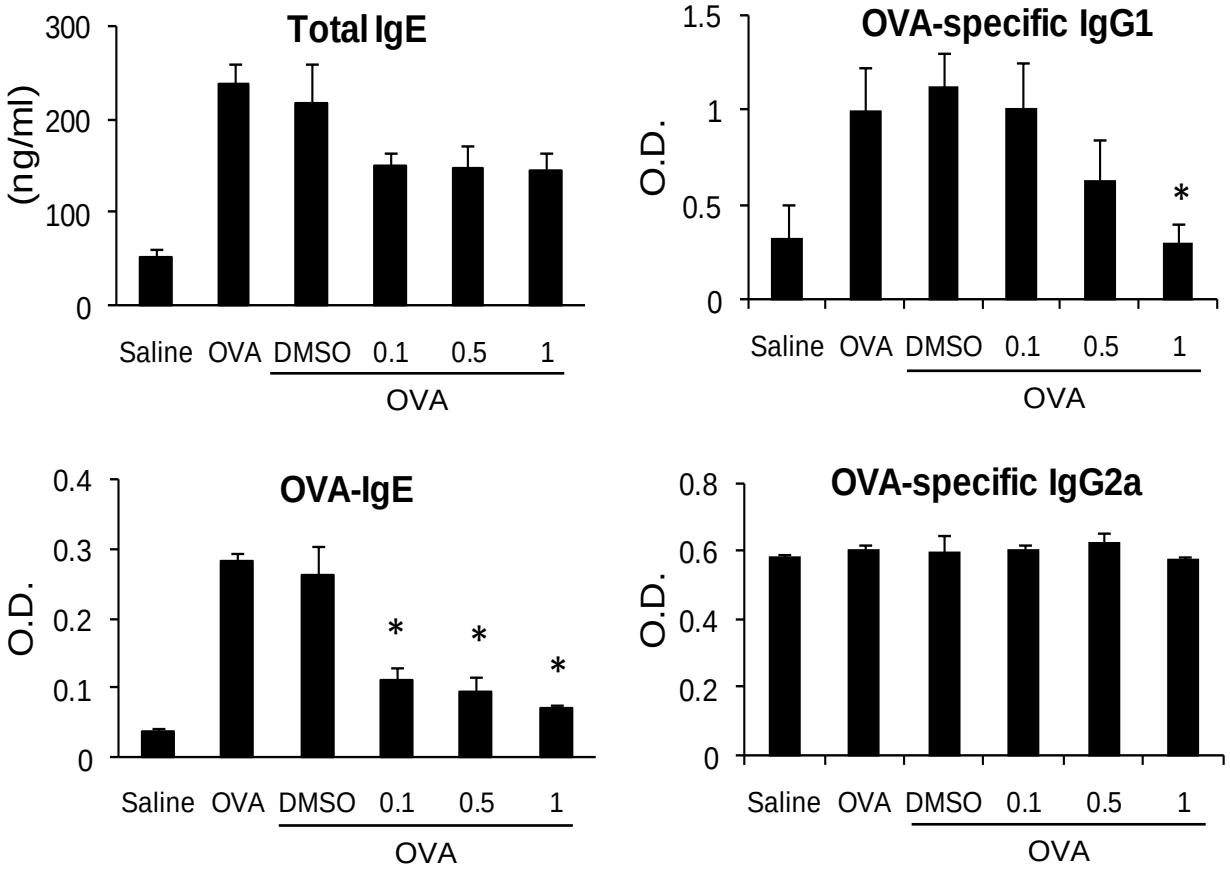


Figure 4.5. Effects of 14-deoxy-11,12-didehydroandrographolide (DDAG) on serum immunoglobulins. Mouse serum was obtained 24 hours after the last OVA aerosol challenge. The levels of total IgE, OVA-specific IgE, OVA-specific IgG1, and OVA-specific IgG2a were analysed using ELISA (n = 6–9 mice). Values shown are the means ± SEM. *Significant difference from DMSO control, $p < 0.05$.

4.1.4. DDAG reduces lung inflammatory biomarkers

To study the effects of DDAG on the profile of expression of inflammatory biomarkers, lungs were harvested 24 hours after the last aerosol challenge. The reverse transcription PCR data shows that OVA challenge markedly up-regulated lung mRNA levels of the inducible endothelial cell adhesion molecules, such as vascular cell adhesion molecule 1 (VCAM-1) and E-selectin (or endothelial-leukocyte adhesion molecule 1), and chemokines such as monocyte chemotactic protein-1 (MCP-1/CCL2), which are pivotal for the recruitment of inflammatory cells (Hogan *et al.*, 2008; Kelly *et al.*, 2007). MCP-1 recruits monocytes to sites of tissue injury, infection, and inflammation. Chitinases including acidic mammalian chitinase (AMCase), and chitinase like protein such as Ym1, Ym2, and YKL-40 have been shown recently to play critical roles in airway inflammation and remodelling (Chupp *et al.*, 2007; Guan *et al.*, 2009; Zhao *et al.*, 2005; Zhu *et al.*, 2004). Furthermore, AMCase and YKL-40 are highly associated with allergic bronchial asthma in patients (Shuhui *et al.*, 2009). IL-17 and IL-33, a member of the IL-1 cytokine family, are two effector cytokines that have been shown to be essential for airway inflammation and remodelling (Kearley *et al.*, 2009; Nembrini *et al.*, 2009). Both IL-17 and IL-33 have been associated with severe and steroid insensitive asthma (Alcorn *et al.*, 2010; Prefontaine *et al.*, 2009). Cyclooxygenase-2 (COX-2) is known to be essential for PGD_2-mediated airway eosinophilia and AHR (Shiraishi *et al.*, 2008), and Muc5ac, essential for mucus hypersecretion (Morcillo *et al.*, 2006). Pretreatment with DDAG (1 mg/kg) strongly suppressed the expression of VCAM-1, E-selectin, MCP-1, AMCase, Ym-2, YKL-40, Muc5ac, COX2, IL-17, and IL-33 levels in the allergic airways of OVA sensitize and challenge mice lungs (Figure 4.6). Taken together, these data suggest that DDAG is a potent anti-inflammatory agent.

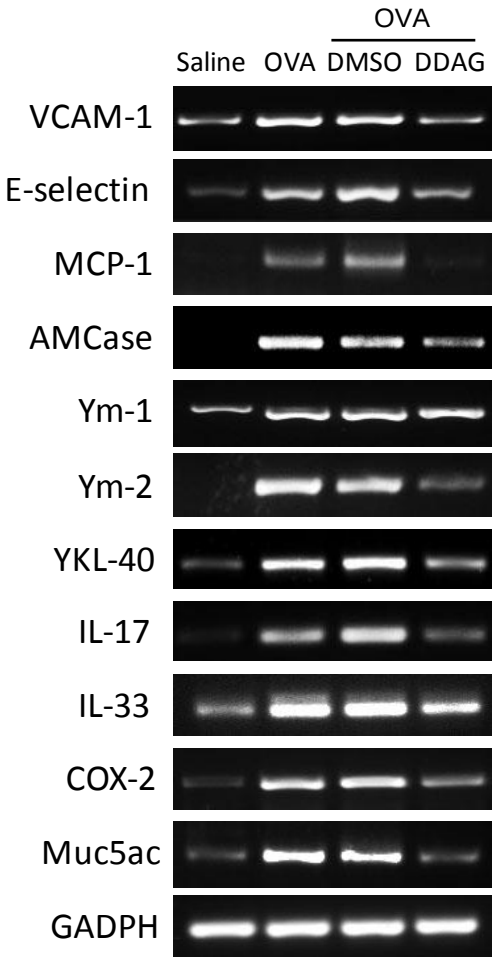


Figure 4.6. Effects of 14-deoxy-11,12-didehydroandrographolide (DDAG) on lung inflammatory biomarkers. Lung tissues were collected 24 h after the last OVA aerosol challenge. Total mRNA was extracted using TriZol reagent, and the PCR products were separated in a 2% agarose gel visualized under UV light. GADPH was used as an internal control. The experiments were repeated three times (n = 3 mice) with a similar pattern of results.

4.1.5. DDAG suppresses allergic airway inflammation

OVA challenge markedly increased total cell and eosinophil counts. A minor but significant increase of lymphocyte and neutrophils upon OVA challenge was also observed (Figure 4.7). DDAG (0.1, 0.5, and 1 mg/kg) significantly decreased total cell and eosinophil counts in BAL fluid in a dose-dependent manner (Figure 4.8). At 1 mg/kg, DDAG also reduced macrophage and lymphocyte counts.

Lung tissue was collected 24 hours after the last OVA challenge. OVA aerosol challenge induced substantial inflammatory cell infiltration into the peribronchiolar and perivascular connective tissues as compared to saline challenge (Figure 4.9). The majority of the infiltrated inflammatory cells were eosinophils. Pre-treatment with DDAG (1 mg/kg) markedly diminished the eosinophil-rich leukocyte infiltration (Figure 4.9). OVA-challenged but not saline challenge mice developed goblet cell hyperplasia and mucus hypersecretion in the bronchi. The OVA-induced mucus secretion was markedly suppressed by DDAG (1 mg/kg) as visualize by PAS staining (Figure 4.10).

4.1.6. DDAG prevents lung mast cell degranulation

OVA aerosol challenge markedly decreased intact mast cell number and increased degranulating mast cells in the lungs. Increase in OVA specific IgE will lead to mast cell activation upon OVA challenge in OVA sensitize mouse as mast cell contain the high affinity IgE binding receptor, FcεR1. Activated mast cell would release score of preform mediators, newly formed lipid mediators and cytokines such as eosinophil chemotactic factors leading to an enhance inflammatory response. DDAG (1 mg/kg) reduced mast cell degranulation and restored intact mast cell number similar to saline aerosol control (Figure 4.11).

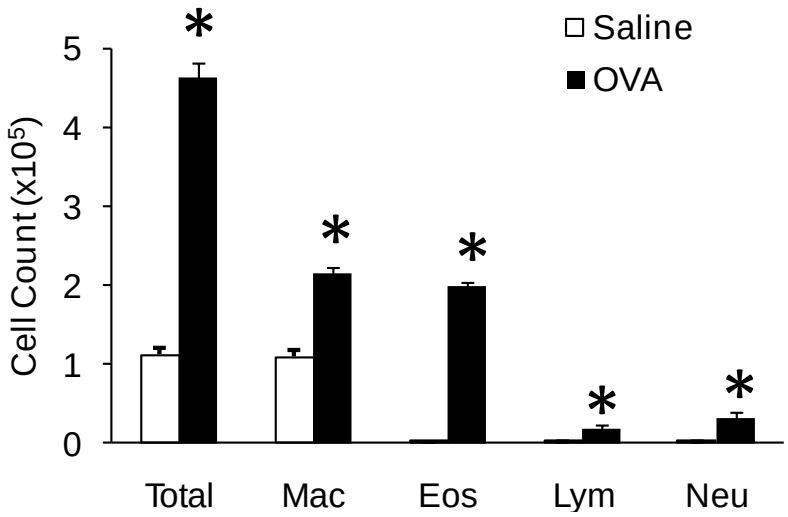


Figure 4.7. Inflammatory cell counts in BAL fluid obtained from sensitized mice 24 h after the last saline aerosol (*n* = 7 mice) or OVA aerosol (*n* = 7 mice) challenge. OVA challenge induced marked infiltration of inflammatory cells into the peribronchiolar and perivascular connective tissues as compared with saline aerosol challenge.

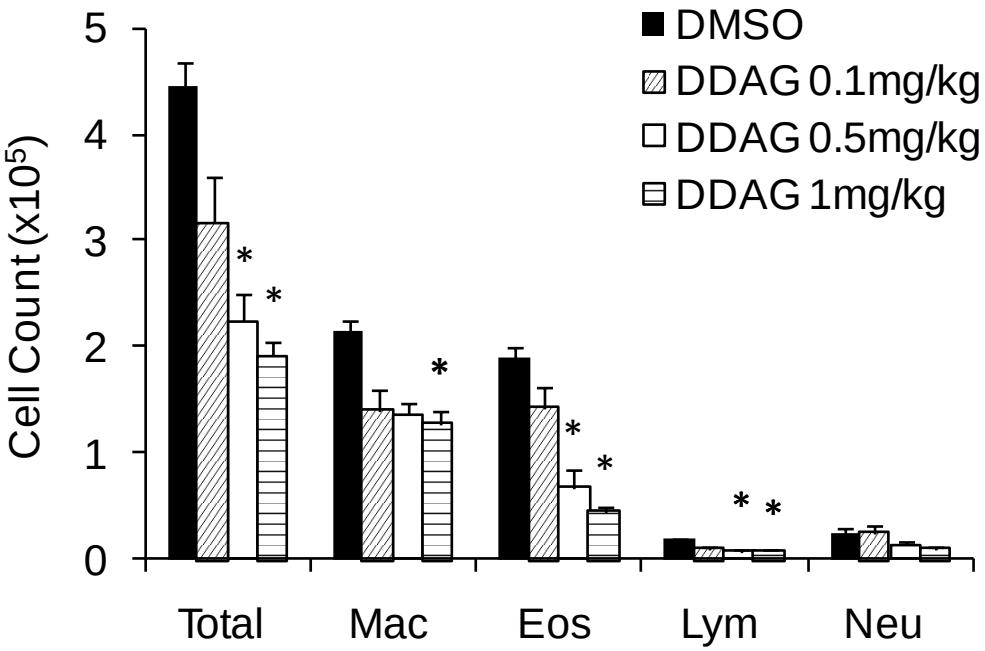


Figure 4.8. Effects of 14-deoxy-11,12-didehydroandrographolide (DDAG) on OVA-induced inflammatory cell recruitment. DDAG dose-dependently reduced OVA-induced inflammatory cell counts in BAL fluid from sensitized mice 24 h after the last OVA aerosol challenge (DMSO, *n* = 7; 0.1 mg/kg, *n* = 8; 0.5 mg/kg, *n* = 7; and 1 mg/kg, *n* = 10). Differential cell counts were performed on a minimum of 500 cells to identify eosinophils (Eos), macrophages (Mac), neutrophils (Neu), and lymphocytes (Lym).

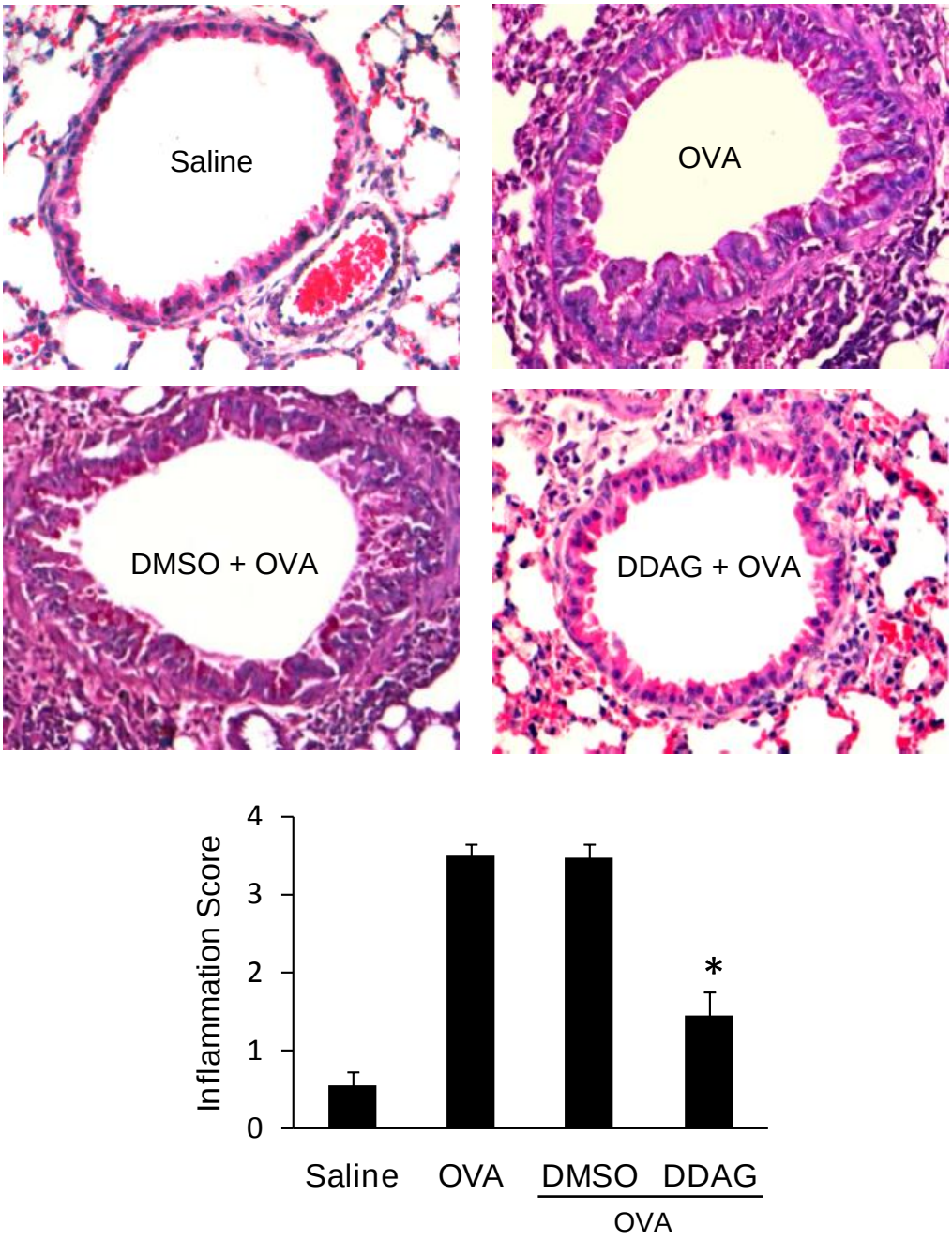


Figure 4.9. Effects of 14-deoxy-11,12-didehydroandrographolide (DDAG) on OVA-induced inflammatory cell recruitment. Histological slides showing lung tissue eosinophilia (magnification 200×) 24 h after the last challenge of saline aerosol, OVA aerosol, OVA aerosol plus DMSO, or OVA aerosol plus 1 mg/kg DDAG are displayed. Quantitative analyses of inflammatory cell infiltration in lung sections were performed as previously described (Bao *et al.*, 2009). Scoring of inflammatory cells was performed in at least three different fields for each lung section. Mean scores were obtained from four animals. *Significant difference from DMSO control, $p < 0.05$.

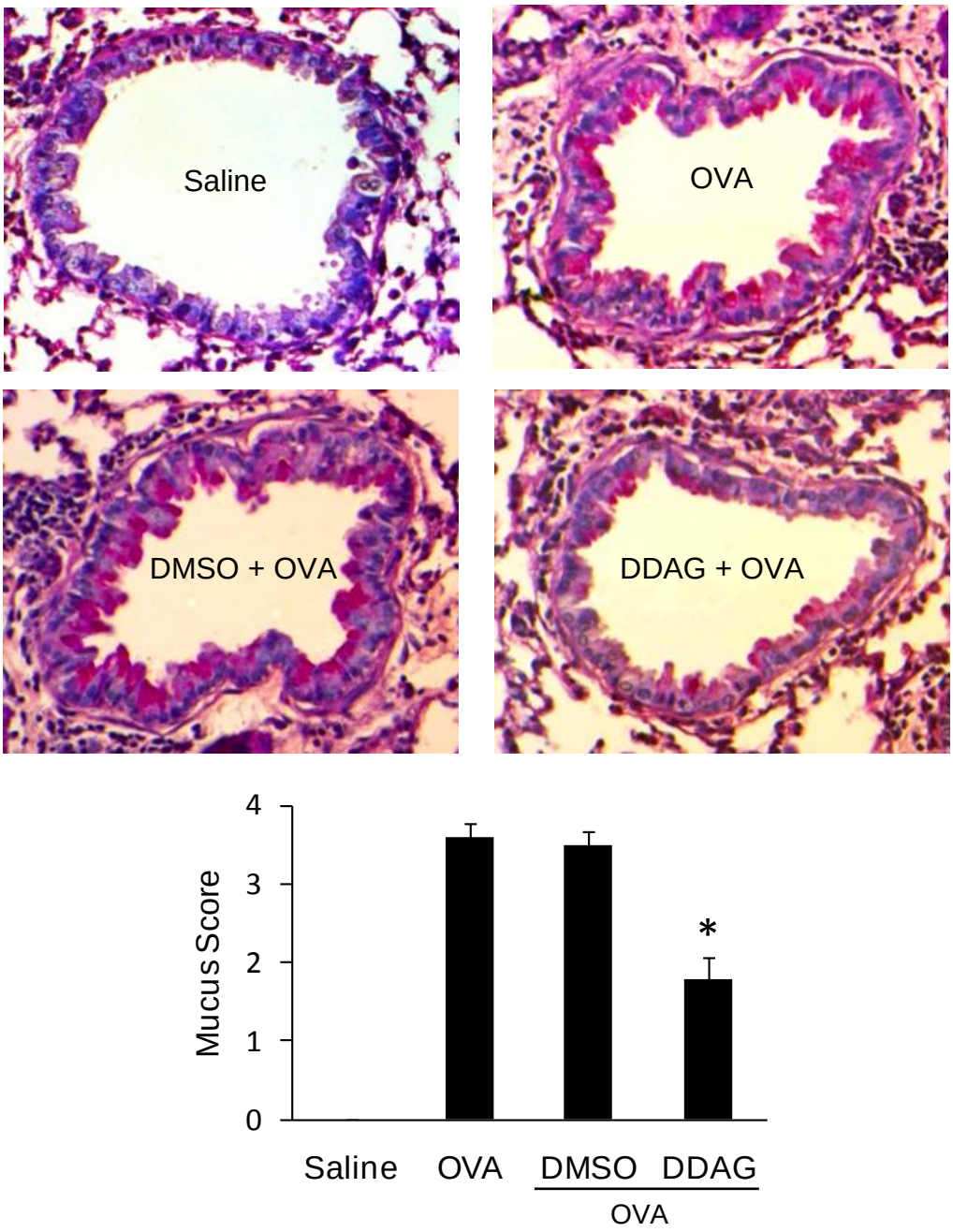
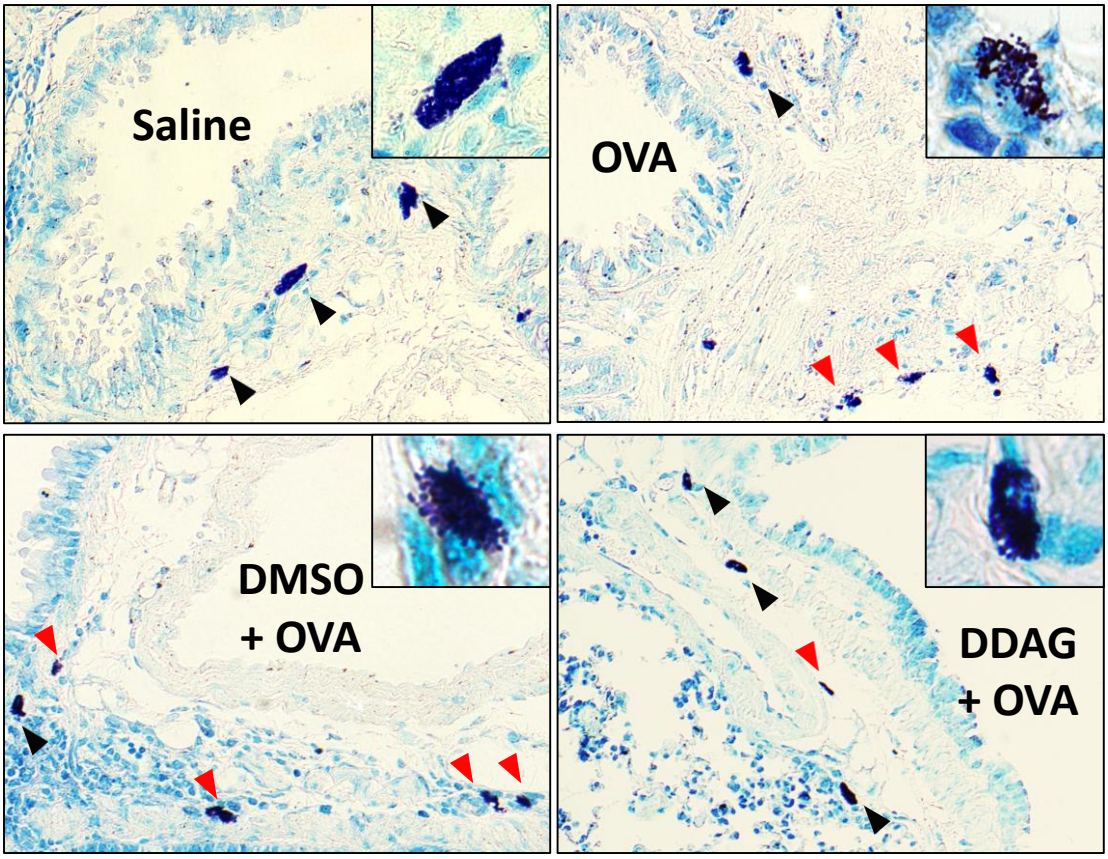


Figure 4.10. Effects of 14-deoxy-11,12-didehydroandrographolide (DDAG) on OVA-induced mucus hypersecretion. Histological slides showing lung mucus secretion (magnification 200×) 24 h after the last challenge of saline aerosol, OVA aerosol, OVA aerosol plus DMSO, or OVA aerosol plus 1 mg/kg DDAG are displayed. Quantitative analyses of inflammatory cell infiltration and mucus production in lung sections were performed as previously described (Bao *et al.*, 2009). Scoring of goblet cells was performed in at least three different fields for each lung section. Mean scores were obtained from four animals. *Significant difference from DMSO control, $p < 0.05$.



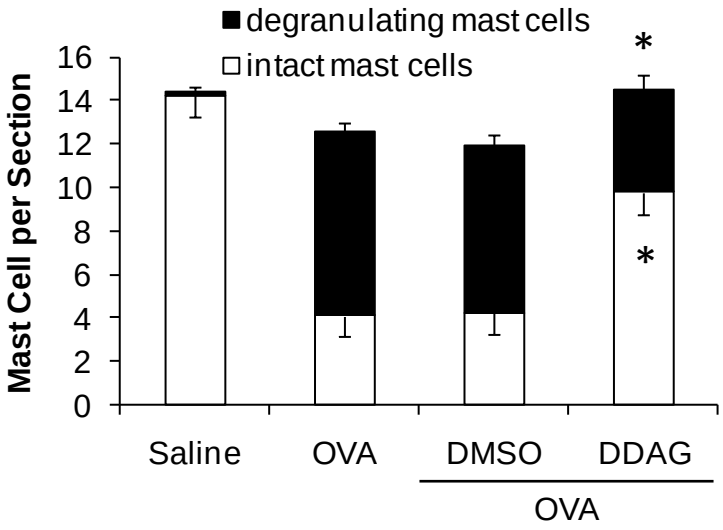


Figure 4.11. Effects of DDAG on OVA-induced lung mast cell degranulation. Lung tissue mast cells were stained using toluidine-blue, and the number of intact and degranulating mast cells was counted (magnification x 200) in the entire lung sections. Black arrow heads point to intact mast cells. Red arrow heads indicate degranulating mast cells. Mean scores were obtained from 4 animals. *Significant difference from DMSO control, $p < 0.05$.

4.1.7. DDAG reduces AHR

OVA-challenged mice developed AHR, which is typically reflected by high lung resistance (Rl) (Figure 4.12) and low dynamic compliance (Cdyn) (Figure 4.13). Rl is defined as the pressure driving respiration divided by flow. Cdyn refers to the distensibility of the lung and is defined as the change in volume of the lung produced by a change in pressure across the lung. DDAG (1 mg/kg) dramatically reduced Rl and restored Cdyn in OVA-challenged mice in response to methacholine, suggesting that the immune-mediated airway pathology in vivo was modified

4.1.8. DDAG inhibits NF-κB activation

To determine if DDAG, like andrographolide, could inhibit NF-κB in ovalbumin (OVA)-challenged mice (Bao *et al.*, 2009), the nuclear translocation of the p65 subunit and p65 DNA-binding activity in lung tissues from mice treated with DDAG were examined. OVA challenge markedly raised the level of p65 subunit in the nuclear extract of lung tissues and promoted nuclear p65 DNA-binding activity (Figure 4.14). DDAG (1 mg/kg) significantly ($p < 0.05$) reduced both nuclear p65 translocation and DNA-binding activity to the basal levels.

The mechanism of action of DDAG in TNF-α-stimulated A549 human lung epithelial cells in vitro was verified. TNF-α plays a critical role in asthma (Brightling *et al.*, 2008; Vroling *et al.*, 2007) and is a potent stimulator of human airway epithelial cells (Newton *et al.*, 2007). A sharp increase in total nuclear p65 level and p65 DNA-binding activity was observed upon TNF-α treatment, and DDAG markedly abated these TNF-α-mediated responses (Figure 4.15).

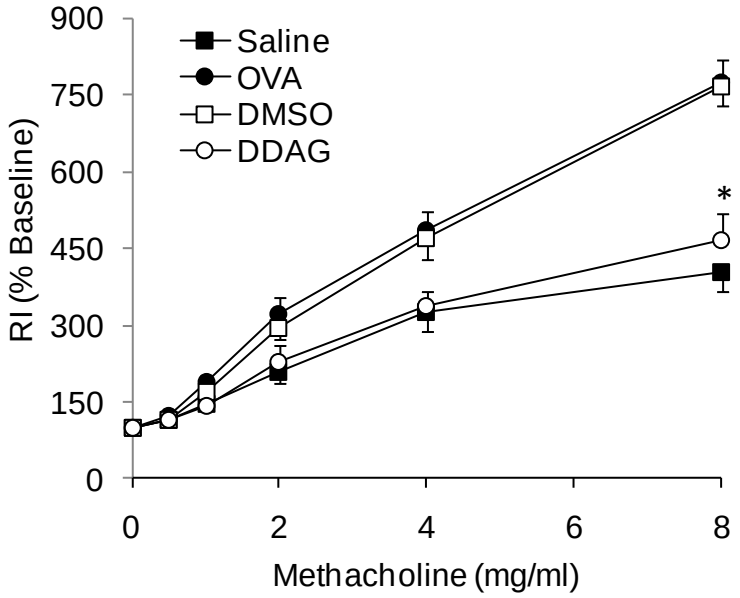


Figure 4.12. Effects of 14-deoxy-11,12-didehydroandrographolide (DDAG) on OVA-induced AHR. Airway responsiveness of mechanically ventilated mice in response to aerosolized methacholine was measured. AHR is expressed as the percent change from the baseline level of lung resistance (Rl, n = 7–9 mice). Rl is defined as the pressure driving respiration divided by flow. *Significant difference from DMSO control, $p < 0.05$.

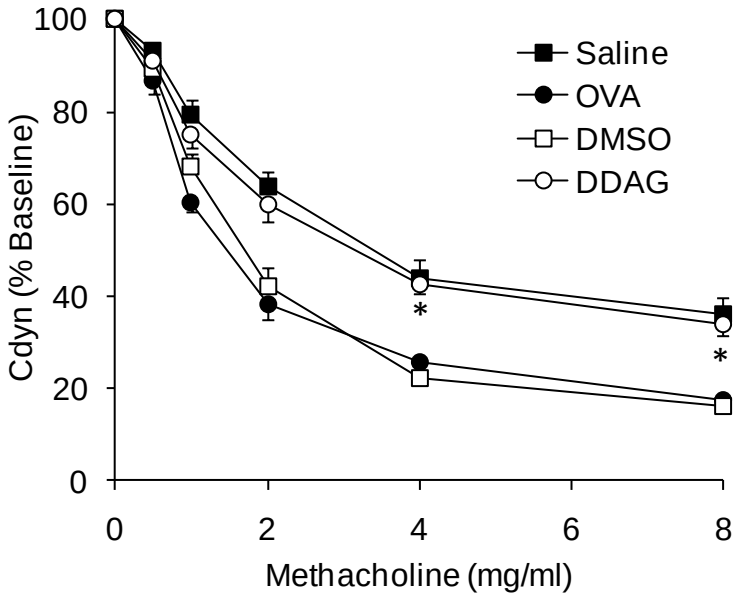


Figure 4.13. Effects of 14-deoxy-11,12-didehydroandrographolide (DDAG) on OVA-induced AHR. Airway responsiveness of mechanically ventilated mice in response to aerosolized methacholine was measured. AHR is expressed as the percent change from the baseline level of dynamic compliance (Cdyn, n = 7–9 mice). Cdyn refers to the distensibility of the lung and is defined as the change in volume of the lung produced by a change in pressure across the lung. *Significant difference from DMSO control, $p < 0.05$.

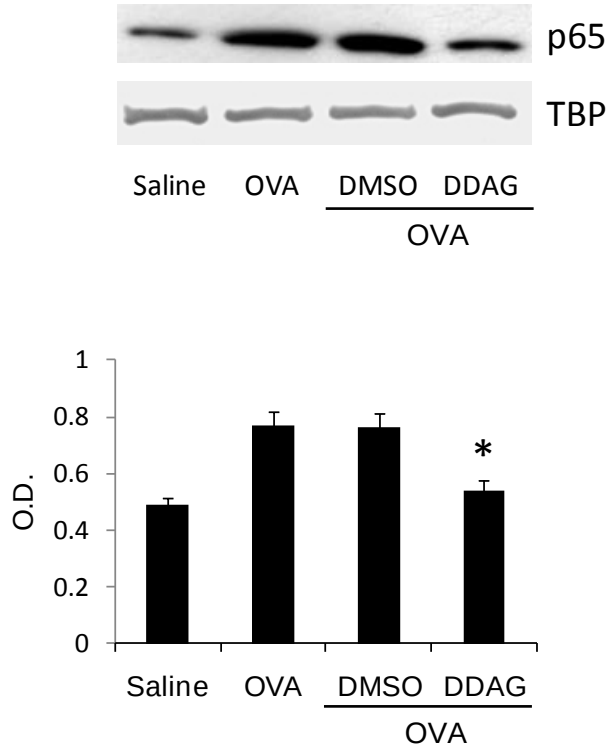


Figure 4.14. Effects of 14-deoxy-11,12-didehydroandrographolide (DDAG) on NF-κB activity in OVA-challenged lungs. Immunoblotting of p65 NF-κB in nuclear extract of lung tissues isolated from mice 24 h after the last OVA challenge. TBP nuclear protein was used as an internal control. The experiments were repeated three times with a similar pattern of results. Nuclear p65 DNA-binding activity was determined using a TransAM p65 transcription factor ELISA kit. Values shown are the mean ± SEM of three separate experiments. *Significant difference from DMSO control, $p < 0.05$.

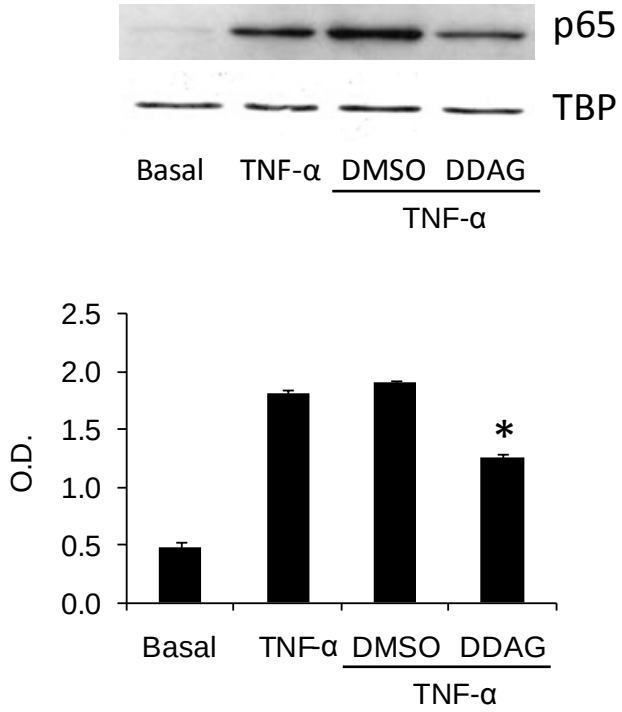


Figure 4.15. Effects of 14-deoxy-11,12-didehydroandrographolide (DDAG) on NF-κB activity in in TNF-α-stimulated A549 human lung epithelial cells. Immunoblotting of p65 NF-κB in nuclear extract of A549 cells stimulated with 10 ng/mL TNF-α for 5 min, in the presence and absence of DDAG (30 μM). TBP nuclear protein was used as an internal control. The experiments were repeated three times with a similar pattern of results. Nuclear p65 DNA-binding activity was determined using a TransAM p65 transcription factor ELISA kit. Values shown are the mean ± SEM of three separate experiments. *Significant difference from DMSO control, $p < 0.05$.

4.2. Discussion

DDAG is the next most abundant of the naturally occurring labdane diterpene lactones in *Andrographis paniculata* besides andrographolide (Koteswara Rao *et al.*, 2004; Pholphana *et al.*, 2004). DDAG has been shown to be a metabolite of andrographolide and can also be synthesized chemically from the parent compound (He, 2003; Nanduri *et al.*, 2004). Nevertheless, the present knowledge of the pharmacological actions of DDAG is much less than for andrographolide. Indeed, a clinical study of andrographolide in upper respiratory tract infection has been reported (Chang *et al.*, 2008a), whereas investigations of DDAG are still at the early preclinical stage. Despite their structurally similarity, there is a notable difference between these two diterpenoids with regard to cytotoxicity. Andrographolide contains an α-alkylidene γ-butyrolactone moiety and three hydroxyls at C-3, C-19 and C-14 that are accountable for the cytotoxic activities of andrographolide against numerous cancer cell lines (Varma *et al.*, 2009). The lacking of an OH group at the C14 position and the double bond at C11 and C12 in DDAG structure might be related to the non-cytotoxic properties. Our present findings reveal that andrographolide exerted a strong dampening effect on the cell viability of human lung epithelial cell lines and rat mast cell line, but DDAG appeared to be non-cytotoxic to these culture cells. These data are consistent with observations reported by other laboratories on other cell lines (Nanduri *et al.*, 2004; Tan *et al.*, 2005) and strongly support the notion that DDAG is a noncytotoxic analogue of andrographolide.

Th2 cytokines play an essential role in the pathogenesis of the allergic airway inflammation (Galli *et al.*, 2008a; Medoff *et al.*, 2008), and NF-κB is a critical transcription factor for Th2 cell differentiation (Das *et al.*, 2001). IL-4 in conjunction with IL-13 are imperative for B cell antibodies isotype switching from IgM to IgE. Whereas IL-5 is vital for the growth, differentiation, recruitment, and survival of eosinophils which contribute to inflammation and even airway remodelling in asthma (Takatsu *et al.*, 2008). Furthermore, IL-

13 also plays a pivotal role in the effector phase of Th2 responses such as eosinophilic inflammation, airway-smooth-muscle hyperplasia, the induction of goblet-cell hyperplasia with mucus hypersecretion, the recruitment of monocytes, macrophages and T cells into the airway spaces, AHR and airway remodelling (Wills-Karp, 2004; Wynn, 2003). IL-4, IL-5 and IL-13 can be produced by various lung resident cells such as bronchial epithelial cells, tissue mast cells and alveolar macrophages as well as infiltrated inflammatory cells such as lymphocytes and eosinophils. Our results show that DDAG significantly reduced the levels of IL-4, IL-5, IL-13 and eotaxin in BAL fluids from OVA-challenged mice. Similar findings were observed in OVA-challenged mice with disrupted NF-κB function via conditional knockout of IKKβ in the airway epithelium (Broide *et al.*, 2005). In addition, repression of the NF-κB signalling pathway has been shown to block IL-13-induced eotaxin production in cultured human airway smooth muscle cells (Li *et al.*, 1999). Therefore, the observed reduction of IL-4, IL-5, IL-13, and eotaxin levels in BAL fluid from DDAG-treated mice may be due to inhibition of NF-κB activation in the inflammatory and airway resident cells.

The most abundant immunoglobulin isotype in the blood is IgG, an immunoglobulin that provides the bulk immunity to most blood borne infectious agents. There are different IgG subclasses such as IgG1, IgG2a, IgG2b, and IgG3. During a T-cell dependent immune response, a progressive change takes place in the predominant immunoglobulin class of the specific antibodies. This subclass switch is influenced by T-cells and their cytokines. In mice, IL-4 preferentially switch activated B cells to the IgG1 isotype (associated with Th2 immune response) while IFN-γ and IL-12 enhances IgG2a responses (associated with Th1 immune response) (Pulendran *et al.*, 1999). The reduction of IgG1 by DDAG treatment in our observation further substantiates the notion that DDAG significantly reduce the Th2 response thereby attenuating the role Th2 cytokines play in orchestrating the complex series of events leading to immunoglobulin E (IgE) production, and the development, recruitment, and

activation of the primary effector cells of the allergic response such as mast cells and eosinophils.

Elevated serum IgE levels are a hallmark of the Th2 immune response. Clinical studies have shown that humanized mAbs against IgE such as omalizumab was effective in patients with poorly controlled, moderate to severe allergic asthma (Busse *et al.*, 2011; Lin *et al.*, 2004). When the serum concentration of unbound IgE were decrease by omalizumab treatment, the expression of high-affinity IgE receptor FcεRI on several cell types were decrease as well (Lin *et al.*, 2004). This will also decrease the amplification of the inflammatory response mediated by T helper cells to prevent IgE-dependent allergen presentation. IgE in turn is produce by B-Cell. NF-κB plays a crucial role in B-cell proliferation and development (Schulze-Luehrmann *et al.*, 2006; Siebenlist *et al.*, 2005). IL-4 and IL-13 are important in directing B-cell growth, differentiation, and secretion of IgE (Li-Weber *et al.*, 2003; Wills-Karp, 2004). Therefore the significantly reduced serum level of OVA-specific IgE in OVA-challenged mice by DDAG treatment may be attributed to the inhibition of NF-κB during B-cell activation and of IL-4- and IL-13-mediated class switching to IgE.

The biological activities of IgE are mediated through its interaction with the high-affinity IgE receptor FcεRI on mast cells and basophils. Once the mice is sensitized to OVA as allergen, subsequent encounters with OVA cause crosslinking of IgE bound to the FcεRI which initiates multiple signaling cascades leading to NF-κB activation and stimulate the release of granule-associated and newly generated mediators that are responsible for the early allergic response (Galli *et al.*, 2008a; Klemm *et al.*, 2006). Subsequently, the release of cytokines and chemokines that recruit macrophages and eosinophils will leads to the late allergic response (Cockcroft *et al.*, 2007).

Eosinophils play a central role in the pathogenesis of allergic inflammation (Hogan *et al.*, 2008; Takatsu *et al.*, 2008). IL-5 is a unique cytokine for the differentiation and survival of eosinophils in response to allergen provocation. Study has demonstrated specific anti-IL-5 therapy is efficacious in severe asthma patients who had frequent exacerbations and showing eosinophilic sputum (Haldar *et al.*, 2009). Their findings suggest that eosinophils have a role as important effector cells in the pathogenesis of severe exacerbation of asthma. The view expressed in Haldar *et al.* were further supported by Nair *et al.* when they show that systemic steroids can be withdrawn in severe asthma patients who had eosinophilic sputum when treated with mepolizumab, a monoclonal antibody against interleukin-5 (Nair *et al.*, 2009). Our present findings demonstrate that DDAG prevented inflammatory cell infiltration into the airways as shown by a significant drop in total cell counts and eosinophil and lymphocyte counts in BAL fluid, and in tissue eosinophilia in lung sections.

Leukocyte transmigration into the airways is orchestrated by cytokines such as IL-4, IL-5, and IL-13 and coordinated by specific chemokines such as eotaxin and RANTES in combination with adhesion molecules as exemplified by VCAM-1 and E-selectin (Hogan *et al.*, 2008; Kelly *et al.*, 2007). Upon OVA provocation, autacoid mediators release by mast cell such as histamine and the cysteinyl leukotrienes (CysLTs) increase endothelial expression of E-selectin which initiate leukocyte rolling, followed by the expression of intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1), which interact with integrin receptors to arrest the leukocyte and assist its passage into the perivascular space (Kelly *et al.*, 2007). The activation of VCAM-1 gene expression is regulated by the transcription factors nuclear factor kappa B (NFκB) as VCAM-1 expression is induced by the cytokines TNF-α and IL-1β (Cook-Mills *et al.*, 2011). Chemokines such as monocyte chemotactic proteins-1 (MCP-1/CCL2) and eotaxin-1 (CCL11) direct and prime leukocytes for mediator secretion (Palmqvist *et al.*, 2007; Velazquez *et al.*, 2011). Apart from

cysteinyl leukotrienes, eotaxin also plays a role in promoting eosinophil recruitment in response to allergic provocation via CCR3 (Luster *et al.*, 1997). IL-13 is by far the most potent inducer of eotaxin expression in airway epithelial cells (Li *et al.*, 1999). IL-17 has been shown to induce eotaxin production from airway smooth muscle cells (Rahman *et al.*, 2006c), whereas IL-33 can promote eotaxin release from macrophages (Liew *et al.*, 2010).

DDAG markedly suppressed E-selectin, VCAM-1, MCP-1, IL-17 and IL-33 mRNA expression and eotaxin production in OVA-challenged lungs. These findings are likely to be due to DDAG-mediated NF-κB inhibition, as the genes for E-selectin, VCAM-1, MCP-1 and eotaxin contain the κB site for NF-κB within their promoters (Kumar *et al.*, 2004a). These finding may also associated with reduced mast cell degranulation with DDAG treatment as mast cells has recently been demonstrated to play a key role in a Th2 cytokine-dependent asthma model through production of adhesion molecules, including ICAM-1 and VCAM-1, by liberation of TNF-α (Chai *et al.*, 2011). Taken together, the suppression of these inflammatory cytokines and chemokines by DDAG treatment in OVA-challenged lungs may be one of the main reasons for the reduction in eosinophil infiltration.

A family of chitinase proteins including AMCase, Ym1, Ym2 and YKL-40 has recently been found to be elevated in allergic airway inflammation in human and in mouse asthma models (Chupp *et al.*, 2007; Zhao *et al.*, 2005; Zhu *et al.*, 2004). They are mainly expressed in airway epithelium and alveolar macrophages. AMCase level is increased in a mouse asthma model and in asthmatic subjects in an IL-13-dependent manner (Zhu *et al.*, 2004). When given intratracheally, IL-13 elevates Ym1 and Ym2 levels in BAL fluid from mice in vivo (Webb *et al.*, 2001). Besides, YKL-40 serum level correlates positively with asthma severity, airway remodelling and deterioration of pulmonary function in asthmatic subjects (Chupp *et al.*, 2007). In a chronic asthma mouse model, only AMCase and Ym2 proteins but not Ym1 were found to be up regulated in the proteomes of lung tissues and/or

BAL fluid (Wong *et al.*, 2008). Overall, chitinases may play a role in airway inflammation and remodelling. Our data show that DDAG markedly down-regulated AMCase, Ym2 and YKL-40 mRNA expression in the lungs of OVA-challenged mice. These may be a consequence of the major drop in IL-4 and IL-13 levels in the airways with DDAG treatment and may contribute to the diminished pulmonary eosinophilia.

We observed a dramatic reduction in airway mucus production in DDAG-treated mice as compared with DMSO control. Cumulative evidence indicates that IL-4, IL-5, IL-13, and IL-17 play a critical role in goblet cell hyperplasia and mucin Muc5ac gene and protein expression in mice (Fujisawa *et al.*, 2009; Justice *et al.*, 2002; Morcillo *et al.*, 2006). Muc5ac gene expression is dependent on the transcriptional activity of NF-κB (Justice *et al.*, 2002; Morcillo *et al.*, 2006). In addition, selective ablation of NF-κB function in the airway epithelium has been shown to reduce OVA-induced mucus production in mice (Birrell *et al.*, 2005a). As such, the reduction in mucus production and Muc5ac mRNA expression in the lungs of DDAG-treated mice may be attributable to a significant drop in IL-4, IL-5, IL-13, and IL-17 levels and an inhibitory action on NF-κB in the airway epithelium.

IL-4, IL-5, and IL-13 have been shown to induce AHR, in which major basic protein and cysteinyl-leukotrienes have been implicated (Hogan *et al.*, 2008; Leigh *et al.*, 2004; Takatsu *et al.*, 2008; Wills-Karp, 2004). Recently, IL-33 was found to directly trigger AHR in mice (Liew *et al.*, 2010). In addition, IgE-mediated mast cell activation may contribute to airway hyperresponsiveness by producing a wide array of mast cell-derived lipid mediators and inflammatory cytokines (Galli *et al.*, 2008a; Klemm *et al.*, 2006; Woodruff *et al.*, 2009). Thus, the observed reduction in AHR by DDAG treatment may be associated with the reduction in Th2 cytokine and IL-33 production, tissue eosinophilia, serum IgE levels and mast cell degranulation.

Persistent NF-κB activation has been observed in allergic airway inflammation in humans and in animal model of asthma (Edwards *et al.*, 2009). p65 protein levels in peripheral blood mononuclear cells (PBMCs) were higher in moderate and severe asthmatics than in normal individuals. In addition, while high amount of IκB phosphorylation were characteristic of asthmatic patients, greater p65 activation was also observe in severe asthmatics (Gagliardo *et al.*, 2003). Similarly, p65 protein abundance and IκB phosphorylation are also higher in PBMCs of children with moderate asthma when compared to normal individuals (La Grutta *et al.*, 2003). Moreover, when compared to non-asthmatic individuals, nuclear extracts from bronchial biopsies and induced sputum cells (Hart *et al.*, 1998) of patients with asthma show higher NF-κB activation as measured by gel shift assays and immunohistochemical examination of bronchial biopsy specimens with an antibody to p65. Likewise, bronchial epithelial cells from stable, corticosteroid naïve asthmatics have also shown greater levels of NF-κB activation (Zhao *et al.*, 2001). The importance of NF-κB in disease pathogenesis has also been highlighted in the mouse models of allergic asthma. Compared to control mice, bronchial epithelium of mice sensitise and challenge with OVA exhibits robust and rapid inhibitory κB kinase α/β (IKK-α/β) activity and p65 nuclear translocation (Poynter *et al.*, 2002). Mice lacking p50 on the other hand have reduced eosinophil infiltration in response to OVA challenge. This effect could be attributed to a lack of Th2 cytokines such as IL-4, IL-5, IL-13 (Das *et al.*, 2001), and the chemokine eotaxin (Yang *et al.*, 1998). This study further demonstrated the importance of NF-κB signalling in both allergic inflammation and mucus production. On the other hand, activation of doxycycline-inducible constitutively active IKK-β transgene not only induces p65 nuclear translocation, inflammatory cell infiltration and concomitant production of pro-inflammatory mediators, it was able to induced AHR, even without allergen sensitization and challenge (Pantano *et al.*, 2008). Besides, antigen receptor activation in T and B lymphocytes and mast

cells culminates in NF-κB activation (Cheng *et al.*, 2011; Galli *et al.*, 2008a; Klemm *et al.*, 2006; Schulze-Luehrmann *et al.*, 2006; Shinohara *et al.*, 2009). In addition, TNF-α stimulation of airway epithelial cells triggers NF-κB activation and gene expression (Newton *et al.*, 2007). These studies further underscore the importance of NF-κB signalling in the generation of pro-inflammatory cytokines, chemokines and adhesion molecules. Furthermore, various therapeutic strategies targeted at the NF-κB activity such as NF-κB-specific decoy oligonucleotide (Desmet *et al.*, 2004), p65-specific antisense oligonucleotide (Choi *et al.*, 2004), p65-targeting siRNA (Platz et al., 2005) and inhibitory κB kinase-β (IKKβ)-selective small molecule inhibitor (Birrell *et al.*, 2005a) have demonstrated beneficial effects in experimental asthma models. Our data revealed a significant inhibition of p65 nuclear translocation and κB DNA-binding activity by DDAG in OVA-challenged lungs *in vivo* and in TNF-α-stimulated human lung epithelial cells *in vitro*. Our findings show for the first time that DDAG, like andrographolide, may exert anti-inflammatory actions via inhibition of NF-κB activity both in OVA-challenged lungs in vivo and in TNF-α-stimulated human lung epithelial cells in vitro.

We report here for the first time that 14-deoxy-11,12-didehydroandrographolide (DDAG), a naturally occurring noncytotoxic analogue of andrographolide, effectively reduced OVA-induced inflammatory cell recruitment into BAL fluid, IL-4, IL-5, IL-13, and eotaxin production, serum IgE synthesis, pulmonary eosinophilia, mucus hypersecretion, mast cell degranulation, and AHR in a mouse asthma model, probably via inhibition of NF-κB activity. These findings support a potential therapeutic role for DDAG in the treatment of asthma subject to further laboratory study.

5. ANTI-OXIDATIVE STRESS EFFECTS OF ANDROGRAPHOLIDE IN CIGARETTE SMOKE INDUCE LUNG INJURY MOUSE MODEL

5.1. Results

5.1.1. Andrographolide attenuates cigarette smoke-induced lung inflammation

Cotinine is a stable metabolite of nicotine and it is often used as a surrogate maker for nicotine exposure (Benowitz, 2009). Plasma cotinine levels in 4% cigarette smoke-exposed mice were sharply elevated above 100 ng mL^{-1}. In contrast, plasma cotinine level in sham air control mice was undetectable (Figure 5.1). The cotinine level was similar with the level observed by others groups (Lu *et al.*, 2007) and in serum of human smokers (Scott *et al.*, 2000). BAL fluid was collected 24 hours after the last cigarette smoke or sham air exposure. Cigarette smoke inhalation markedly increased BAL fluid total cell and neutrophil counts, with moderate but significant elevation in macrophage counts, as compared with sham air control (Figure 5.2). The low level of total BAL fluids' cell count in cigarette smoke inhalation models is reflective of the low grade of inflammation found in COPD patients. To examine the effect of andrographolide in cigarette smoke induce lung injury mouse model, cigarette expose mouse was treated prophylactically with andrographolide. Our study show that andrographolide (0.1, 0.5 and 1 mg kg^{-1}) significantly suppressed the total inflammatory cell and neutrophil counts in BAL fluid in a dose-dependent manner as compared with the DMSO control (Figure 5.3). Andrographolide (1 mg kg^{-1}) also showed a moderate inhibitory effect on macrophage count but did not reach statistical significance.

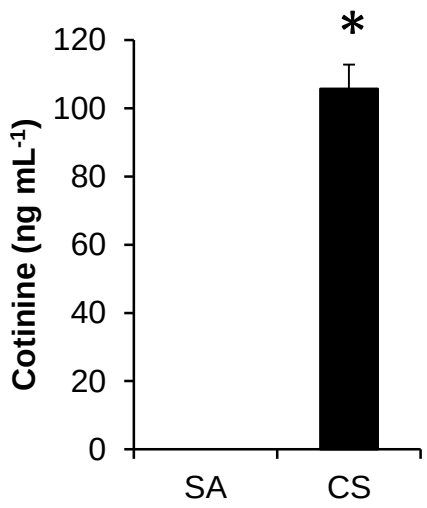


Figure 5.1. Effects of cigarette smoke in mice. Blood was collected immediately after 1 h 4% cigarette smoke exposure and plasma cotinine levels were measured using ELISA (n = 6). Values shown are the mean ± SEM. *Significant difference from Sham Air (SA), $P < 0.05$.

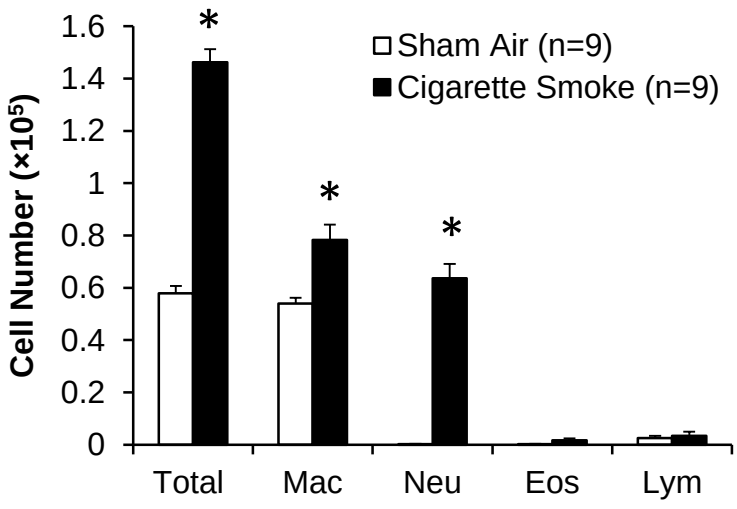


Figure 5.2. Effects of cigarette smoke-induced inflammatory cell recruitment. Inflammatory cell counts in BAL fluid obtained from mice 24 h after the last sham air (n = 9 mice per group) or cigarette smoke (n = 9 mice per group) exposure. Values shown are the mean ± SEM. *Significant difference from Sham Air, $P < 0.05$.

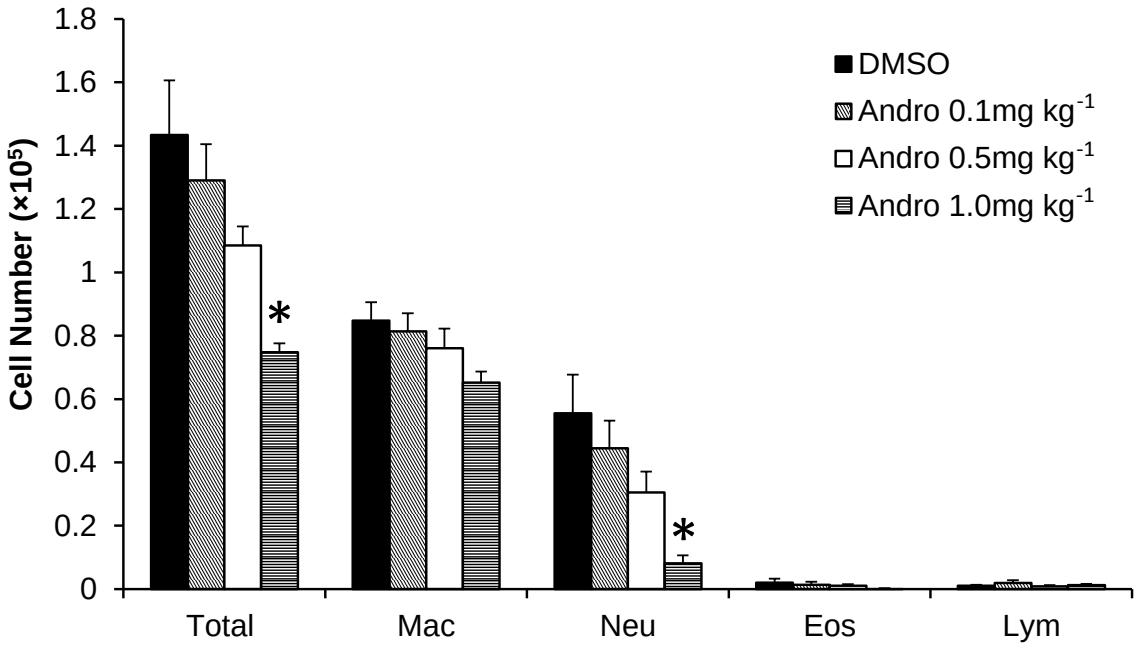


Figure 5.3. Effects of andrographolide on cigarette smoke-induced inflammatory cell recruitment. Andrographolide dose-dependently reduced cigarette smoke-induced inflammatory cell counts in BAL fluid from mice 24 h after the last cigarette smoke challenge (DMSO, n = 6; 0.1 mg kg^{-1}, n = 6; 0.5 mg kg^{-1}, n = 6; and 1 mg kg^{-1}, n = 8 mice per group). Differential cell counts were performed on a minimum of 500 cells to identify eosinophil (Eos), macrophage (Mac), neutrophil (Neu), and lymphocyte (Lym). Values shown are the mean ± SEM. *Significant difference from DMSO, $P < 0.05$.

5.1.2. Andrographolide attenuates cigarette smoke-induced inflammatory cytokine and chemokine Level

IL-1β plays a critical role in the inflammatory response induced by cigarette smoke exposure. KC (CXCL1), IP-10 (CXCL10) and MCP-1 (CCL2) are key chemokines in attracting macrophages and neutrophils. BAL fluid levels of IL-1β, IP-10 (CXCL10), MCP-1 (CCL2) and KC (CXCL1) were significantly raised in cigarette smoke-exposed mice as compared to sham air control mice (Figure 5.4). Andrographolide was able to abate the BAL fluid levels of IL-1β, IP-10, MCP-1 and KC in a dose-dependent manner, reaching significant inhibition at the dose of 1 mg kg^{-1}.

5.1.3. Andrographolide attenuates cigarette smoke-induced inflammatory and proteolytic mediators' gene expression

Andrographolide (1 mg kg^{-1}) significantly suppressed the elevated gene expression of lung tissue GM-CSF, TNF-α and MIP-2α (as known as GRO-β or CXCL2) induced by cigarette smoking (Figure 5.5). Cigarette smoke also up-regulated the gene expression of inducible nitric oxide synthase (iNOS), a pro-oxidant enzyme responsible for NO production and subsequent oxidative lung damage. Andrographolide markedly reversed the iNOS gene expression down to basal level (Figure 5.5). Furthermore, the increase in gene expression of matrix metalloproteinase-12 (MMP-12) and tissue inhibitor of metalloproteinase-1 (TIMP-1), a metalloproteinase and an anti-metalloproteinase critical for lung remodelling and repair in COPD, by cigarette smoke was significantly inhibited by 1 mg kg^{-1} andrographolide (Figure 5.5).

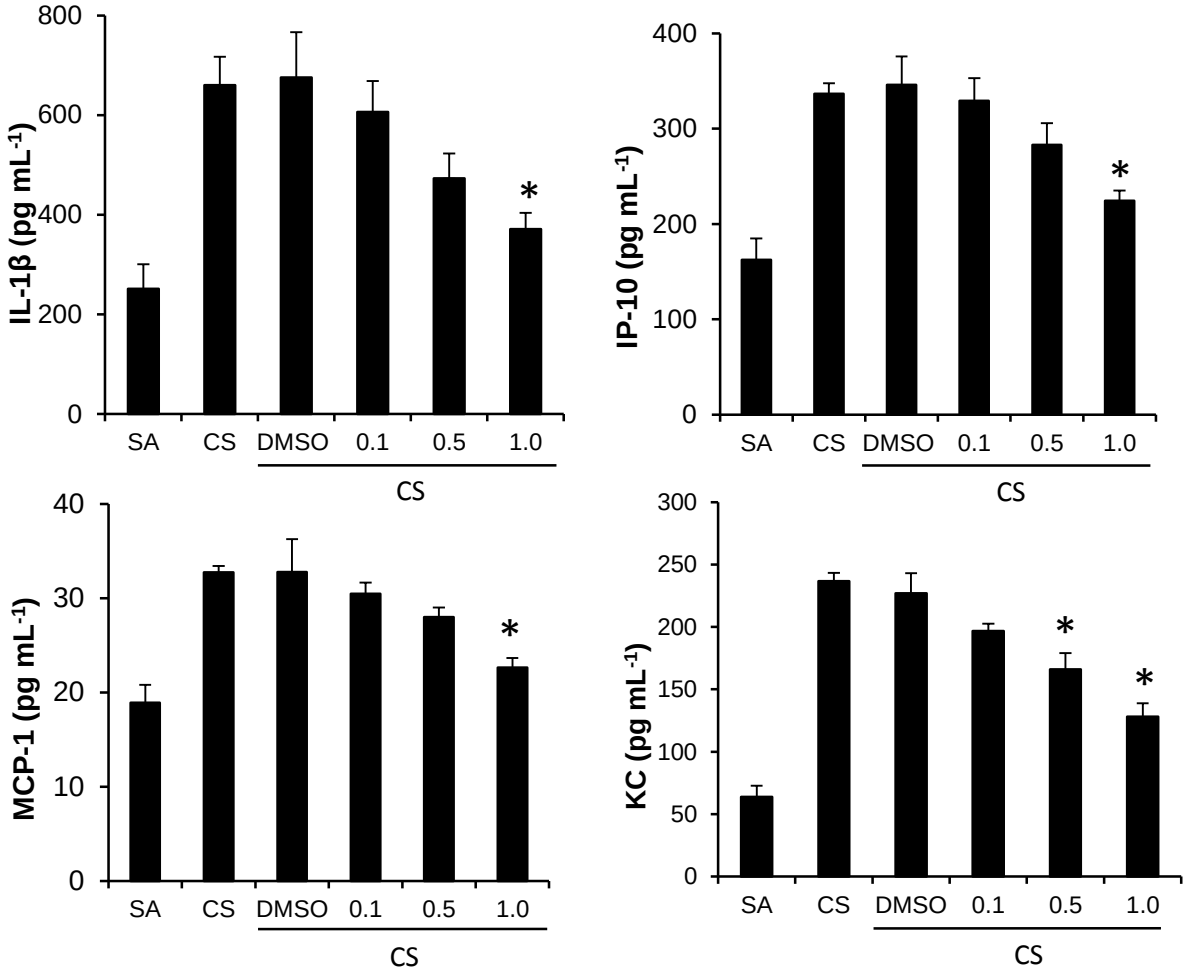


Figure 5.4. Effects of andrographolide on cigarette smoke BAL fluid cytokine and chemokine levels. BAL fluid levels of IL-1β, MCP-1, KC, and IP-10 were analyzed using ELISA (n = 6 mice per group). Lower limits of detection were as follows: IL-1β at 9 pg mL^{-1}; IP-10 at 15 pg mL^{-1}; and MCP-1 and KC at 16 pg mL^{-1}. Values shown are the mean ± SEM. *Significant difference from DMSO, $P < 0.05$.

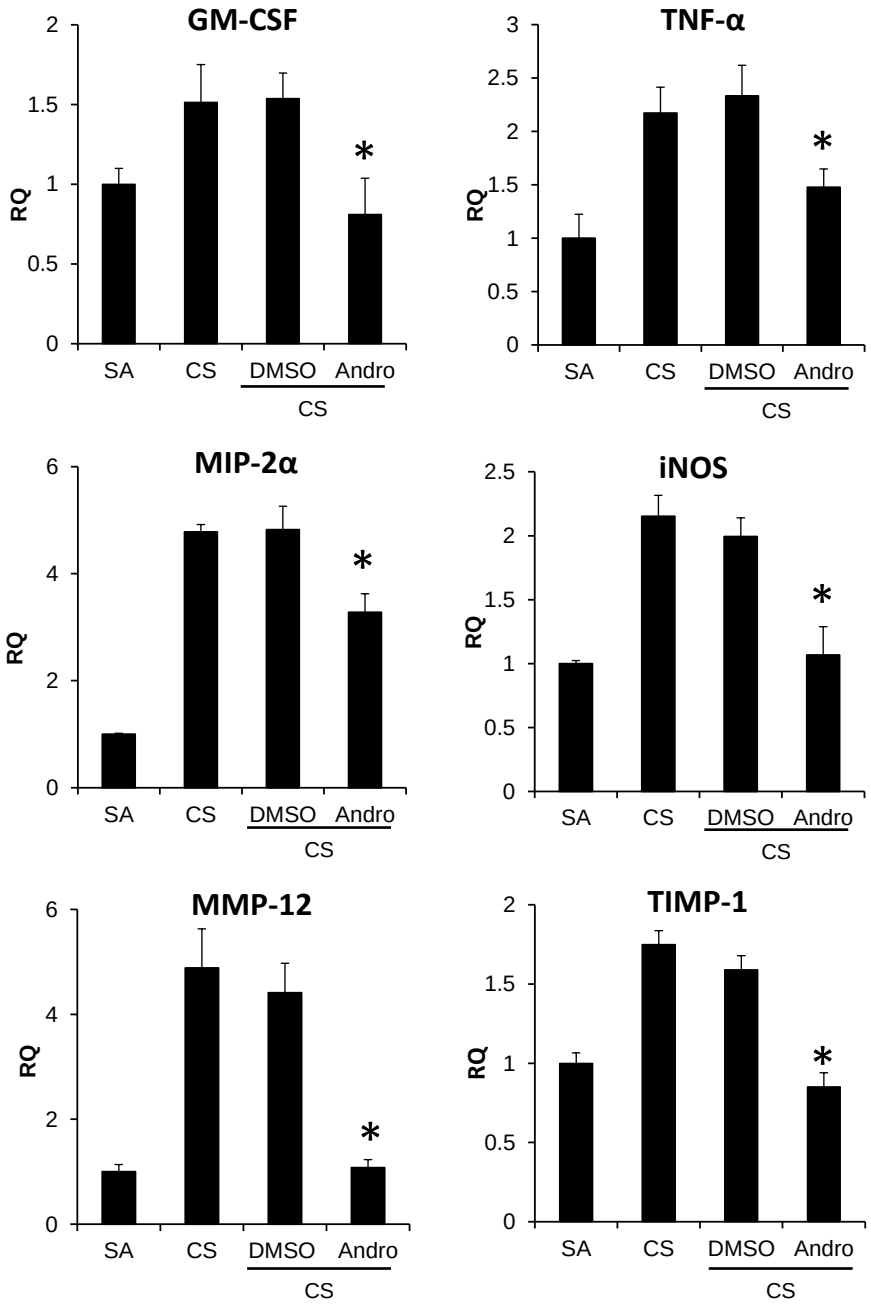


Figure 5.5. Effects of andrographolide on cigarette smoke-induced lung tissue pro-inflammatory and proteolytic mediator gene expression in mice. Real-time PCR analyses of cytokine, chemokine and protease gene expressions in lung tissues (n = 6 mice per group). The mRNA expression levels for all samples were normalized to the level of the housekeeping gene 18S. Values shown are the mean ± SEM. *Significant difference from DMSO, $P < 0.05$.

5.1.4. Andrographolide protects against cigarette smoke-induced oxidative lung damage

BAL fluid was used to assay for 3-NT, a product of protein nitration indicative of oxidative protein damage; 8-OHdG, a marker for oxidative DNA damage; and 8-isoprostane, an indicator for lipid peroxidation, using ELISA. Cigarette smoking significantly elevated BAL fluid levels of 3-NT, 8-OHdG and 8-isoprostane as compared to the sham air control (Figure 5.6). Andrographolide markedly suppressed the levels of 3-NT, 8-OHdG and 8-isoprostane at all three doses used, ameliorating oxidative damage to proteins, DNA and lipids induced by cigarette smoking.

5.1.5. Andrographolide augments the GPx and GR activities

Cigarette smoke exposure induced a marked adaptive increase in the lung catalase activity as compared to sham air. Andrographolide (1 mg kg^{-1}) significantly reduced the catalase activity. In contrast, lung SOD activity was not altered by cigarette smoke challenge or by andrographolide treatment (Figure 5.7). The activity of GPx, an antioxidant enzyme capable of reducing H_2O_2 to H_2O by oxidizing GSH, was not altered by cigarette smoke but was substantially augmented in cigarette smoke-exposed mouse lungs by andrographolide. On the other hand, lung GR activity was markedly abated by cigarette smoke exposure, but andrographolide treatment totally restored GR activity (Figure 5.7). In brief, andrographolide promoted the GSH-related enzyme activities, which may be attributable to its antioxidant effects.

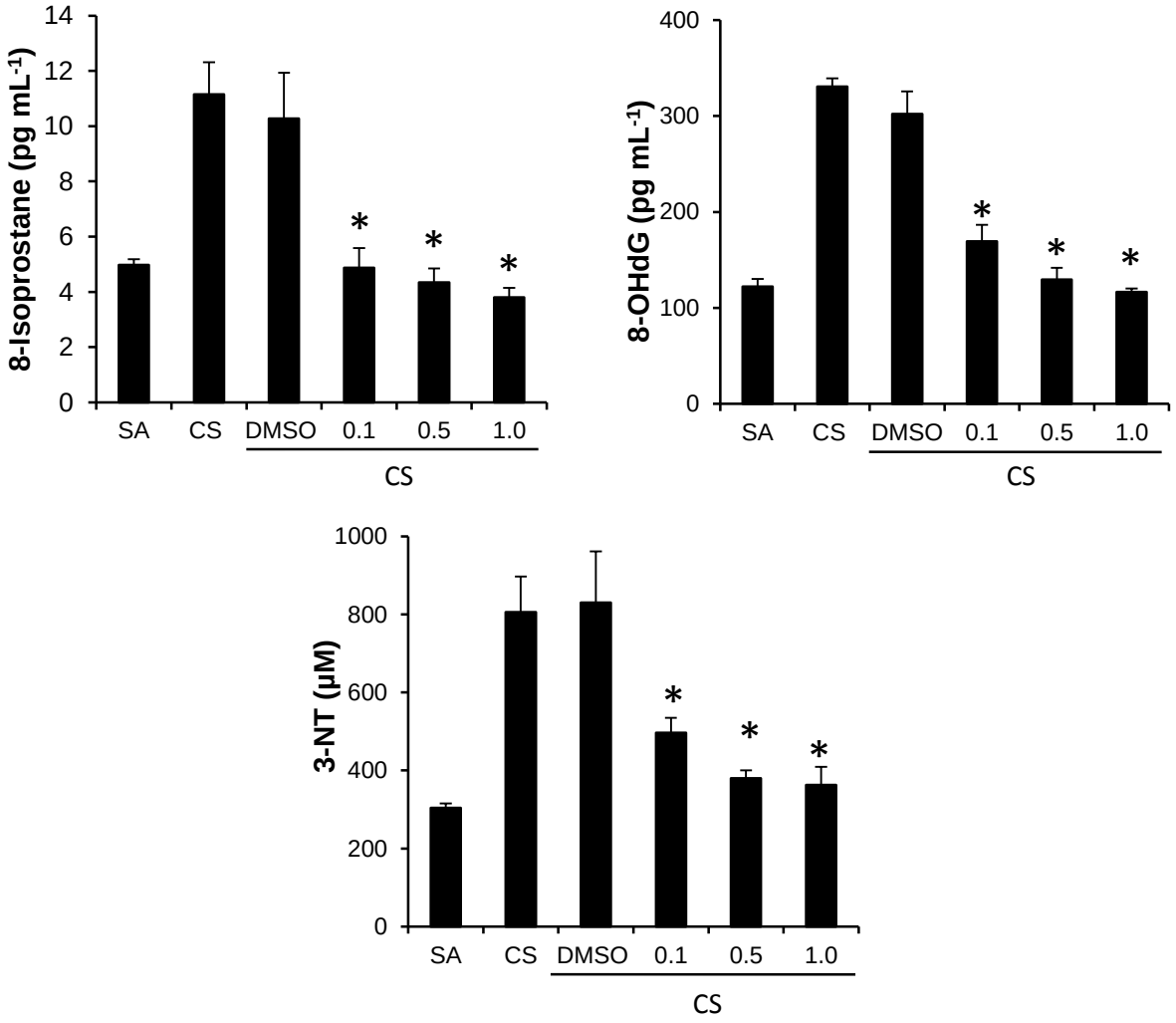


Figure 5.6. Effects of andrographolide on cigarette smoke-induced BAL fluid oxidative damage marker levels. 8-Isoprostane, 8-OHdG and 3-NT levels were measured using ELISA. Lower limits of detection were as follows: 8-isoprostane at 2.7 pg mL^{-1}; 8-OHdG at 33 pg mL^{-1}; and 3-NT at 0.1 μM. Values are means ± SEM for 6 mice per group. *Significant difference from DMSO, $P < 0.05$.

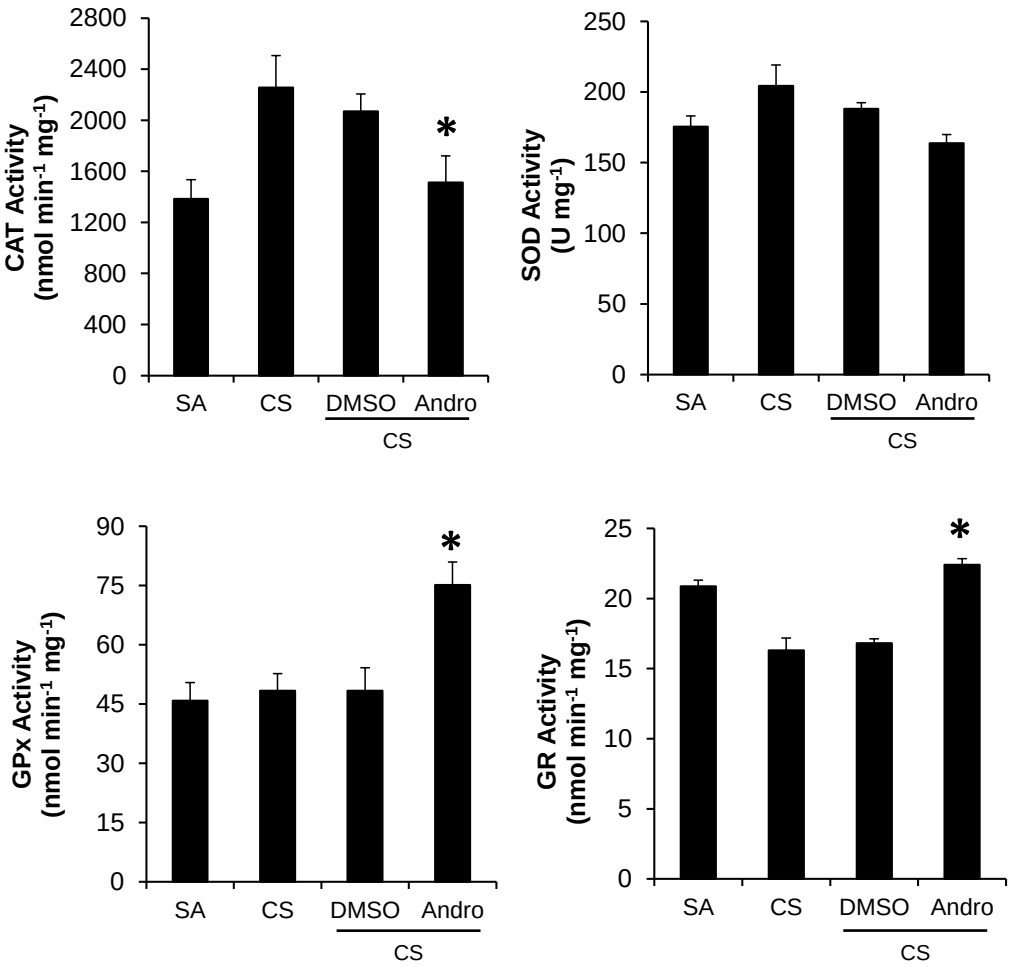


Figure 5.7. Effects of andrographolide on cigarette smoke-induced lung antioxidant enzymatic activities. Mice were exposed to 4% cigarette smoke for 1 h daily for 5 consecutive days with or without andrographolide treatment. Enzymatic activities of SOD, catalase, GPx and GR in lung tissues obtained 24 h after the last cigarette smoke challenge were measured using ELISA. Values are means ± SEM for 6 mice per group. *Significant difference from DMSO, $P < 0.05$.

5.1.6. Andrographolide promotes nuclear Nrf2 accumulation

To investigate the molecular mechanism of andrographolide anti-oxidative effects, we studied nuclear Nrf2 translocation and transactivation in BEAS-2B human bronchial epithelial cells. Addition of 2% CSE to BEAS-2B cells which don't have an impair Nrf2 system like those in COPD patients, resulted in marked nuclear translocation of Nrf2 at 4 hours, and andrographolide (30 μM) does not affect the nuclear Nrf2 level at this time point. Thus, the data suggest that andrographolide does not affect normal Nrf2 activation induced by cigarette smoke. In contrast, at 24 hours after CSE exposure, nuclear Nrf2 of CSE only and DMSO treated cells dropped to the basal level, but andrographolide treatment maintained the nuclear Nrf2 level elevated in BEAS-2B cells (Figure 5.8). On the other hand, cytoplasmic levels of Nrf2 were scarcely detectable in control and all treatment groups (Figure 5.8). This observation is expected as under basal conditions, Keap1 constantly targets Nrf2 for ubiquitination and proteasomal degradation. As a consequence, there are minimal cytoplasmic levels of Nrf2. To further substantiate the finding, Nrf2 ARE binding activities were studied using Nrf2 transactivation assay. The transactivation activities of Nrf2 were found to be significantly higher in andrographolide-treated BEAS-2B 24 hours after 2% CSE exposure (Figure 5.9).

5.1.7. Andrographolide promotes GSH level

Glutathione (GSH), a ubiquitous tripeptide thiol, is a vital cellular protective antioxidant in the lungs as the cellular stress response induced by cigarette smoke extract is critically dependent on the intracellular GSH concentration (Muller *et al.*, 1998). Cellular GSH level was also strongly elevated by andrographolide in BEAS-2B cells exposed to 2% CSE for 24 h (Figure 5.10). Our data suggest that andrographolide is able to sustain nuclear Nrf2 accumulation and activation in cells exposed to cigarette smoke, leading to augmented production of GSH.

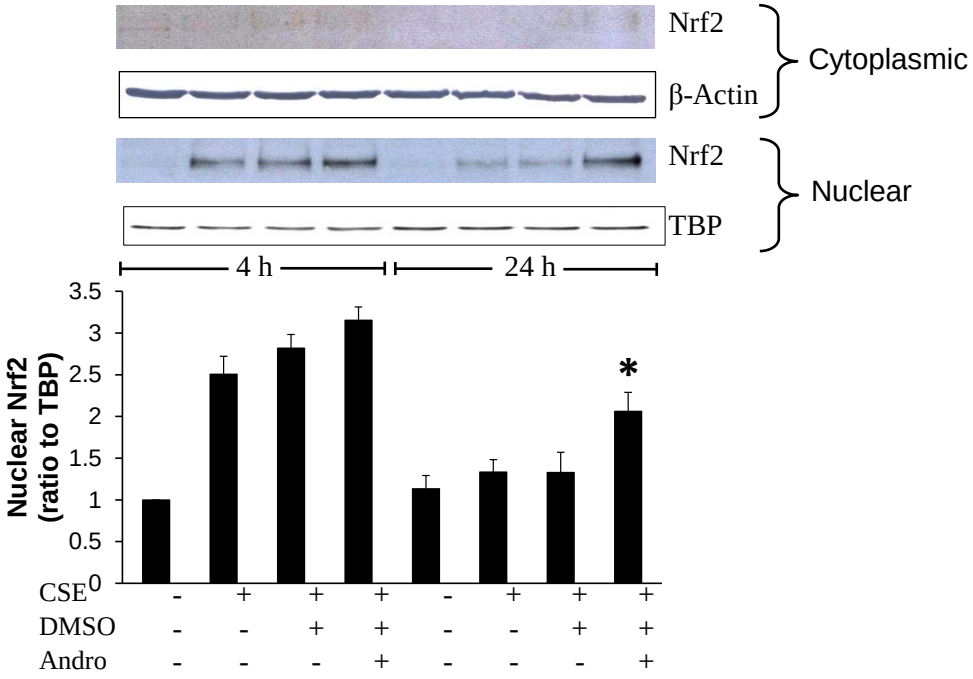


Figure 5.8. Effects of andrographolide on nuclear Nrf2 level. Cytoplasmic and nuclear extracts of BEAS-2B cells treated with 2% CSE in the presence and absence of 30 µM andrographolide for 4h and 24h were separated in 10% SDS-PAGE. Immunoblots were probed for Nrf2. β-actin and TBP were used as internal controls for cytosolic and nuclear proteins, respectively. The experiments were repeated four times with similar pattern of results. Protein band intensities were quantified using ImageJ software and were normalized to β-actin controls. Values shown are the mean ± SEM. *significant difference from DMSO, $P < 0.05$.

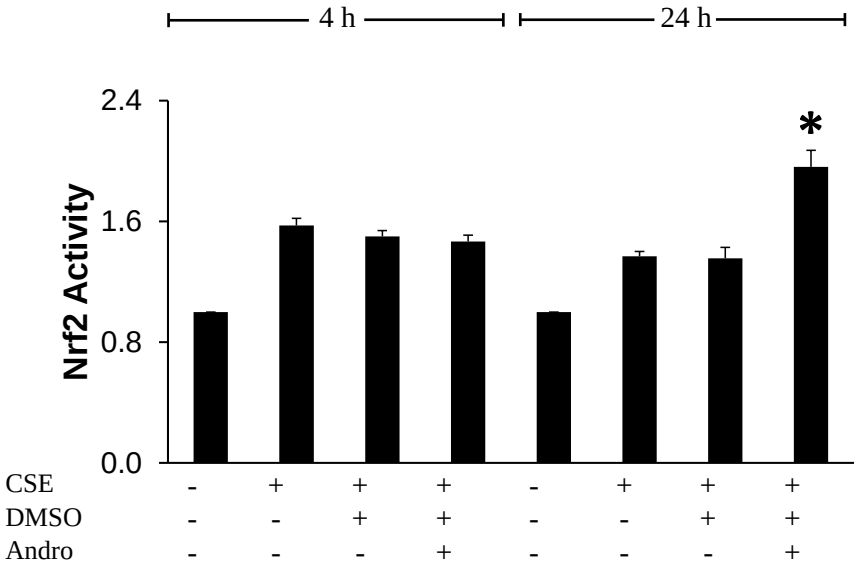


Figure 5.9. Effects of andrographolide on nuclear Nrf2 level. Nuclear extracts of BEAS-2B cells treated with 2% CSE in the presence and absence of 30 μM andrographolide for 4h and 24h ARE-binding activity of Nrf2 in nuclear extracts of BEAS-2B cells stimulated with 2% CSE in the presence and absence of 30 μM andrographolide for 4 h and 24 h was determined using a TransAM™ Nrf2 ELISA kit. *significant difference from DMSO, $P < 0.05$.

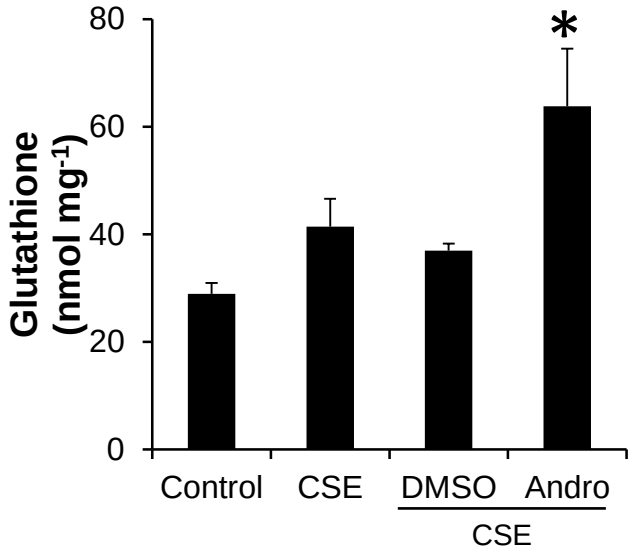


Figure 5.10. Effects of andrographolide on cellular GSH levels. Cellular GSH levels at 24 h after exposure to 2% CSE in the presence and absence of 30 μM andrographolide were measured using a GSH assay kit. Experiments were repeated four times and values are expressed as means ± SEM. *significant difference from DMSO, $P < 0.05$.

5.1.8. Andrographolide augments Nrf2-regulated gene targets

Nrf2 is the primary transcription factor that plays an important role in cellular defence against oxidative stress (Kobayashi *et al.*, 2005). Nrf2 is critical for maintaining the GSH redox state via transcriptional regulation of GR and in GSH level by regulating gene that essential in the de novo synthesis of GSH (Harvey *et al.*, 2009). To investigate biological responses to nuclear Nrf2 accumulation and activation, we examined Nrf2-specific antioxidant gene expressions including GR, GPx-2 and HO-1 as well as biosynthetic enzymes of the glutathione such as glutamate-cysteine ligase catalytic (GCLC) subunit and glutamate-cysteine ligase modifier (GCLM) subunit in BEAS-2B cells exposed to 2% CSE for 12 hours and 24 hours. Andrographolide treatment significantly enhanced the expression of antioxidants GCLM, GCLC, GR, GPx-2 and HO-1 in CSE-exposed BEAS-2B cells as compare to DMSO or CSE-exposed only BEAS-2B cells (Figure 5.11 and 5.12). Specifically, both GCLM and GCLC gene expression level was significantly increase at both 12 and 24 hours. Notably, andrographolide augmented gene expression of both GPx-2 by about 12 folds as compare to DMSO controls at 24 hours of CSE exposure and HO-1 by about 8 folds as compared to DMSO controls at 12 hours of CSE exposure. The observation further substantiate the notion that andrographolide treatment active Nrf2 as both GPx-2 and HO-1 are highly regulated by Nrf2 transactivational activities. Taken together, our results clearly indicate that andrographolide is a potent enhancer of antioxidant gene expression in response to cigarette smoke.

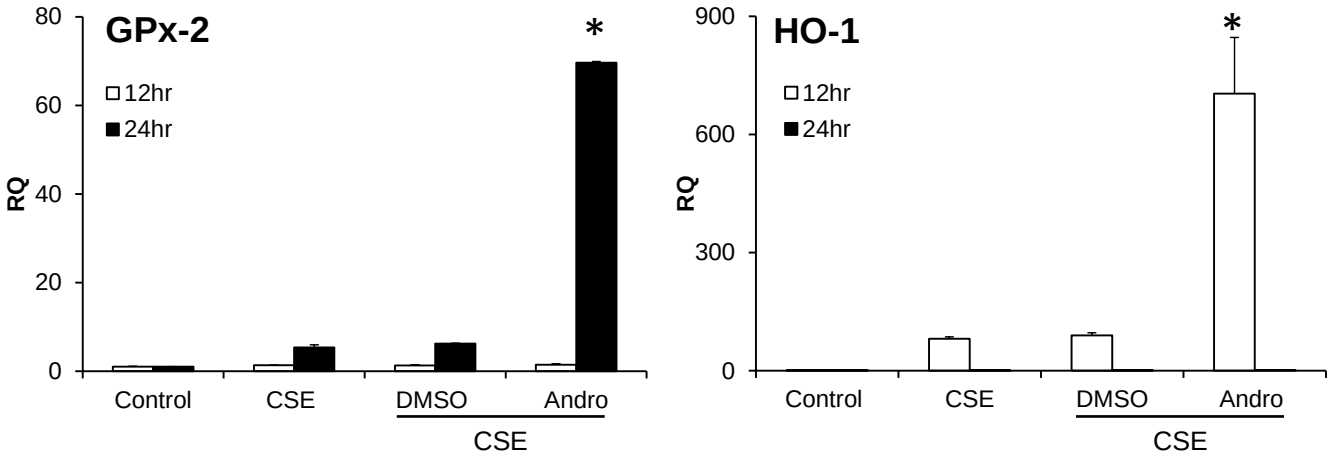


Figure 5.11. Effects of andrographolide on antioxidant gene expression. Total RNA was extracted from BEAS-2B cells treated with 2% CSE in the presence and absence of 30 μM andrographolide for 12 h and 24 h, and gene expression was quantified using real-time PCR and normalized to 18S control gene. Experiments were repeated three times and values are expressed as means ± SEM. *significant difference from DMSO, $P < 0.05$.

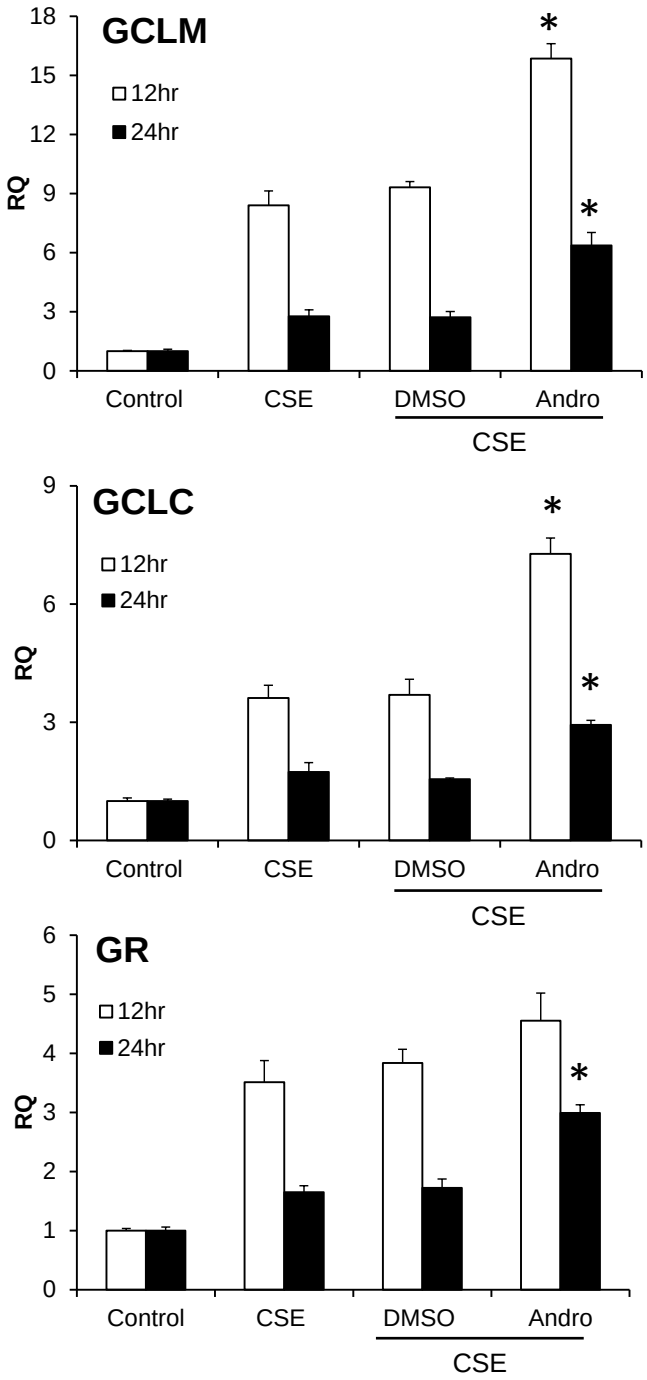


Figure 5.12. Effects of andrographolide on antioxidant gene expression. Total RNA was extracted from BEAS-2B cells treated with 2% CSE in the presence and absence of 30 μM andrographolide for 12 h and 24 h, and gene expression was quantified using real-time PCR and normalized to 18S control gene. Experiments were repeated three times and values are expressed as means ± SEM. *significant difference from DMSO, $P < 0.05$.

5.2. Discussion

Cigarette smoke contains high concentrations of potent oxidants and free radicals including reactive aldehyde, quinone, hydroquinone, semiquinone and superoxide (Cantin, 2010; Yao *et al.*, 2011). Exposure to cigarette smoke causes lung epithelial cell damage and triggers production of pro-inflammatory cytokines and chemokines, leading to inflammatory cell infiltration and activation, especially the neutrophils and macrophages. Both neutrophils and macrophages are rich sources of endogenous reactive oxygen species (ROS), reactive nitrogen species (RNS) and tissue proteases such as elastase and metalloproteinases, entailing additional oxidant burden to the lungs and facilitating destruction of alveolar walls. Our findings reveal for the first time that andrographolide prevented cigarette smoke-induced lung infiltration of neutrophils and, to a lesser extent, macrophages in a dose-dependent manner. The decrease in inflammatory cell accumulation could be associated with a decrease in BAL fluid cytokine and chemotactic factor.

Keratinocyte-derived chemokine (KC/CXCL1; an IL-8 homologue in mice) and macrophage inflammatory protein-2α (MIP-2α/CXCL2) are pivotal for neutrophil lung infiltration while monocyte chemotactic protein-1 (MCP-1/CCL2) and interferon-γ-inducible protein 10 (IP-10/CXCL10) are essential for recruitment of macrophages and monocytes (Brusselle *et al.*, 2011; Conti *et al.*, 1995; Cosio *et al.*, 2009; Dufour *et al.*, 2002; Lee *et al.*, 1995; Louhelainen *et al.*, 2008). Granulocyte-macrophage colony stimulating factor (GM-CSF) is another direct neutrophil chemotactic factor (Gomez-Cambronero *et al.*, 2003) and survival factor in the respiratory tract (Barnes *et al.*, 2003) that is up regulated by cigarette smoke (Vlahos *et al.*, 2006). Our findings demonstrated that andrographolide was able to suppress the increase of KC, IP-10, MCP-1, MIP-2α and GM-CSF in lungs from cigarette smoke-exposed mice.

Among the chemokine studied, MCP-1, KC, MIP-2α, IP-10, and GM-CSF can be stimulated by tumour necrosis factor-α (TNF-α) (Becker *et al.*, 1994; De Plaen *et al.*, 2006; Ohmori *et al.*, 1993; Standiford *et al.*, 1991). Cigarette smoke has been reported to induces the alveolar macrophages to release TNF-α (Keatings *et al.*, 1996), which is considered to be a key inflammatory mediator in lung pathology (Barnes, 2004) as the activation of TNF-α receptor also induces the production and release of several inflammatory mediators, such as Interleukin-1β (IL-1β), and protease such as MMP-12. Andrographolide treatment significantly attenuated the mRNA expression of both TNF-α and IL-1β.

We also observed a sharp rise in lung matrix metallopeptidase-12 (MMP-12) expression together with an adaptive increase in lung TIMP-1 expression in cigarette smoke-exposed mice. MMP-12, which is produced by macrophages and induced by the presence of IP-10 (Grumelli *et al.*, 2004), is able to degrade elastin, disrupting lung architecture leading to airspace enlargement (Hautamaki *et al.*, 1997; Mocchegiani *et al.*, 2011). The resulting elastin fragments can also lead to a positive feedback loop that further increasing macrophage recruitment (Hunninghake *et al.*, 2009). Mice deficient in MMP-12 were protected against cigarette smoke-induced lung inflammation and emphysema (Hautamaki *et al.*, 1997). An association study has strongly suggested that MMP12 plays a role in determining lung function and susceptibility to COPD in adult smokers (Hunninghake *et al.*, 2009). Nonetheless, histological examination of cigarette smoke exposed mouse lung was not performed as acute cigarette smoke exposure was shown not to cause a significant air space enlargement, a canonical feature in emphysema (Rajendrasozhan *et al.*, 2010). Andrographolide reversed the elevation of lung MMP-12 expression and the adaptive increase in TIMP-1 in cigarette smoke-exposed mice. These findings implicate a protective role of andrographolide against airway inflammation, remodeling and emphysema in COPD.

Oxidative stress emitted by the cigarette smoke and generated from the infiltrated macrophages and neutrophils play a pivotal role in the pathogenesis in COPD (Rahman *et al.*, 2006b). Excessive accumulation of ROS and RNS in the lungs results in protein denaturation, lipid peroxidation, and DNA damage as determined by the levels of 3-nitrotyrosine (3-NT) (Sugiura *et al.*, 2011), 8-isoprostane (Louhelainen *et al.*, 2008) and 8-hydroxydeoxyguanosine (8-OHdG) (Aoshiba *et al.*, 2012; Tzortzaki *et al.*, 2012), respectively in BAL fluid or in induced sputum. Lipid peroxidation, which leads to destruction of membrane lipid, is a well-established mechanism of cellular injury (Rahman *et al.*, 1996; Rahman *et al.*, 2002). Oxidative stress leads to peroxidation of arachidonic acid form a unique series of prostaglandin-like compound knows as isoprostanes. The isoprostanes possess potent biological activities, which includes bronchoconstriction and plasma exudation (Janssen, 2008). Moreover, patients with COPD have increased exhaled breath condensate 8-isoprostanes compared to healthy smokers and non-smokers (Montuschi *et al.*, 2000). Reactive oxygen species (ROS) attack guanine bases in DNA easily and form 8-OHdG (Cheng *et al.*, 2003) which can cross the cell membrane and is usually detected in the urine or serum of patients who have diseases associated with oxidative stress (Svoboda *et al.*, 2008). Indeed, sputum 8-OHdG was statistically significant increase in COPD when compared with non-COPD smokers and healthy subjects (Tzortzaki *et al.*, 2011). Nitrotyrosine on the other hand is an indicator of the involvement of reactive nitrogen species in irreversible oxidative reactions and has been associated with altered protein function (Davis *et al.*, 2002; Murata *et al.*, 2004). 3-nitrotyrosine is one of the most common products of the action of reactive nitrogen species on proteins. As peroxynitrite from cigarette smoke is able to nitrate tyrosine (Yamaguchi *et al.*, 2007), nitrotyrosine levels are increased in the sputum of patients with COPD compared to healthy subjects and those with asthma (Ichinose *et al.*, 2000). We observe that andrographolide completely blocked the increase of all three oxidant biomarkers

in BAL fluid from cigarette smoke-exposed mice in a dose-dependent manner. The suppression of 3-NT level can be partly explained by the inhibitory effect of andrographolide on the inducible nitric oxide synthase (iNOS) expression. The reduction in iNOS, partly due to the down regulation of cytokines such as TNF-α and IL-1β which regulate is expression (Radomski *et al.*, 1993), may leads to a drop in nitric oxide (NO) production, peroxynitrite ($ONOO^-$) formation, and protein nitration of tyrosine residues (Sugiura *et al.*, 2011). When nitric oxide is produced in high concentrations, such as with inducible NOS, it can react with oxygen or superoxide to form the highly reactive compounds nitrogen dioxide and peroxynitrite, which shifts the cellular redox potential to a more oxidized state. In addition, andrographolide has also demonstrated potent antioxidant activity by enhancing both glutathione peroxidase (GPx) and glutathione reductase (GR) activities in lung tissues from cigarette smoke-exposed mice, which further augment the lung antioxidant capacity. In agreement with our results, studies by Neogy et al.(Neogy *et al.*, 2008) demonstrated that intraperitoneal treatment of andrographolide significantly increase the glutathione peroxidase activities.

Cigarette smoke exposures induce both oxidative stress and inflammatory response. Administration of andrographolide in mice ameliorated oxidative stress in lungs, suppressing the subsequent up-regulation of chemokines and eventually reducing the neutrophil and macrophage influx. To investigate the molecular mechanism of antioxidant action for andrographolide, we studied the effects of andrographolide on nuclear translocation and activation of the redox-sensitive transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2) and the gene expression of Nrf2-regulated antioxidant gene targets in cigarette smoke extract (CSE)-treated human bronchial epithelial cells (BEAS-2B). Nrf2 are studied because it plays a predominant role in antioxidant protection against oxidative damage in the lungs incurred by cigarette smoking (Boutten *et al.*, 2011). Nrf2 gene disruption in mice resulted in

enhanced susceptibility to emphysema and oxidative stress induced by cigarette smoke exposure (Rangasamy *et al.*, 2004). More significantly, Nrf2 activities in patients with advanced COPD are reduced in peripheral lungs, resulting in reduced antioxidant responses and persistent oxidative stress. The reduction of Nrf2 activity may be due to a drop in the Nrf2-positive regulator DJ-1 level, which permits destabilization of Nrf2 protein via rapid proteasomal degradation by Keap1 (Malhotra *et al.*, 2008). Nonetheless, even in DJ-1-disrupted human epithelial cells, Nrf2 activator such as sulforaphane was able to enhance Nrf2 antioxidant defence in response to cigarette smoke (Malhotra *et al.*, 2008), indicating that Nrf2 activation is an attractive therapeutic approach to the treatment of COPD (Boutten *et al.*, 2011).

In line with other studies (Lee *et al.*, 2011; Taguchi *et al.*, 2011), our data show that cytoplasmic levels of Nrf2 in BEAS-2B cells of control and all treatment groups were almost undetectable. It is mainly due to rapid ubiquitination and proteasomal degradation of Nrf2 targeted by Kelch-like ECH-associated protein 1 (Keap1), a E3 ubiquitin ligase. At nuclear fraction however, andrographolide strongly promoted Nrf2 nuclear stabilization and accumulation, and binding to antioxidant response element (ARE) in BEAS-2B cells exposed to CSE for 24 hours. Indeed, andrographolide has been shown to elevate Nrf2 nuclear level in human endothelial cells (Yu *et al.*, 2010). Furthermore, in the process of developing a reporter gene assay for monitoring Nrf2 activation, andrographolide was found to be the most potent Nrf2 activator among 2000 biologically active compounds tested (Smirnova *et al.*, 2011). By enhancing Nrf2 protein nuclear accumulation and transactivational activity, our finding further substantiate the notion that andrographolide is an effective Nrf2 activator.

Glutathione (GSH) is an endogenous non-protein thiol with potent free radical scavenging capacity (Biljak *et al.*, 2010; Cantin, 2010; Gould *et al.*, 2011). GSH is an important intra- and extracellular lung antioxidant involved in maintenance of epithelial

integrity, and its deficiency leads to airway injury and epithelial damage (Rahman *et al.*, 1999). Andrographolide noticeably enhanced cellular level of GSH in BEAS-2B cells in response to CSE, providing a powerful first line antioxidant defence against cigarette smoke-induced epithelial damage. In agreement with our studies, andrographolide has also been shown to promote GSH level in nicotine-stimulated peripheral blood lymphocytes (Das *et al.*, 2009).

Consistent with the Nrf2 activation by andrographolide in BEAS-2B cells, we have also observed robust inductions of Nrf2-regulated gene targets including GCLC, GCLM, GR, GPx-2 and HO-1 (Adair-Kirk *et al.*, 2008; Hubner *et al.*, 2009) by andrographolide in response to CSE.

Glutamate-cycteine ligase (GCL), which is the rate-limiting enzyme in the GSH biosynthesis pathway, is a heterodimer comprising of the catalytic subunit (GCLC) and the regulatory subunit (GCLM) (Biljak *et al.*, 2010; Gould *et al.*, 2011). The increase of GSH level may be attributed to the increase in GCLC and GCLM expression as GCL contribute to the de-novo synthesis of GSH. In agreement with this result, overexpression of GCLM increases the cellular GSH, rendering cells resistant to oxidative stress (Tipnis *et al.*, 1999). Similarly, up-regulation of GCLC alone has also been reported to support high levels of intracellular GSH (Mulcahy *et al.*, 1995). Recently, overexpression of GCL in human granulosa tumor cells has been shown to increase total GSH levels and protect against H_2O_2-induced cell death (Cortes-Wanstreet *et al.*, 2009).

In addition, GSH also functions as a reducing substrate in the redox cycle to facilitate the reduction of H_2O_2 by GPx to H_2O and glutathione disulphide (GSSG), to prevent peroxide-induced DNA damage, lipid peroxidation and protein degradation. As the oxidative burden in the lungs of smokers has been estimated to be on the order of 10^{14} free radicals per puff (Church *et al.*, 1985) and many of these oxidants, such as relatively long-lived tar

semiquinone that can generate H_2O_2, GSH and GSH-related enzymes present in the lower respiratory tract are believed to act as a first line of defense against attacks by cigarette smoke (DeLeve *et al.,* 1990). GPx also have been shown to reduce of peroxynitrite to nitrite (Sies *et al.*, 1997) and thus further attenuating the free radical stress impose by cigarette smoke.

Beside de novo synthesis, GSH level are also sustain in the redox cycle by GR, which play an important role in reducing glutathione disulfide (GSSG) to the sulfhydryl form of glutathione (GSH) (Biljak *et al.,* 2010; Cantin, 2010). GPx and GR are important endogenous antioxidant enzymes responsible for the oxidative balance in the lungs in response to cigarette smoke (Biljak *et al.,* 2010; Gould *et al.,* 2011). It has been reported that GPx activity was markedly decreased in patients with COPD (Biljak *et al.,* 2010; Kluchova *et al.,* 2007; Santos *et al.,* 2004; Vibhuti *et al.,* 2007). It is noteworthy that andrographolide was able to augment the GPx-2 expression level by at least 12 folds in BEAS-2B cells in response to CSE stimulation. The observed slow rate of GPx-2 gene induction starting at 24 h is consistent with that reported by Singh *et al.* (Singh *et al.*, 2006b) that Nrf2 activation-induced GPx-2 expression occurred after 24 h and peaked at 72 h. In their studies, activation of Nrf2 by specific knock down of Keap1 by siRNA upregulated the expression of GPx-2, whereas Nrf2 siRNA down-regulated the expression of GPX2 in lung epithelial cells (Singh *et al.,* 2006b). The slow induction of GPx-2 might be due to the slow degradation rate of this protein (Chu *et al.*, 1999). Our findings clearly indicate that activation of Nrf2 activity leading to up-regulation of GSH level is an antioxidant mechanism of action for andrographolide against cigarette smoking.

Aside from the GSH cycle, andrographolide also induced a rapid 8-fold increase in HO-1 gene expression in BEAS-2B cells exposed to CSE within 12 h. Our findings are in line with those reported that HO-1 mRNA induction peaked at 6-8 h and returned to basal level at 24 h in human lung fibroblasts stimulated with CSE (Baglole *et al.,* 2008) and in human

bronchial epithelial cells treated with Nrf2 activator (Kumar *et al.*, 2011b). Under oxidative stress condition, free heme, a pro-oxidant catalysing production of free radicals, is released from hemoproteins to induce oxidative damages. HO-1 can be rapidly induced under the control of the Nrf2 transcription factor to catabolize heme into labile iron (Fe), carbon monoxide and biliverdin. The latter end product can be further converted by biliverdin reductase into bilirubin. All three reactive products have been shown to possess antioxidant and anti-inflammatory actions (Fredenburgh *et al.*, 2007; Gozzelino *et al.*, 2010). HO-1 overexpression in macrophages reduces TNF-α (Otterbein *et al.*, 2000) secretion and inhibits GM-CSF production in response to LPS stimulation (Sarady *et al.*, 2002). In addition, transgenic mice overexpressing HO-1 have reduced levels of macrophage inflammatory protein (MIP)-2 in BAL fluid during endotoxemia (Ryter *et al.*, 2007). More importanly, patients with COPD showed reduced levels of HO-1 in BAL fluid alveolar macrophages (Fredenburgh *et al.*, 2007). Adenovirus-mediated gene transfer of HO-1 in mice ameliorated elastase-induced inflammation and airspace enlargement (Shinohara *et al.*, 2005). Polymorphisms of the HO-1 promoter associated with reduced HO-1 expression have been linked to increased susceptibility to emphysema development (Exner *et al.*, 2004). The antioxidant actions of andrographolide against cigarette smoke-induced lung injury may also be linked to augmented HO-1 expression.

In conclusion, this study reveals for the first time that andrographolide can augment antioxidant gene expression and activities probably via up regulation of Nrf2 activity in experimental models of cigarette smoke exposure. Besides, inhibition of nuclear factor-κB (NF-κB) is another well-established mechanism of action for andrographolide to bring about anti-inflammatory effects (Bao *et al.*, 2009; Lim *et al.*, 2012), which may contribute to some of the protective effects observed in the present study. The reduction in cigarette smoke-induced lung oxidative damage afforded by andrographolide is likely associated with the

significant decline in BAL fluid inflammatory cell counts, in chemokine, cytokine and protease productions, and the up-regulation of GSH redox defence system and HO-1 expression. These findings support a novel therapeutic value for andrographolide in the treatment of COPD.

Although our COPD is an acute model, the study provides us with positive preliminary data. For future study, we should proceed with a chronic cigarette smoke-induced COPD mouse model with clinical features similar to COPD patients, such as reductions in Nrf2 protein and activities to further validate the therapeutic value of andrographolide in COPD.

6. CONCLUSIONS

General Discussion

Andrographolide and DDAG are both biologically active constituents that can be extracted from *Andrographis paniculata* (Lim *et al.*, 2012; Pholphana *et al.*, 2004). The plant extract have been traditionally used in upper respiratory tract infections. Current studies have further expanded our understanding on the effects of both of these compounds in an allergic asthma and COPD mouse models and the molecular targets involved.

Although DDAG and andrographolide are structurally similar, our present data showed that DDAG was less cytotoxic to tumour cell lines but still maintained similar anti-inflammatory effects as andrographolide in an allergic asthma mouse model (Bao *et al.*, 2009). There was however subtle difference between the anti-inflammatory effects of andrographolide and DDAG treatment in the allergic mouse models and the difference could be further explore to enhance our understanding on the mechanism of action for both of these compounds. Additionally, our studies also demonstrated that andrographolide have a strong anti-oxidative and anti-inflammatory effects on cigarette smoke induce oxidative damage and inflammation and thus position andrographolide as a multi-potential drug candidate.

Nonetheless, while DDAG was shown to be less cytotoxic than andrographolide, the effects of DDAG was not examined in the COPD mouse models as studies has demonstrated that andrographolide but not DDAG treatment protects cardiomyocyte against hypoxia and reoxygenation injury. Furthermore, only andrographolide but not DDAG treatment enhances the antioxidant potential of cardiomyocyte through the activities of a number of antioxidant enzymes and the GSH level (Woo *et al.*, 2008). Andrographolide also cost less than DDAG and therefore made a better drug candidate. Hence, only the effects of andrographolide in oxidative damage models were studied.

The differences between andrographolide and DDAG are very intriguing and could be further examined in future studies.

Conclusion

COPD and asthma are both obstructive respiratory diseases that have high mortality and morbidity but currently lack effective treatments. The effects of andrographolide on cigarette smoke induced lung injury model and the effects of DDAG on allergic asthma mouse model has been explore on this study.

This study demonstrates that andrographolide possesses anti-oxidative properties against cigarette smoke-induced lung injury possibly via the augmentation of Nrf2 activity. Upon andrographolide treatment, cigarette smoke induced oxidative stress markers are markedly reduced while both GR and GPx activities were augmented. Andrographolide also markedly reduced neutrophil and macrophages infiltration, inflammatory cytokine and protease production. Additionally, the significant increase of GPx-2 and HO-1 expression as well as the cellular GSH level by andrographolide treatment strongly exemplified a Nrf2 pathway activation.

This study also shown that DDAG as a non-cytotoxic analogue of andrographolide, retains the anti-inflammatory activities of andrographolide for the treatments of OVA induce allergic asthma via the inhibition of NF-κB. DDAG effectively reduce OVA induced inflammatory cell infiltration, BAL fluid Th2 cytokine and eotaxin level as well as serum total and OVA specific IgE level. Together with the reduction or AHR, the anti-inflammatory effects of DDAG were further reinforced by the reduction in pulmonary eosinophilia, mucus hypersecretion and mast cell degranulation observed histologically.

This study demonstrated that Nrf2 activation by andrographolide may have therapeutic potential for treating COPD while DDAG may be considered as a safer analogue of andrographolide for the potential treatment of asthma. Thus, it is crucial to further substantiate the effects of both of these compounds in chronic and exacerbation models, ideally under therapeutic dosing regimens, for a more complete assessment of these compounds'

therapeutic potential. These findings also warrant future research into both andrographolide and DDAG derivative as to further elucidate the active functional group in this compound so a more potent and safer therapeutic drug for both asthma and COPD patients can be developed.

References

(2011). Retraction of four articles and abstracts. *Am J Respir Crit Care Med* **183**(3)**:** 418.

Abboud RT, Vimalanathan S (2008). Pathogenesis of COPD. Part I. The role of protease-antiprotease imbalance in emphysema. *Int J Tuberc Lung Dis* **12**(4)**:** 361-367.

Abu-Ghefreh AA, Canatan H, Ezeamuzie CI (2009). In vitro and in vivo anti-inflammatory effects of andrographolide. *Int Immunopharmacol* **9**(3)**:** 313-318.

Ackerman V, Marini M, Vittori E, Bellini A, Vassali G, Mattoli S (1994). Detection of cytokines and their cell sources in bronchial biopsy specimens from asthmatic patients. Relationship to atopic status, symptoms, and level of airway hyperresponsiveness. *Chest* **105**(3)**:** 687-696.

Adair-Kirk TL, Atkinson JJ, Griffin GL, Watson MA, Kelley DG, DeMello D, *et al.* (2008). Distal airways in mice exposed to cigarette smoke: Nrf2-regulated genes are increased in Clara cells. *Am J Respir Cell Mol Biol* **39**(4)**:** 400-411.

Agusti A, Soriano JB (2008). COPD as a systemic disease. *Copd* **5**(2)**:** 133-138.

Akinbami LJ, Moorman JE, Bailey C, Zahran HS, King M, Johnson CA, *et al.* (2012). Trends in asthma prevalence, health care use, and mortality in the United States, 2001-2010. *NCHS Data Brief*(94)**:** 1-8.

Akinbami LJ, Moorman JE, Liu X (2011). Asthma prevalence, health care use, and mortality: United States, 2005-2009. *Natl Health Stat Report*(32)**:** 1-14.

Alcorn JF, Crowe CR, Kolls JK (2010). TH17 cells in asthma and COPD. *Annual review of physiology* **72:** 495-516.

Alon R, Kassner PD, Carr MW, Finger EB, Hemler ME, Springer TA (1995). The integrin VLA-4 supports tethering and rolling in flow on VCAM-1. *J Cell Biol* **128**(6)**:** 1243-1253.

Alsaeedi A, Sin DD, McAlister FA (2002). The effects of inhaled corticosteroids in chronic obstructive pulmonary disease: a systematic review of randomized placebo-controlled trials. *The American journal of medicine* **113**(1)**:** 59-65.

An L, Liu CT, Yu MJ, Chen ZH, Guo XG, Peng W, *et al.* (2012). Heme oxygenase-1 system, inflammation and ventilator-induced lung injury. *Eur J Pharmacol* **677**(1-3)**:** 1-4.

Angkasekwinai P, Chang SH, Thapa M, Watarai H, Dong C (2010). Regulation of IL-9 expression by IL-25 signaling. *Nat Immunol* **11**(3)**:** 250-256.

Aoshiba K, Zhou F, Tsuji T, Nagai A (2012). DNA damage as a molecular link in the pathogenesis of COPD in smokers. *Eur Respir J* **39**(6)**:** 1368-1376.

Aromdee C (2012). Modifications of andrographolide to increase some biological activities: a patent review (2006 - 2011). *Expert Opin Ther Pat* **22**(2)**:** 169-180.

Aromdee C, Sriubolmas N, Wiyakrutta S, Suebsasna S, Khunkitti W (2011a). Effect of the derivatives of andrographolide on the morphology of Bacillus subtilis. *Archives of pharmacal research* **34**(1)**:** 71-77.

Aromdee C, Suebsasana S, Ekalaksananan T, Pientong C, Thongchai S (2011b). Stage of action of naturally occurring andrographolides and their semisynthetic analogues against herpes simplex virus type 1 in vitro. *Planta medica* **77**(9)**:** 915-921.

Ather JL, Hodgkins SR, Janssen-Heininger YM, Poynter ME (2011). Airway epithelial NF-kappaB activation promotes allergic sensitization to an innocuous inhaled antigen. *Am J Respir Cell Mol Biol* **44**(5)**:** 631-638.

Baggiolini M, Dewald B, Moser B (1997). Human chemokines: an update. *Annual review of immunology* **15:** 675-705.

Baglole CJ, Sime PJ, Phipps RP (2008). Cigarette smoke-induced expression of heme oxygenase-1 in human lung fibroblasts is regulated by intracellular glutathione. *Am J Physiol Lung Cell Mol Physiol* **295**(4)**:** L624-636.

Balbi B, Bason C, Balleari E, Fiasella F, Pesci A, Ghio R, *et al.* (1997). Increased bronchoalveolar granulocytes and granulocyte/macrophage colony-stimulating factor during exacerbations of chronic bronchitis. *Eur Respir J* **10**(4)**:** 846-850.

Balhara J, Gounni AS (2012). The alveolar macrophages in asthma: a double-edged sword. *Mucosal immunology*.

Bao Z, Guan S, Cheng C, Wu S, Wong SH, Kemeny DM, *et al.* (2009). A novel antiinflammatory role for andrographolide in asthma via inhibition of the nuclear factor-kappaB pathway. *Am J Respir Crit Care Med* **179**(8)**:** 657-665.

Baraldo S, Turato G, Badin C, Bazzan E, Beghe B, Zuin R, *et al.* (2004). Neutrophilic infiltration within the airway smooth muscle in patients with COPD. *Thorax* **59**(4)**:** 308-312.

Barnes PJ (2000). Chronic obstructive pulmonary disease. *N Engl J Med* **343**(4)**:** 269-280.

Barnes PJ (2008a). The cytokine network in asthma and chronic obstructive pulmonary disease. *The Journal of clinical investigation* **118**(11)**:** 3546-3556.

Barnes PJ (2008b). Future treatments for chronic obstructive pulmonary disease and its comorbidities. *Proceedings of the American Thoracic Society* **5**(8)**:** 857-864.

Barnes PJ (2008c). Immunology of asthma and chronic obstructive pulmonary disease. *Nat Rev Immunol* **8**(3)**:** 183-192.

Barnes PJ (2004). Mediators of chronic obstructive pulmonary disease. *Pharmacol Rev* **56**(4)**:** 515-548.

Barnes PJ (2003). New concepts in chronic obstructive pulmonary disease. *Annu Rev Med* **54:** 113-129.

Barnes PJ (2010). New therapies for chronic obstructive pulmonary disease. *Med Princ Pract* **19**(5)**:** 330-338.

Barnes PJ, Chowdhury B, Kharitonov SA, Magnussen H, Page CP, Postma D, *et al.* (2006). Pulmonary biomarkers in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* **174**(1)**:** 6-14.

Barnes PJ, Shapiro SD, Pauwels RA (2003). Chronic obstructive pulmonary disease: molecular and cellular mechanisms. *Eur Respir J* **22**(4)**:** 672-688.

Bathoorn E, Slebos DJ, Postma DS, Koeter GH, van Oosterhout AJ, van der Toorn M, *et al.* (2007). Anti-inflammatory effects of inhaled carbon monoxide in patients with COPD: a pilot study. *Eur Respir J* **30**(6)**:** 1131-1137.

Battaglia S, Mauad T, van Schadewijk AM, Vignola AM, Rabe KF, Bellia V, *et al.* (2007). Differential distribution of inflammatory cells in large and small airways in smokers. *Journal of clinical pathology* **60**(8)**:** 907-911.

Becker S, Quay J, Koren HS, Haskill JS (1994). Constitutive and stimulated MCP-1, GRO alpha, beta, and gamma expression in human airway epithelium and bronchoalveolar macrophages. *Am J Physiol* **266**(3 Pt 1)**:** L278-286.

Beeh KM, Kornmann O, Buhl R, Culpitt SV, Giembycz MA, Barnes PJ (2003). Neutrophil chemotactic activity of sputum from patients with COPD: role of interleukin 8 and leukotriene B4. *Chest* **123**(4)**:** 1240-1247.

Belaaouaj A (2002). Neutrophil elastase-mediated killing of bacteria: lessons from targeted mutagenesis. *Microbes and infection / Institut Pasteur* **4**(12)**:** 1259-1264.

Benowitz NL (2009). Pharmacology of nicotine: addiction, smoking-induced disease, and therapeutics. *Annu Rev Pharmacol Toxicol* **49:** 57-71.

Berry M, Brightling C, Pavord I, Wardlaw A (2007). TNF-alpha in asthma. *Curr Opin Pharmacol* **7**(3)**:** 279-282.

Berry MA, Hargadon B, Shelley M, Parker D, Shaw DE, Green RH, *et al.* (2006). Evidence of a role of tumor necrosis factor alpha in refractory asthma. *N Engl J Med* **354**(7)**:** 697-708.

Besnard AG, Togbe D, Guillou N, Erard F, Quesniaux V, Ryffel B (2011). IL-33-activated dendritic cells are critical for allergic airway inflammation. *European journal of immunology* **41**(6)**:** 1675-1686.

Biljak VR, Rumora L, Cepelak I, Pancirov D, Popovic-Grle S, Soric J, *et al.* (2010). Glutathione cycle in stable chronic obstructive pulmonary disease. *Cell biochemistry and function* **28**(6)**:** 448-453.

Birrell MA, Hardaker E, Wong S, McCluskie K, Catley M, De Alba J, *et al.* (2005a). Ikappa-B kinase-2 inhibitor blocks inflammation in human airway smooth muscle and a rat model of asthma. *Am J Respir Crit Care Med* **172**(8)**:** 962-971.

Birrell MA, McCluskie K, Wong S, Donnelly LE, Barnes PJ, Belvisi MG (2005b). Resveratrol, an extract of red wine, inhibits lipopolysaccharide induced airway neutrophilia and inflammatory mediators through an NF-kappaB-independent mechanism. *Faseb J* **19**(7)**:** 840-841.

Birrell MA, Wong S, Hele DJ, McCluskie K, Hardaker E, Belvisi MG (2005c). Steroid-resistant inflammation in a rat model of chronic obstructive pulmonary disease is associated with a lack of nuclear factor-kappaB pathway activation. *American journal of respiratory and critical care medicine* **172**(1)**:** 74-84.

Bisgaard H, Hermansen MN, Loland L, Halkjaer LB, Buchvald F (2006). Intermittent inhaled corticosteroids in infants with episodic wheezing. *N Engl J Med* **354**(19)**:** 1998-2005.

Biswas SK, Rahman I (2009). Environmental toxicity, redox signaling and lung inflammation: the role of glutathione. *Mol Aspects Med* **30**(1-2)**:** 60-76.

Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL, Wolfson MF, *et al.* (1997). A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. *Nature* **385**(6618)**:** 729-733.

Blakey JD, Wardlaw AJ (2012). What is severe asthma? *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* **42**(5)**:** 617-624.

Bloemen K, Verstraelen S, Van Den Heuvel R, Witters H, Nelissen I, Schoeters G (2007). The allergic cascade: review of the most important molecules in the asthmatic lung. *Immunol Lett* **113**(1)**:** 6-18.

Boguniewicz M, Martin RJ, Martin D, Gibson U, Celniker A, Williams M, *et al.* (1995). The effects of nebulized recombinant interferon-gamma in asthmatic airways. *J Allergy Clin Immunol* **95**(1 Pt 1)**:** 133-135.

Borregaard N, Sorensen OE, Theilgaard-Monch K (2007). Neutrophil granules: a library of innate immunity proteins. *Trends Immunol* **28**(8)**:** 340-345.

Bousquet J, Clark TJ, Hurd S, Khaltaev N, Lenfant C, O'Byrne P, *et al.* (2007). GINA guidelines on asthma and beyond. *Allergy* **62**(2)**:** 102-112.

Boutten A, Goven D, Artaud-Macari E, Boczkowski J, Bonay M (2011). NRF2 targeting: a promising therapeutic strategy in chronic obstructive pulmonary disease. *Trends Mol Med* **17**(7)**:** 363-371.

Boyer L, Plantier L, Dagouassat M, Lanone S, Goven D, Caramelle P, *et al.* (2011). Role of nitric oxide synthases in elastase-induced emphysema. *Laboratory investigation; a journal of technical methods and pathology* **91**(3)**:** 353-362.

Braber S, Koelink PJ, Henricks PA, Jackson PL, Nijkamp FP, Garssen J, *et al.* (2011). Cigarette smoke-induced lung emphysema in mice is associated with prolyl endopeptidase, an enzyme involved in collagen breakdown. *American journal of physiology. Lung cellular and molecular physiology* **300**(2)**:** L255-265.

Brightling C, Berry M, Amrani Y (2008). Targeting TNF-alpha: a novel therapeutic approach for asthma. *J Allergy Clin Immunol* **121**(1)**:** 5-10; quiz 11-12.

Brightling CE, Ammit AJ, Kaur D, Black JL, Wardlaw AJ, Hughes JM, *et al.* (2005). The CXCL10/CXCR3 axis mediates human lung mast cell migration to asthmatic airway smooth muscle. *Am J Respir Crit Care Med* **171**(10)**:** 1103-1108.

Broide DH, Lawrence T, Doherty T, Cho JY, Miller M, McElwain K, *et al.* (2005). Allergen-induced peribronchial fibrosis and mucus production mediated by IkappaB kinase beta-dependent genes in airway epithelium. *Proc Natl Acad Sci U S A* **102**(49)**:** 17723-17728.

Brunnemann KD, Hoffmann D (1991). Analytical studies on tobacco-specific N-nitrosamines in tobacco and tobacco smoke. *Crit Rev Toxicol* **21**(4)**:** 235-240.

Brusselle GG, Joos GF, Bracke KR (2011). New insights into the immunology of chronic obstructive pulmonary disease. *Lancet* **378**(9795)**:** 1015-1026.

Busse WW (2010). The relationship of airway hyperresponsiveness and airway inflammation: Airway hyperresponsiveness in asthma: its measurement and clinical significance. *Chest* **138**(2 Suppl)**:** 4S-10S.

Busse WW, Morgan WJ, Gergen PJ, Mitchell HE, Gern JE, Liu AH, *et al.* (2011). Randomized trial of omalizumab (anti-IgE) for asthma in inner-city children. *The New England journal of medicine* **364**(11)**:** 1005-1015.

Byers DE, Holtzman MJ (2011). Alternatively activated macrophages and airway disease. *Chest* **140**(3)**:** 768-774.

Bystrom J, Amin K, Bishop-Bailey D (2011). Analysing the eosinophil cationic protein--a clue to the function of the eosinophil granulocyte. *Respir Res* **12:** 10.

Calabrese C, Berman SH, Babish JG, Ma X, Shinto L, Dorr M, *et al.* (2000). A phase I trial of andrographolide in HIV positive patients and normal volunteers. *Phytother Res* **14**(5)**:** 333-338.

Calabrese F, Giacometti C, Beghe B, Rea F, Loy M, Zuin R, *et al.* (2005). Marked alveolar apoptosis/proliferation imbalance in end-stage emphysema. *Respir Res* **6:** 14.

Calverley PM, Anderson JA, Celli B, Ferguson GT, Jenkins C, Jones PW, *et al.* (2007). Salmeterol and fluticasone propionate and survival in chronic obstructive pulmonary disease. *N Engl J Med* **356**(8)**:** 775-789.

Cantin AM (2010). Cellular response to cigarette smoke and oxidants: adapting to survive. *Proc Am Thorac Soc* **7**(6)**:** 368-375.

Caramori G, Lim S, Ito K, Tomita K, Oates T, Jazrawi E, *et al.* (2001). Expression of GATA family of transcription factors in T-cells, monocytes and bronchial biopsies. *Eur Respir J* **18**(3)**:** 466-473.

Cassol E, Cassetta L, Rizzi C, Alfano M, Poli G (2009). M1 and M2a polarization of human monocyte-derived macrophages inhibits HIV-1 replication by distinct mechanisms. *J Immunol* **182**(10)**:** 6237-6246.

Castaldi PJ, Demeo DL, Hersh CP, Lomas DA, Soerheim IC, Gulsvik A, *et al.* (2011). Impact of non-linear smoking effects on the identification of gene-by-smoking interactions in COPD genetics studies. *Thorax* **66**(10)**:** 903-909.

Cavarra E, Lucattelli M, Gambelli F, Bartalesi B, Fineschi S, Szarka A, *et al.* (2001). Human SLPI inactivation after cigarette smoke exposure in a new in vivo model of pulmonary oxidative stress. *Am J Physiol Lung Cell Mol Physiol* **281**(2)**:** L412-417.

Cazzola M, Calzetta L, Matera MG (2011). beta(2) -adrenoceptor agonists: current and future direction. *Br J Pharmacol* **163**(1)**:** 4-17.

Cederbom L, Hall H, Ivars F (2000). CD4+CD25+ regulatory T cells down-regulate co-stimulatory molecules on antigen-presenting cells. *European journal of immunology* **30**(6)**:** 1538-1543.

Cembrzynska-Nowak M, Szklarz E, Inglot AD, Teodorczyk-Injeyan JA (1993). Elevated release of tumor necrosis factor-alpha and interferon-gamma by bronchoalveolar leukocytes from patients with bronchial asthma. *The American review of respiratory disease* **147**(2)**:** 291-295.

Chai OH, Han EH, Lee HK, Song CH (2011). Mast cells play a key role in Th2 cytokine-dependent asthma model through production of adhesion molecules by liberation of TNF-alpha. *Exp Mol Med* **43**(1)**:** 35-43.

Chan KH, Ho SP, Yeung SC, So WH, Cho CH, Koo MW, *et al.* (2009). Chinese green tea ameliorates lung injury in cigarette smoke-exposed rats. *Respir Med* **103**(11)**:** 1746-1754.

Chang HC, Sehra S, Goswami R, Yao W, Yu Q, Stritesky GL, *et al.* (2010). The transcription factor PU.1 is required for the development of IL-9-producing T cells and allergic inflammation. *Nat Immunol* **11**(6)**:** 527-534.

Chang J, Zhang RM, Zhang Y, Chen ZB, Zhang ZM, Xu Q, *et al.* (2008a). [Andrographolide drop-pill in treatment of acute upper respiratory tract infection with external wind-heat syndrome: a multicenter and randomized controlled trial]. *Zhong Xi Yi Jie He Xue Bao* **6**(12)**:** 1238-1245.

Chang KT, Lii CK, Tsai CW, Yang AJ, Chen HW (2008b). Modulation of the expression of the pi class of glutathione S-transferase by Andrographis paniculata extracts and andrographolide. *Food Chem Toxicol* **46**(3)**:** 1079-1088.

Chang Y, Al-Alwan L, Risse PA, Halayko AJ, Martin JG, Baglole CJ, *et al.* (2012). Th17-associated cytokines promote human airway smooth muscle cell proliferation. *Faseb J*.

Chao WW, Kuo YH, Lin BF (2010a). Anti-inflammatory activity of new compounds from Andrographis paniculata by NF-kappaB transactivation inhibition. *Journal of agricultural and food chemistry* **58**(4)**:** 2505-2512.

Chao WW, Lin BF (2010b). Isolation and identification of bioactive compounds in Andrographis paniculata (Chuanxinlian). *Chin Med* **5:** 17.

Chapman RW, Minnicozzi M, Celly CS, Phillips JE, Kung TT, Hipkin RW, *et al.* (2007). A novel, orally active CXCR1/2 receptor antagonist, Sch527123, inhibits neutrophil recruitment, mucus production, and goblet cell hyperplasia in animal models of pulmonary inflammation. *J Pharmacol Exp Ther* **322**(2)**:** 486-493.

Chaudhuri R, Livingston E, McMahon AD, Thomson L, Borland W, Thomson NC (2003). Cigarette smoking impairs the therapeutic response to oral corticosteroids in chronic asthma. *Am J Respir Crit Care Med* **168**(11)**:** 1308-1311.

Chen X, Oppenheim JJ (2011). Contrasting effects of TNF and anti-TNF on the activation of effector T cells and regulatory T cells in autoimmunity. *FEBS Lett* **585**(23)**:** 3611-3618.

Cheng J, Montecalvo A, Kane LP (2011). Regulation of NF-kappaB induction by TCR/CD28. *Immunol Res* **50**(2-3)**:** 113-117.

Cheng TJ, Kao HP, Chan CC, Chang WP (2003). Effects of ozone on DNA single-strand breaks and 8-oxoguanine formation in A549 cells. *Environmental research* **93**(3)**:** 279-284.

Cho HY, Reddy SP, Kleeberger SR (2006). Nrf2 defends the lung from oxidative stress. *Antioxidants & redox signaling* **8**(1-2)**:** 76-87.

Cho SH, Stanciu LA, Holgate ST, Johnston SL (2005). Increased interleukin-4, interleukin-5, and interferon-gamma in airway CD4+ and CD8+ T cells in atopic asthma. *Am J Respir Crit Care Med* **171**(3)**:** 224-230.

Choi IW, Kim DK, Ko HM, Lee HK (2004). Administration of antisense phosphorothioate oligonucleotide to the p65 subunit of NF-kappaB inhibits established asthmatic reaction in mice. *Int Immunopharmacol* **4**(14)**:** 1817-1828.

Chu FF, Esworthy RS, Lee L, Wilczynski S (1999). Retinoic acid induces Gpx2 gene expression in MCF-7 human breast cancer cells. *J Nutr* **129**(10)**:** 1846-1854.

Chung KF (2006). Cytokines as targets in chronic obstructive pulmonary disease. *Curr Drug Targets* **7**(6)**:** 675-681.

Chupp GL, Lee CG, Jarjour N, Shim YM, Holm CT, He S, *et al.* (2007). A chitinase-like protein in the lung and circulation of patients with severe asthma. *N Engl J Med* **357**(20)**:** 2016-2027.

Church DF, Pryor WA (1985). Free-radical chemistry of cigarette smoke and its toxicological implications. *Environ Health Perspect* **64:** 111-126.

Church LD, McDermott MF (2009). Canakinumab, a fully-human mAb against IL-1beta for the potential treatment of inflammatory disorders. *Current opinion in molecular therapeutics* **11**(1)**:** 81-89.

Churg A, Wang RD, Tai H, Wang X, Xie C, Dai J, *et al.* (2003). Macrophage metalloelastase mediates acute cigarette smoke-induced inflammation via tumor necrosis factor-alpha release. *American journal of respiratory and critical care medicine* **167**(8)**:** 1083-1089.

Churg A, Wright JL (2007). Animal models of cigarette smoke-induced chronic obstructive lung disease. *Contrib Microbiol* **14:** 113-125.

Chustz RT, Nagarkar DR, Poposki JA, Favoreto S, Jr., Avila PC, Schleimer RP, *et al.* (2011). Regulation and function of the IL-1 family cytokine IL-1F9 in human bronchial epithelial cells. *Am J Respir Cell Mol Biol* **45**(1)**:** 145-153.

Circu ML, Aw TY (2010). Reactive oxygen species, cellular redox systems, and apoptosis. *Free Radic Biol Med* **48**(6)**:** 749-762.

Cockcroft DW, Hargreave FE, O'Byrne PM, Boulet LP (2007). Understanding allergic asthma from allergen inhalation tests. *Canadian respiratory journal : journal of the Canadian Thoracic Society* **14**(7)**:** 414-418.

Cohn L, Elias JA, Chupp GL (2004). Asthma: mechanisms of disease persistence and progression. *Annu Rev Immunol* **22:** 789-815.

Conti P, Boucher W, Letourneau R, Feliciani C, Reale M, Barbacane RC, *et al.* (1995). Monocyte chemotactic protein-1 provokes mast cell aggregation and [3H]5HT release. *Immunology* **86**(3)**:** 434-440.

Cook-Mills JM, Marchese ME, Abdala-Valencia H (2011). Vascular cell adhesion molecule-1 expression and signaling during disease: regulation by reactive oxygen species and antioxidants. *Antioxidants & redox signaling* **15**(6)**:** 1607-1638.

Coon JT, Ernst E (2004). Andrographis paniculata in the treatment of upper respiratory tract infections: a systematic review of safety and efficacy. *Planta Med* **70**(4)**:** 293-298.

Corry DB, Irvin CG (2006). Promise and pitfalls in animal-based asthma research: building a better mousetrap. *Immunol Res* **35**(3)**:** 279-294.

Cortes-Wanstreet MM, Giedzinski E, Limoli CL, Luderer U (2009). Overexpression of glutamate-cysteine ligase protects human COV434 granulosa tumour cells against oxidative and gamma-radiation-induced cell death. *Mutagenesis* **24**(3)**:** 211-224.

Cosio MG, Saetta M, Agusti A (2009). Immunologic aspects of chronic obstructive pulmonary disease. *N Engl J Med* **360**(23)**:** 2445-2454.

Covar RA (2007). Bronchoprovocation testing in asthma. *Immunology and allergy clinics of North America* **27**(4)**:** 633-649; vi-vii.

Culpitt SV, Rogers DF, Shah P, De Matos C, Russell RE, Donnelly LE, *et al.* (2003). Impaired inhibition by dexamethasone of cytokine release by alveolar macrophages from patients with chronic obstructive pulmonary disease. *American journal of respiratory and critical care medicine* **167**(1)**:** 24-31.

Culver BH (2011). Obstructive? Restrictive? Or a ventilatory impairment? *Chest* **140**(3)**:** 568-569.

Dahl M, Bowler RP, Juul K, Crapo JD, Levy S, Nordestgaard BG (2008). Superoxide dismutase 3 polymorphism associated with reduced lung function in two large populations. *Am J Respir Crit Care Med* **178**(9)**:** 906-912.

Dai GF, Xu HW, Wang JF, Liu FW, Liu HM (2006). Studies on the novel alpha-glucosidase inhibitory activity and structure-activity relationships for andrographolide analogues. *Bioorg Med Chem Lett* **16**(10)**:** 2710-2713.

Das J, Chen CH, Yang L, Cohn L, Ray P, Ray A (2001). A critical role for NF-kappa B in GATA3 expression and TH2 differentiation in allergic airway inflammation. *Nat Immunol* **2**(1)**:** 45-50.

Das S, Neogy S, Gautam N, Roy S (2009). In vitro nicotine induced superoxide mediated DNA fragmentation in lymphocytes: protective role of Andrographis paniculata Nees. *Toxicol In Vitro* **23**(1)**:** 90-98.

Davis IC, Zhu S, Sampson JB, Crow JP, Matalon S (2002). Inhibition of human surfactant protein A function by oxidation intermediates of nitrite. *Free Radic Biol Med* **33**(12)**:** 1703-1713.

De Plaen IG, Han XB, Liu X, Hsueh W, Ghosh S, May MJ (2006). Lipopolysaccharide induces CXCL2/macrophage inflammatory protein-2 gene expression in enterocytes via NF-kappaB activation: independence from endogenous TNF-alpha and platelet-activating factor. *Immunology* **118**(2)**:** 153-163.

Decramer M, Rutten-van Molken M, Dekhuijzen PN, Troosters T, van Herwaarden C, Pellegrino R, *et al.* (2005). Effects of N-acetylcysteine on outcomes in chronic obstructive pulmonary disease (Bronchitis Randomized on NAC Cost-Utility Study, BRONCUS): a randomised placebo-controlled trial. *Lancet* **365**(9470)**:** 1552-1560.

Deguchi JO, Aikawa E, Libby P, Vachon JR, Inada M, Krane SM, *et al.* (2005). Matrix metalloproteinase-13/collagenase-3 deletion promotes collagen accumulation and organization in mouse atherosclerotic plaques. *Circulation* **112**(17)**:** 2708-2715.

Dekhuijzen PN, van Beurden WJ (2006). The role for N-acetylcysteine in the management of COPD. *International journal of chronic obstructive pulmonary disease* **1**(2)**:** 99-106.

DeLeve LD, Kaplowitz N (1990). Importance and regulation of hepatic glutathione. *Semin Liver Dis* **10**(4)**:** 251-266.

Demedts IK, Demoor T, Bracke KR, Joos GF, Brusselle GG (2006). Role of apoptosis in the pathogenesis of COPD and pulmonary emphysema. *Respir Res* **7:** 53.

Dentener MA, Creutzberg EC, Pennings HJ, Rijkers GT, Mercken E, Wouters EF (2008). Effect of infliximab on local and systemic inflammation in chronic obstructive pulmonary disease: a pilot study. *Respiration; international review of thoracic diseases* **76**(3)**:** 275-282.

Desai D, Brightling C (2009). Cytokine and anti-cytokine therapy in asthma: ready for the clinic? *Clin Exp Immunol* **158**(1)**:** 10-19.

Deshmane SL, Kremlev S, Amini S, Sawaya BE (2009). Monocyte chemoattractant protein-1 (MCP-1): an overview. *J Interferon Cytokine Res* **29**(6)**:** 313-326.

Desmet C, Gosset P, Pajak B, Cataldo D, Bentires-Alj M, Lekeux P, *et al.* (2004). Selective blockade of NF-kappa B activity in airway immune cells inhibits the effector phase of experimental asthma. *J Immunol* **173**(9)**:** 5766-5775.

Di Stefano A, Capelli A, Lusuardi M, Balbo P, Vecchio C, Maestrelli P, *et al.* (1998). Severity of airflow limitation is associated with severity of airway inflammation in smokers. *Am J Respir Crit Care Med* **158**(4)**:** 1277-1285.

Di Stefano A, Caramori G, Gnemmi I, Contoli M, Bristot L, Capelli A, *et al.* (2009). Association of increased CCL5 and CXCL7 chemokine expression with neutrophil activation in severe stable COPD. *Thorax* **64**(11)**:** 968-975.

Di Stefano A, Maestrelli P, Roggeri A, Turato G, Calabro S, Potena A, *et al.* (1994). Upregulation of adhesion molecules in the bronchial mucosa of subjects with chronic obstructive bronchitis. *Am J Respir Crit Care Med* **149**(3 Pt 1)**:** 803-810.

Doganci A, Sauer K, Karwot R, Finotto S (2005). Pathological role of IL-6 in the experimental allergic bronchial asthma in mice. *Clinical reviews in allergy & immunology* **28**(3)**:** 257-270.

Donohue JF, Menjoge S, Kesten S (2003). Tolerance to bronchodilating effects of salmeterol in COPD. *Respir Med* **97**(9)**:** 1014-1020.

Dournes G, Laurent F (2012). Airway Remodelling in Asthma and COPD: Findings, Similarities, and Differences Using Quantitative CT. *Pulm Med* **2012:** 670414.

Drannik AG, Pouladi MA, Robbins CS, Goncharova SI, Kianpour S, Stampfli MR (2004). Impact of cigarette smoke on clearance and inflammation after Pseudomonas aeruginosa infection. *Am J Respir Crit Care Med* **170**(11)**:** 1164-1171.

Drost EM, Skwarski KM, Sauleda J, Soler N, Roca J, Agusti A, *et al.* (2005). Oxidative stress and airway inflammation in severe exacerbations of COPD. *Thorax* **60**(4)**:** 293-300.

Dufour JH, Dziejman M, Liu MT, Leung JH, Lane TE, Luster AD (2002). IFN-gamma-inducible protein 10 (IP-10; CXCL10)-deficient mice reveal a role for IP-10 in effector T cell generation and trafficking. *Journal of immunology* **168**(7)**:** 3195-3204.

Edwards MR, Bartlett NW, Clarke D, Birrell M, Belvisi M, Johnston SL (2009). Targeting the NF-kappaB pathway in asthma and chronic obstructive pulmonary disease. *Pharmacology & therapeutics* **121**(1)**:** 1-13.

Egan PJ, van Nieuwenhuijze A, Campbell IK, Wicks IP (2008). Promotion of the local differentiation of murine Th17 cells by synovial macrophages during acute inflammatory arthritis. *Arthritis Rheum* **58**(12)**:** 3720-3729.

El-Zein RA, Young RP, Hopkins RJ, Etzel CJ (2012). Genetic predisposition to chronic obstructive pulmonary disease and/or lung cancer: important considerations when evaluating risk. *Cancer Prev Res (Phila)* **5**(4)**:** 522-527.

Erin EM, Leaker BR, Nicholson GC, Tan AJ, Green LM, Neighbour H, *et al.* (2006). The effects of a monoclonal antibody directed against tumor necrosis factor-alpha in asthma. *American journal of respiratory and critical care medicine* **174**(7)**:** 753-762.

Exner M, Minar E, Wagner O, Schillinger M (2004). The role of heme oxygenase-1 promoter polymorphisms in human disease. *Free radical biology & medicine* **37**(8)**:** 1097-1104.

Fabbri LM, Romagnoli M, Corbetta L, Casoni G, Busljetic K, Turato G, *et al.* (2003). Differences in airway inflammation in patients with fixed airflow obstruction due to asthma or chronic obstructive pulmonary disease. *American journal of respiratory and critical care medicine* **167**(3)**:** 418-424.

Finotto S, Neurath MF, Glickman JN, Qin S, Lehr HA, Green FH, *et al.* (2002). Development of spontaneous airway changes consistent with human asthma in mice lacking T-bet. *Science* **295**(5553)**:** 336-338.

Flood-Page P, Swenson C, Faiferman I, Matthews J, Williams M, Brannick L, *et al.* (2007). A study to evaluate safety and efficacy of mepolizumab in patients with moderate persistent asthma. *Am J Respir Crit Care Med* **176**(11)**:** 1062-1071.

Foronjy R, Wallace A, D'Armiento J (2008). The pharmokinetic limitations of antioxidant treatment for COPD. *Pulm Pharmacol Ther* **21**(2)**:** 370-379.

Foster PS, Hogan SP, Ramsay AJ, Matthaei KI, Young IG (1996). Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. *J Exp Med* **183**(1)**:** 195-201.

Fredenburgh LE, Perrella MA, Mitsialis SA (2007). The role of heme oxygenase-1 in pulmonary disease. *American journal of respiratory cell and molecular biology* **36**(2)**:** 158-165.

Fujisawa T, Velichko S, Thai P, Hung LY, Huang F, Wu R (2009). Regulation of airway MUC5AC expression by IL-1beta and IL-17A; the NF-kappaB paradigm. *J Immunol* **183**(10)**:** 6236-6243.

Gaffen SL (2009). Structure and signalling in the IL-17 receptor family. *Nat Rev Immunol* **9**(8)**:** 556-567.

Gagliardo R, Chanez P, Mathieu M, Bruno A, Costanzo G, Gougat C, *et al.* (2003). Persistent activation of nuclear factor-kappaB signaling pathway in severe uncontrolled asthma. *Am J Respir Crit Care Med* **168**(10)**:** 1190-1198.

Gallelli L, Falcone D, Pelaia G, Renda T, Terracciano R, Malara N, *et al.* (2008). Interleukin-6 receptor superantagonist Sant7 inhibits TGF-beta-induced proliferation of human lung fibroblasts. *Cell proliferation* **41**(3)**:** 393-407.

Galli SJ, Grimbaldeston M, Tsai M (2008a). Immunomodulatory mast cells: negative, as well as positive, regulators of immunity. *Nat Rev Immunol* **8**(6)**:** 478-486.

Galli SJ, Kalesnikoff J, Grimbaldeston MA, Piliponsky AM, Williams CM, Tsai M (2005). Mast cells as "tunable" effector and immunoregulatory cells: recent advances. *Annual review of immunology* **23:** 749-786.

Galli SJ, Tsai M, Piliponsky AM (2008b). The development of allergic inflammation. *Nature* **454**(7203)**:** 445-454.

Gan WQ, Man SF, Postma DS, Camp P, Sin DD (2006). Female smokers beyond the perimenopausal period are at increased risk of chronic obstructive pulmonary disease: a systematic review and meta-analysis. *Respir Res* **7:** 52.

Gaschler GJ, Skrtic M, Zavitz CC, Lindahl M, Onnervik PO, Murphy TF, *et al.* (2009). Bacteria challenge in smoke-exposed mice exacerbates inflammation and skews the inflammatory profile. *Am J Respir Crit Care Med* **179**(8)**:** 666-675.

Gaschler GJ, Zavitz CC, Bauer CM, Stampfli MR (2010). Mechanisms of clearance of nontypeable Haemophilus influenzae from cigarette smoke-exposed mouse lungs. *Eur Respir J* **36**(5)**:** 1131-1142.

Geiser T, Dewald B, Ehrengruber MU, Clark-Lewis I, Baggiolini M (1993). The interleukin-8-related chemotactic cytokines GRO alpha, GRO beta, and GRO gamma activate human neutrophil and basophil leukocytes. *The Journal of biological chemistry* **268**(21)**:** 15419-15424.

Georas SN, Guo J, De Fanis U, Casolaro V (2005). T-helper cell type-2 regulation in allergic disease. *The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology* **26**(6)**:** 1119-1137.

Gernez Y, Tirouvanziam R, Chanez P (2010). Neutrophils in chronic inflammatory airway diseases: can we target them and how? *Eur Respir J* **35**(3)**:** 467-469.

GINA (2011). From the Global Strategy for Asthma Management and Prevention, Global Initiative for Asthma (GINA).

Goh FY, Upton N, Guan S, Cheng C, Shanmugam MK, Sethi G, *et al.* (2012). Fisetin, a bioactive flavonol, attenuates allergic airway inflammation through negative regulation of NF-kappaB. *Eur J Pharmacol* **679**(1-3)**:** 109-116.

GOLD (2011). Global strategy for the diagnosis, management and prevention of chronic obstructive pulmonary disease (GOLD).

Gomez-Cambronero J, Horn J, Paul CC, Baumann MA (2003). Granulocyte-macrophage colony-stimulating factor is a chemoattractant cytokine for human neutrophils: involvement of the ribosomal p70 S6 kinase signaling pathway. *J Immunol* **171**(12)**:** 6846-6855.

Gosset P, Tsicopoulos A, Wallaert B, Vannimenus C, Joseph M, Tonnel AB, *et al.* (1991). Increased secretion of tumor necrosis factor alpha and interleukin-6 by alveolar macrophages consecutive to the development of the late asthmatic reaction. *The Journal of allergy and clinical immunology* **88**(4)**:** 561-571.

Gould HJ, Sutton BJ (2008). IgE in allergy and asthma today. *Nat Rev Immunol* **8**(3)**:** 205-217.

Gould NS, Min E, Gauthier S, Martin RJ, Day BJ (2011). Lung glutathione adaptive responses to cigarette smoke exposure. *Respir Res* **12:** 133.

Gozzelino R, Jeney V, Soares MP (2010). Mechanisms of cell protection by heme oxygenase-1. *Annu Rev Pharmacol Toxicol* **50:** 323-354.

Gross P, Pfitzer EA, Tolker E, Babyak MA, Kaschak M (1965). Experimental Emphysema: Its Production with Papain in Normal and Silicotic Rats. *Arch Environ Health* **11:** 50-58.

Grumelli S, Corry DB, Song LZ, Song L, Green L, Huh J, *et al.* (2004). An immune basis for lung parenchymal destruction in chronic obstructive pulmonary disease and emphysema. *PLoS Med* **1**(1)**:** e8.

Grunig G, Warnock M, Wakil AE, Venkayya R, Brombacher F, Rennick DM, *et al.* (1998). Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* **282**(5397)**:** 2261-2263.

Gualano RC, Hansen MJ, Vlahos R, Jones JE, Park-Jones RA, Deliyannis G, *et al.* (2008). Cigarette smoke worsens lung inflammation and impairs resolution of influenza infection in mice. *Respir Res* **9:** 53.

Guan SP, Mok YK, Koo KN, Chu KL, Wong WS (2009). Chitinases: biomarkers for human diseases. *Protein and peptide letters* **16**(5)**:** 490-498.

Guilbert TW, Morgan WJ, Zeiger RS, Mauger DT, Boehmer SJ, Szefler SJ, *et al.* (2006). Long-term inhaled corticosteroids in preschool children at high risk for asthma. *N Engl J Med* **354**(19)**:** 1985-1997.

Gyllfors P, Dahlen SE, Kumlin M, Larsson K, Dahlen B (2006). Bronchial responsiveness to leukotriene D4 is resistant to inhaled fluticasone propionate. *The Journal of allergy and clinical immunology* **118**(1)**:** 78-83.

Hackett TL, Holloway R, Holgate ST, Warner JA (2008). Dynamics of pro-inflammatory and anti-inflammatory cytokine release during acute inflammation in chronic obstructive pulmonary disease: an ex vivo study. *Respir Res* **9:** 47.

Haldar P, Brightling CE, Hargadon B, Gupta S, Monteiro W, Sousa A, *et al.* (2009). Mepolizumab and exacerbations of refractory eosinophilic asthma. *The New England journal of medicine* **360**(10)**:** 973-984.

Hamelmann E, Oshiba A, Loader J, Larsen GL, Gleich G, Lee J, *et al.* (1997). Antiinterleukin-5 antibody prevents airway hyperresponsiveness in a murine model of airway sensitization. *Am J Respir Crit Care Med* **155**(3)**:** 819-825.

Hamilton JA (2002). GM-CSF in inflammation and autoimmunity. *Trends Immunol* **23**(8)**:** 403-408.

Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN (2009). House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat Med* **15**(4)**:** 410-416.

Hammad H, Lambrecht BN (2008). Dendritic cells and epithelial cells: linking innate and adaptive immunity in asthma. *Nat Rev Immunol* **8**(3)**:** 193-204.

Hancke J, Burgos R, Caceres D, Wikman G (1995). A double-blind study with a new monodrug Kan Jang: Decrease of symptoms and improvement in the recovery from common colds. *Phytotherapy Research* **9**(8)**:** 559-562.

Handa SS, Sharma A (1990). Hepatoprotective activity of andrographolide against galactosamine & paracetamol intoxication in rats. *The Indian journal of medical research* **92:** 284-292.

Hansbro PM, Kaiko GE, Foster PS (2011). Cytokine/anti-cytokine therapy - novel treatments for asthma? *Br J Pharmacol* **163**(1)**:** 81-95.

Hansel TT, Tennant RC, Tan AJ, Higgins LA, Neighbour H, Erin EM, *et al.* (2004). Theophylline: mechanism of action and use in asthma and chronic obstructive pulmonary disease. *Drugs Today (Barc)* **40**(1)**:** 55-69.

Hansen NC, Skriver A, Brorsen-Riis L, Balslov S, Evald T, Maltbaek N, *et al.* (1994). Orally administered N-acetylcysteine may improve general well-being in patients with mild chronic bronchitis. *Respir Med* **88**(7)**:** 531-535.

Hargreave FE (2007). Quantitative sputum cell counts as a marker of airway inflammation in clinical practice. *Curr Opin Allergy Clin Immunol* **7**(1)**:** 102-106.

Harju T, Mazur W, Merikallio H, Soini Y, Kinnula VL (2008). Glutathione-S-transferases in lung and sputum specimens, effects of smoking and COPD severity. *Respir Res* **9:** 80.

Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, *et al.* (2005). Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nature immunology* **6**(11)**:** 1123-1132.

Harrison TW, Oborne J, Newton S, Tattersfield AE (2004). Doubling the dose of inhaled corticosteroid to prevent asthma exacerbations: randomised controlled trial. *Lancet* **363**(9405)**:** 271-275.

Hart LA, Krishnan VL, Adcock IM, Barnes PJ, Chung KF (1998). Activation and localization of transcription factor, nuclear factor-kappaB, in asthma. *Am J Respir Crit Care Med* **158**(5 Pt 1)**:** 1585-1592.

Hartl D, Koller B, Mehlhorn AT, Reinhardt D, Nicolai T, Schendel DJ, *et al.* (2007). Quantitative and functional impairment of pulmonary CD4+CD25hi regulatory T cells in pediatric asthma. *J Allergy Clin Immunol* **119**(5)**:** 1258-1266.

Harvey CJ, Thimmulappa RK, Singh A, Blake DJ, Ling G, Wakabayashi N, *et al.* (2009). Nrf2-regulated glutathione recycling independent of biosynthesis is critical for cell survival during oxidative stress. *Free radical biology & medicine* **46**(4)**:** 443-453.

Hattesohl AD, Jorres RA, Dressel H, Schmid S, Vogelmeier C, Greulich T, *et al.* (2011). Discrimination between COPD patients with and without alpha 1-antitrypsin deficiency using an electronic nose. *Respirology* **16**(8)**:** 1258-1264.

Hautamaki RD, Kobayashi DK, Senior RM, Shapiro SD (1997). Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice. *Science* **277**(5334)**:** 2002-2004.

He X, Li, J. K., Gao, H., Qiu, F., Hu, K., Cui, X. M. and Yao, X. S. (2003). Six new andrographolide metabolites in rats. *Chem. Pharm. Bull. (Tokyo)* **51:** 586-589.

Hellermann GR, Nagy SB, Kong X, Lockey RF, Mohapatra SS (2002). Mechanism of cigarette smoke condensate-induced acute inflammatory response in human bronchial epithelial cells. *Respir Res* **3:** 22.

Hellings PW, Kasran A, Liu Z, Vandekerckhove P, Wuyts A, Overbergh L, *et al.* (2003). Interleukin-17 orchestrates the granulocyte influx into airways after allergen inhalation in a mouse model of allergic asthma. *Am J Respir Cell Mol Biol* **28**(1)**:** 42-50.

Herbert C, Scott MM, Scruton KH, Keogh RP, Yuan KC, Hsu K, *et al.* (2010). Alveolar macrophages stimulate enhanced cytokine production by pulmonary CD4+ T-lymphocytes in an exacerbation of murine chronic asthma. *Am J Pathol* **177**(4)**:** 1657-1664.

Hirai H, Kubo H, Yamaya M, Nakayama K, Numasaki M, Kobayashi S, *et al.* (2003). Microsatellite polymorphism in heme oxygenase-1 gene promoter is associated with susceptibility to oxidant-induced apoptosis in lymphoblastoid cell lines. *Blood* **102**(5)**:** 1619-1621.

Ho IC, Pai SY (2007). GATA-3 - not just for Th2 cells anymore. *Cellular & molecular immunology* **4**(1)**:** 15-29.

Ho WE, Cheng C, Peh HY, Xu F, Tannenbaum SR, Ong CN, *et al.* (2012). Anti-malarial drug artesunate ameliorates oxidative lung damage in experimental allergic asthma. *Free radical biology & medicine* **53**(3)**:** 498-507.

Hogan SP, Rosenberg HF, Moqbel R, Phipps S, Foster PS, Lacy P, *et al.* (2008). Eosinophils: biological properties and role in health and disease. *Clin Exp Allergy* **38**(5)**:** 709-750.

Hogg JC, Chu F, Utokaparch S, Woods R, Elliott WM, Buzatu L, *et al.* (2004). The nature of small-airway obstruction in chronic obstructive pulmonary disease. *The New England journal of medicine* **350**(26)**:** 2645-2653.

Holgate ST (2008). Pathogenesis of asthma. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* **38**(6)**:** 872-897.

Hoshino T, Toda R, Aizawa H (2009). Pharmacological treatment in asthma and COPD. *Allergol Int* **58**(3)**:** 341-346.

Hu L, Dixit VD, de Mello-Coelho V, Taub DD (2004). Age-associated alterations in CXCL1 chemokine expression by murine B cells. *BMC immunology* **5:** 15.

Huang TJ, MacAry PA, Kemeny DM, Chung KF (1999). Effect of CD8+ T-cell depletion on bronchial hyper-responsiveness and inflammation in sensitized and allergen-exposed Brown-Norway rats. *Immunology* **96**(3)**:** 416-423.

Hubner RH, Schwartz JD, De Bishnu P, Ferris B, Omberg L, Mezey JG, *et al.* (2009). Coordinate control of expression of Nrf2-modulated genes in the human small airway epithelium is highly responsive to cigarette smoking. *Mol Med* **15**(7-8)**:** 203-219.

Hunninghake GM, Cho MH, Tesfaigzi Y, Soto-Quiros ME, Avila L, Lasky-Su J, *et al.* (2009). MMP12, lung function, and COPD in high-risk populations. *N Engl J Med* **361**(27)**:** 2599-2608.

Huvenne W, Lanckacker EA, Krysko O, Bracke KR, Demoor T, Hellings PW, *et al.* (2011). Exacerbation of cigarette smoke-induced pulmonary inflammation by Staphylococcus aureus enterotoxin B in mice. *Respir Res* **12:** 69.

Ichinose M (2009). Differences of inflammatory mechanisms in asthma and COPD. *Allergol Int* **58**(3)**:** 307-313.

Ichinose M, Sugiura H, Yamagata S, Koarai A, Shirato K (2000). Increase in reactive nitrogen species production in chronic obstructive pulmonary disease airways. *American journal of respiratory and critical care medicine* **162**(2 Pt 1)**:** 701-706.

Ilmarinen-Salo P, Moilanen E, Kankaanranta H (2010). Nitric oxide induces apoptosis in GM-CSF-treated eosinophils via caspase-6-dependent lamin and DNA fragmentation. *Pulm Pharmacol Ther* **23**(4)**:** 365-371.

Imai K, Mercer BA, Schulman LL, Sonett JR, D'Armiento JM (2005). Correlation of lung surface area to apoptosis and proliferation in human emphysema. *Eur Respir J* **25**(2)**:** 250-258.

Inonu H, Doruk S, Sahin S, Erkorkmaz U, Celik D, Celikel S, *et al.* (2012). Oxidative stress levels in exhaled breath condensate associated with COPD and smoking. *Respir Care* **57**(3)**:** 413-419.

Iruretagoyena MI, Tobar JA, Gonzalez PA, Sepulveda SE, Figueroa CA, Burgos RA, *et al.* (2005). Andrographolide interferes with T cell activation and reduces experimental autoimmune encephalomyelitis in the mouse. *J Pharmacol Exp Ther* **312**(1)**:** 366-372.

Isogai S, Jedrzkiewicz S, Taha R, Hamid Q, Martin JG (2005). Resident CD8+ T cells suppress CD4+ T cell-dependent late allergic airway responses. *J Allergy Clin Immunol* **115**(3)**:** 521-526.

Isogai S, Rubin A, Maghni K, Ramos-Barbon D, Taha R, Yoshizawa Y, *et al.* (2003). The effects of CD8+gammadelta T cells on late allergic airway responses and airway inflammation in rats. *J Allergy Clin Immunol* **112**(3)**:** 547-555.

Isogai S, Taha R, Tamaoka M, Yoshizawa Y, Hamid Q, Martin JG (2004). CD8+ alphabeta T cells can mediate late airway responses and airway eosinophilia in rats. *J Allergy Clin Immunol* **114**(6)**:** 1345-1352.

Janssen LJ (2008). Isoprostanes and lung vascular pathology. *American journal of respiratory cell and molecular biology* **39**(4)**:** 383-389.

Jiang H, Xie Y, Abel PW, Toews ML, Townley RG, Casale TB, *et al.* (2012). Targeting Phosphoinositide 3-Kinase gamma in Airway Smooth Muscle Cells to Suppress Interleukin-13-Induced Mouse Airway Hyperresponsiveness. *J Pharmacol Exp Ther* **342**(2)**:** 305-311.

Jiang X, Yu P, Jiang J, Zhang Z, Wang Z, Yang Z, *et al.* (2009). Synthesis and evaluation of antibacterial activities of andrographolide analogues. *Eur J Med Chem* **44**(7)**:** 2936-2943.

Joetham A, Takeda K, Taube C, Miyahara N, Matsubara S, Koya T, *et al.* (2007). Naturally occurring lung CD4(+)CD25(+) T cell regulation of airway allergic responses depends on IL-10 induction of TGF-beta. *J Immunol* **178**(3)**:** 1433-1442.

John AE, Zhu YM, Brightling CE, Pang L, Knox AJ (2009). Human airway smooth muscle cells from asthmatic individuals have CXCL8 hypersecretion due to increased NF-kappa B p65, C/EBP beta, and RNA polymerase II binding to the CXCL8 promoter. *J Immunol* **183**(7)**:** 4682-4692.

Joshi AD, Oak SR, Hartigan AJ, Finn WG, Kunkel SL, Duffy KE, *et al.* (2010). Interleukin-33 contributes to both M1 and M2 chemokine marker expression in human macrophages. *BMC immunology* **11:** 52.

Juhn YJ (2012). Influence of asthma epidemiology on the risk for other diseases. *Allergy Asthma Immunol Res* **4**(3)**:** 122-131.

Jung CH, Jung H, Shin YC, Park JH, Jun CY, Kim HM, *et al.* (2007). Eleutherococcus senticosus extract attenuates LPS-induced iNOS expression through the inhibition of Akt and JNK pathways in murine macrophage. *Journal of ethnopharmacology* **113**(1)**:** 183-187.

Justice JP, Crosby J, Borchers MT, Tomkinson A, Lee JJ, Lee NA (2002). CD4(+) T cell-dependent airway mucus production occurs in response to IL-5 expression in lung. *Am J Physiol Lung Cell Mol Physiol* **282**(5)**:** L1066-1074.

Kaur D, Saunders R, Berger P, Siddiqui S, Woodman L, Wardlaw A, *et al.* (2006). Airway smooth muscle and mast cell-derived CC chemokine ligand 19 mediate airway smooth muscle migration in asthma. *American journal of respiratory and critical care medicine* **174**(11)**:** 1179-1188.

Kearley J, Barker JE, Robinson DS, Lloyd CM (2005). Resolution of airway inflammation and hyperreactivity after in vivo transfer of CD4+CD25+ regulatory T cells is interleukin 10 dependent. *J Exp Med* **202**(11)**:** 1539-1547.

Kearley J, Buckland KF, Mathie SA, Lloyd CM (2009). Resolution of allergic inflammation and airway hyperreactivity is dependent upon disruption of the T1/ST2-IL-33 pathway. *Am J Respir Crit Care Med* **179**(9)**:** 772-781.

Keatings VM, Collins PD, Scott DM, Barnes PJ (1996). Differences in interleukin-8 and tumor necrosis factor-alpha in induced sputum from patients with chronic obstructive pulmonary disease or asthma. *Am J Respir Crit Care Med* **153**(2)**:** 530-534.

Kelly M, Hwang JM, Kubes P (2007). Modulating leukocyte recruitment in inflammation. *J Allergy Clin Immunol* **120**(1)**:** 3-10.

Klemm S, Ruland J (2006). Inflammatory signal transduction from the Fc epsilon RI to NF-kappa B. *Immunobiology* **211**(10)**:** 815-820.

Kluchova Z, Petrasova D, Joppa P, Dorkova Z, Tkacova R (2007). The association between oxidative stress and obstructive lung impairment in patients with COPD. *Physiol Res* **56**(1)**:** 51-56.

Ko HC, Wei BL, Chiou WF (2006). The effect of medicinal plants used in Chinese folk medicine on RANTES secretion by virus-infected human epithelial cells. *Journal of ethnopharmacology* **107**(2)**:** 205-210.

Kobayashi M, Yamamoto M (2005). Molecular mechanisms activating the Nrf2-Keap1 pathway of antioxidant gene regulation. *Antioxidants & redox signaling* **7**(3-4)**:** 385-394.

Kode A, Rajendrasozhan S, Caito S, Yang SR, Megson IL, Rahman I (2008). Resveratrol induces glutathione synthesis by activation of Nrf2 and protects against cigarette smoke-mediated oxidative stress in human lung epithelial cells. *American journal of physiology. Lung cellular and molecular physiology* **294**(3)**:** L478-488.

Kohnlein T, Welte T (2008). Alpha-1 antitrypsin deficiency: pathogenesis, clinical presentation, diagnosis, and treatment. *The American journal of medicine* **121**(1)**:** 3-9.

Kolls JK, Linden A (2004). Interleukin-17 family members and inflammation. *Immunity* **21**(4)**:** 467-476.

Komai-Koma M, Xu D, Li Y, McKenzie AN, McInnes IB, Liew FY (2007). IL-33 is a chemoattractant for human Th2 cells. *European journal of immunology* **37**(10)**:** 2779-2786.

Koteswara Rao Y, Vimalamma G, Rao CV, Tzeng YM (2004). Flavonoids and andrographolides from Andrographis paniculata. *Phytochemistry* **65**(16)**:** 2317-2321.

Koya T, Miyahara N, Takeda K, Matsubara S, Matsuda H, Swasey C, *et al.* (2007). CD8+ T cell-mediated airway hyperresponsiveness and inflammation is dependent on CD4+IL-4+ T cells. *J Immunol* **179**(5)**:** 2787-2796.

Kuestner RE, Taft DW, Haran A, Brandt CS, Brender T, Lum K, *et al.* (2007). Identification of the IL-17 receptor related molecule IL-17RC as the receptor for IL-17F. *J Immunol* **179**(8)**:** 5462-5473.

Kumar A, Takada Y, Boriek AM, Aggarwal BB (2004a). Nuclear factor-kappaB: its role in health and disease. *J Mol Med* **82**(7)**:** 434-448.

Kumar RA, Sridevi K, Kumar NV, Nanduri S, Rajagopal S (2004b). Anticancer and immunostimulatory compounds from Andrographis paniculata. *J Ethnopharmacol* **92**(2-3)**:** 291-295.

Kumar S, Naqvi RA, Khanna N, Pathak P, Rao DN (2011a). Th3 immune responses in the progression of leprosy via molecular cross-talks of TGF-beta, CTLA-4 and Cbl-b. *Clin Immunol* **141**(2)**:** 133-142.

Kumar V, Kumar S, Hassan M, Wu H, Thimmulappa RK, Kumar A, *et al.* (2011b). Novel chalcone derivatives as potent Nrf2 activators in mice and human lung epithelial cells. *Journal of medicinal chemistry* **54**(12)**:** 4147-4159.

Kuperman DA, Huang X, Koth LL, Chang GH, Dolganov GM, Zhu Z, *et al.* (2002). Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. *Nat Med* **8**(8)**:** 885-889.

Kurowska-Stolarska M, Kewin P, Murphy G, Russo RC, Stolarski B, Garcia CC, *et al.* (2008). IL-33 induces antigen-specific IL-5+ T cells and promotes allergic-induced airway inflammation independent of IL-4. *Journal of immunology* **181**(7)**:** 4780-4790.

La Grutta S, Gagliardo R, Mirabella F, Pajno GB, Bonsignore G, Bousquet J, *et al.* (2003). Clinical and biological heterogeneity in children with moderate asthma. *American journal of respiratory and critical care medicine* **167**(11)**:** 1490-1495.

Lach-Trifilieff E, McKay RA, Monia BP, Karras JG, Walker C (2001). In vitro and in vivo inhibition of interleukin (IL)-5-mediated eosinopoiesis by murine IL-5Ralpha antisense oligonucleotide. *Am J Respir Cell Mol Biol* **24**(2)**:** 116-122.

Lajoie S, Lewkowich IP, Suzuki Y, Clark JR, Sproles AA, Dienger K, *et al.* (2010). Complement-mediated regulation of the IL-17A axis is a central genetic determinant of the severity of experimental allergic asthma. *Nat Immunol* **11**(10)**:** 928-935.

Lambrecht BN, De Veerman M, Coyle AJ, Gutierrez-Ramos JC, Thielemans K, Pauwels RA (2000). Myeloid dendritic cells induce Th2 responses to inhaled antigen, leading to eosinophilic airway inflammation. *J Clin Invest* **106**(4)**:** 551-559.

Lambrecht BN, Hammad H (2012). The airway epithelium in asthma. *Nat Med* **18**(5)**:** 684-692.

Lambrecht BN, Hammad H (2009). Biology of lung dendritic cells at the origin of asthma. *Immunity* **31**(3)**:** 412-424.

Lampton PW, Crooker RJ, Newmark JA, Warner CM (2008). Expression of major histocompatibility complex class I proteins and their antigen processing chaperones in mouse embryonic stem cells from fertilized and parthenogenetic embryos. *Tissue antigens* **72**(5)**:** 448-457.

Langhammer A, Johnsen R, Gulsvik A, Holmen TL, Bjermer L (2003). Sex differences in lung vulnerability to tobacco smoking. *The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology* **21**(6)**:** 1017-1023.

Langier S, Sade K, Kivity S (2012). Regulatory T cells in allergic asthma. *The Israel Medical Association journal : IMAJ* **14**(3)**:** 180-183.

Lanone S, Zheng T, Zhu Z, Liu W, Lee CG, Ma B, *et al.* (2002). Overlapping and enzyme-specific contributions of matrix metalloproteinases-9 and -12 in IL-13-induced inflammation and remodeling. *The Journal of clinical investigation* **110**(4)**:** 463-474.

Lapperre TS, Postma DS, Gosman MM, Snoeck-Stroband JB, ten Hacken NH, Hiemstra PS, *et al.* (2006). Relation between duration of smoking cessation and bronchial inflammation in COPD. *Thorax* **61**(2)**:** 115-121.

Larsson K (2007). Aspects on pathophysiological mechanisms in COPD. *J Intern Med* **262**(3)**:** 311-340.

Leckie MJ, ten Brinke A, Khan J, Diamant Z, O'Connor BJ, Walls CM, *et al.* (2000). Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet* **356**(9248)**:** 2144-2148.

Lee J, Cacalano G, Camerato T, Toy K, Moore MW, Wood WI (1995). Chemokine binding and activities mediated by the mouse IL-8 receptor. *Journal of immunology* **155**(4)**:** 2158-2164.

Lee JJ, McGarry MP, Farmer SC, Denzler KL, Larson KA, Carrigan PE, *et al.* (1997). Interleukin-5 expression in the lung epithelium of transgenic mice leads to pulmonary changes pathognomonic of asthma. *J Exp Med* **185**(12)**:** 2143-2156.

Lee MJ, Rao YK, Chen K, Lee YC, Chung YS, Tzeng YM (2010). Andrographolide and 14-deoxy-11,12-didehydroandrographolide from Andrographis paniculata attenuate high glucose-induced fibrosis and apoptosis in murine renal mesangeal cell lines. *Journal of ethnopharmacology* **132**(2)**:** 497-505.

Lee YJ, Lee SH (2011). Sulforaphane induces antioxidative and antiproliferative responses by generating reactive oxygen species in human bronchial epithelial BEAS-2B cells. *J Korean Med Sci* **26**(11)**:** 1474-1482.

Leech MD, Benson RA, De Vries A, Fitch PM, Howie SE (2007). Resolution of Der p1-induced allergic airway inflammation is dependent on CD4+CD25+Foxp3+ regulatory cells. *J Immunol* **179**(10)**:** 7050-7058.

Leigh R, Ellis R, Wattie JN, Hirota JA, Matthaei KI, Foster PS, *et al.* (2004). Type 2 cytokines in the pathogenesis of sustained airway dysfunction and airway remodeling in mice. *Am J Respir Crit Care Med* **169**(7)**:** 860-867.

Lemaitre V, D'Armiento J (2006). Matrix metalloproteinases in development and disease. *Birth Defects Res C Embryo Today* **78**(1)**:** 1-10.

Li-Weber M, Krammer PH (2003). Regulation of IL4 gene expression by T cells and therapeutic perspectives. *Nat Rev Immunol* **3**(7)**:** 534-543.

Li L, Xia Y, Nguyen A, Lai YH, Feng L, Mosmann TR, *et al.* (1999). Effects of Th2 cytokines on chemokine expression in the lung: IL-13 potently induces eotaxin expression by airway epithelial cells. *J Immunol* **162**(5)**:** 2477-2487.

Liew FY, Pitman NI, McInnes IB (2010). Disease-associated functions of IL-33: the new kid in the IL-1 family. *Nat Rev Immunol* **10**(2)**:** 103-110.

Lim JC, Chan TK, Ng DS, Sagineedu SR, Stanslas J, Wong WS (2012). Andrographolide and its analogues: versatile bioactive molecules for combating inflammation and cancer. *Clin Exp Pharmacol Physiol* **39**(3)**:** 300-310.

Lin H, Boesel KM, Griffith DT, Prussin C, Foster B, Romero FA, *et al.* (2004). Omalizumab rapidly decreases nasal allergic response and FcepsilonRI on basophils. *The Journal of allergy and clinical immunology* **113**(2)**:** 297-302.

Lin JL, Thomas PS (2010). Current perspectives of oxidative stress and its measurement in chronic obstructive pulmonary disease. *Copd* **7**(4)**:** 291-306.

Lin TP, Chen SY, Duh PD, Chang LK, Liu YN (2008). Inhibition of the epstein-barr virus lytic cycle by andrographolide. *Biological & pharmaceutical bulletin* **31**(11)**:** 2018-2023.

Liu G, Ma H, Qiu L, Li L, Cao Y, Ma J, *et al.* (2011). Phenotypic and functional switch of macrophages induced by regulatory CD4+CD25+ T cells in mice. *Immunology and cell biology* **89**(1)**:** 130-142.

Lloyd CM, Hessel EM (2010). Functions of T cells in asthma: more than just T(H)2 cells. *Nat Rev Immunol* **10**(12)**:** 838-848.

Lopez AD, Murray CC (1998). The global burden of disease, 1990-2020. *Nat Med* **4**(11)**:** 1241-1243.

Loppow D, Schleiss MB, Kanniess F, Taube C, Jorres RA, Magnussen H (2001). In patients with chronic bronchitis a four week trial with inhaled steroids does not attenuate airway inflammation. *Respir Med* **95**(2)**:** 115-121.

Lordan JL, Bucchieri F, Richter A, Konstantinidis A, Holloway JW, Thornber M, *et al.* (2002). Cooperative effects of Th2 cytokines and allergen on normal and asthmatic bronchial epithelial cells. *J Immunol* **169**(1)**:** 407-414.

Louhelainen N, Myllarniemi M, Rahman I, Kinnula VL (2008). Airway biomarkers of the oxidant burden in asthma and chronic obstructive pulmonary disease: current and future perspectives. *International journal of chronic obstructive pulmonary disease* **3**(4)**:** 585-603.

Lu LM, Zavitz CC, Chen B, Kianpour S, Wan Y, Stampfli MR (2007). Cigarette smoke impairs NK cell-dependent tumor immune surveillance. *J Immunol* **178**(2)**:** 936-943.

Lukacs NW, Strieter RM, Chensue SW, Widmer M, Kunkel SL (1995). TNF-alpha mediates recruitment of neutrophils and eosinophils during airway inflammation. *Journal of immunology* **154**(10)**:** 5411-5417.

Lund R, Aittokallio T, Nevalainen O, Lahesmaa R (2003). Identification of novel genes regulated by IL-12, IL-4, or TGF-beta during the early polarization of CD4+ lymphocytes. *J Immunol* **171**(10)**:** 5328-5336.

Lundblad LK, Thompson-Figueroa J, Leclair T, Sullivan MJ, Poynter ME, Irvin CG, *et al.* (2005). Tumor necrosis factor-alpha overexpression in lung disease: a single cause behind a complex phenotype. *Am J Respir Crit Care Med* **171**(12)**:** 1363-1370.

Luster AD, Rothenberg ME (1997). Role of the monocyte chemoattractant protein and eotaxin subfamily of chemokines in allergic inflammation. *J Leukoc Biol* **62**(5)**:** 620-633.

Macintyre NR (2009). Spirometry for the diagnosis and management of chronic obstructive pulmonary disease. *Respiratory care* **54**(8)**:** 1050-1057.

MacNee W, Rahman I (2001). Is oxidative stress central to the pathogenesis of chronic obstructive pulmonary disease? *Trends Mol Med* **7**(2)**:** 55-62.

Mak JC (2008). Pathogenesis of COPD. Part II. Oxidative-antioxidative imbalance. *Int J Tuberc Lung Dis* **12**(4)**:** 368-374.

Mak JC, Ho SP, Yu WC, Choo KL, Chu CM, Yew WW, *et al.* (2007). Polymorphisms and functional activity in superoxide dismutase and catalase genes in smokers with COPD. *Eur Respir J* **30**(4)**:** 684-690.

Makris D, Moschandreas J, Damianaki A, Ntaoukakis E, Siafakas NM, Milic Emili J, *et al.* (2007). Exacerbations and lung function decline in COPD: new insights in current and ex-smokers. *Respir Med* **101**(6)**:** 1305-1312.

Malhotra D, Thimmulappa R, Navas-Acien A, Sandford A, Elliott M, Singh A, *et al.* (2008). Decline in NRF2-regulated antioxidants in chronic obstructive pulmonary disease lungs due to loss of its positive regulator, DJ-1. *American journal of respiratory and critical care medicine* **178**(6)**:** 592-604.

Maneechotesuwan K, Xin Y, Ito K, Jazrawi E, Lee KY, Usmani OS, *et al.* (2007). Regulation of Th2 cytokine genes by p38 MAPK-mediated phosphorylation of GATA-3. *J Immunol* **178**(4)**:** 2491-2498.

Mannino DM, Buist AS (2007). Global burden of COPD: risk factors, prevalence, and future trends. *Lancet* **370**(9589)**:** 765-773.

Manoury B, Nenan S, Guenon I, Boichot E, Planquois JM, Bertrand CP, *et al.* (2006). Macrophage metalloelastase (MMP-12) deficiency does not alter bleomycin-induced pulmonary fibrosis in mice. *J Inflamm (Lond)* **3:** 2.

Mantovani A, Garlanda C, Locati M (2009). Macrophage diversity and polarization in atherosclerosis: a question of balance. *Arteriosclerosis, thrombosis, and vascular biology* **29**(10)**:** 1419-1423.

March TH, Wilder JA, Esparza DC, Cossey PY, Blair LF, Herrera LK, *et al.* (2006). Modulators of cigarette smoke-induced pulmonary emphysema in A/J mice. *Toxicol Sci* **92**(2)**:** 545-559.

Martinez FJ, Curtis JL, Sciurba F, Mumford J, Giardino ND, Weinmann G, *et al.* (2007). Sex differences in severe pulmonary emphysema. *American journal of respiratory and critical care medicine* **176**(3)**:** 243-252.

Marwick JA, Caramori G, Stevenson CS, Casolari P, Jazrawi E, Barnes PJ, *et al.* (2009). Inhibition of PI3Kdelta restores glucocorticoid function in smoking-induced airway inflammation in mice. *American journal of respiratory and critical care medicine* **179**(7)**:** 542-548.

Mashreghi MF, Klemz R, Knosalla IS, Gerstmayer B, Janssen U, Buelow R, *et al.* (2008). Inhibition of dendritic cell maturation and function is independent of heme oxygenase 1 but requires the activation of STAT3. *J Immunol* **180**(12)**:** 7919-7930.

Masoli M, Fabian D, Holt S, Beasley R (2004). The global burden of asthma: executive summary of the GINA Dissemination Committee report. *Allergy* **59**(5)**:** 469-478.

Matera MG, Calzetta L, Cazzola M (2010). TNF-alpha inhibitors in asthma and COPD: we must not throw the baby out with the bath water. *Pulm Pharmacol Ther* **23**(2)**:** 121-128.

Mathers CD, Loncar D (2006). Projections of global mortality and burden of disease from 2002 to 2030. *PLoS Med* **3**(11)**:** e442.

Matsumoto M, Kunimitsu S, Wada K, Ikeda M, Keyama A, Kodama H (2007). Mast cell distribution, activation, and phenotype in xanthoma. *Journal of the American Academy of Dermatology* **56**(6)**:** 1006-1012.

McAllister F, Henry A, Kreindler JL, Dubin PJ, Ulrich L, Steele C, *et al.* (2005). Role of IL-17A, IL-17F, and the IL-17 receptor in regulating growth-related oncogene-alpha and granulocyte colony-stimulating factor in bronchial epithelium: implications for airway inflammation in cystic fibrosis. *J Immunol* **175**(1)**:** 404-412.

McElhinney B, Poynter ME, Shrivastava P, Hazen SL, Janssen-Heininger YM (2003). Eosinophil peroxidase catalyzes JNK-mediated membrane blebbing in a Rho kinase-dependent manner. *J Leukoc Biol* **74**(5)**:** 897-907.

McEver RP (2002). Selectins: lectins that initiate cell adhesion under flow. *Curr Opin Cell Biol* **14**(5)**:** 581-586.

McKinley L, Alcorn JF, Peterson A, Dupont RB, Kapadia S, Logar A, *et al.* (2008). TH17 cells mediate steroid-resistant airway inflammation and airway hyperresponsiveness in mice. *Journal of immunology* **181**(6)**:** 4089-4097.

Medicherla S, Fitzgerald MF, Spicer D, Woodman P, Ma JY, Kapoun AM, *et al.* (2008). p38alpha-selective mitogen-activated protein kinase inhibitor SD-282 reduces inflammation in a subchronic model of tobacco smoke-induced airway inflammation. *J Pharmacol Exp Ther* **324**(3)**:** 921-929.

Medoff BD, Thomas SY, Luster AD (2008). T cell trafficking in allergic asthma: the ins and outs. *Annu Rev Immunol* **26:** 205-232.

Melendez-Zajgla J, Del Pozo L, Ceballos G, Maldonado V (2008). Tissue inhibitor of metalloproteinases-4. The road less traveled. *Molecular cancer* **7:** 85.

Melgarejo E, Medina MA, Sanchez-Jimenez F, Urdiales JL (2009). Monocyte chemoattractant protein-1: a key mediator in inflammatory processes. *The international journal of biochemistry & cell biology* **41**(5)**:** 998-1001.

Meshi B, Vitalis TZ, Ionescu D, Elliott WM, Liu C, Wang XD, *et al.* (2002). Emphysematous lung destruction by cigarette smoke. The effects of latent adenoviral infection on the lung inflammatory response. *Am J Respir Cell Mol Biol* **26**(1)**:** 52-57.

Mills CD, Kincaid K, Alt JM, Heilman MJ, Hill AM (2000). M-1/M-2 macrophages and the Th1/Th2 paradigm. *J Immunol* **164**(12)**:** 6166-6173.

Minai-Fleminger Y, Levi-Schaffer F (2009). Mast cells and eosinophils: the two key effector cells in allergic inflammation. *Inflammation research : official journal of the European Histamine Research Society ... [et al.]* **58**(10)**:** 631-638.

Minematsu N, Blumental-Perry A, Shapiro SD (2011). Cigarette smoke inhibits engulfment of apoptotic cells by macrophages through inhibition of actin rearrangement. *Am J Respir Cell Mol Biol* **44**(4)**:** 474-482.

Mishra K, Dash AP, Dey N (2011). Andrographolide: A Novel Antimalarial Diterpene Lactone Compound from Andrographis paniculata and Its Interaction with Curcumin and Artesunate. *Journal of tropical medicine* **2011:** 579518.

Miyahara N, Takeda K, Kodama T, Joetham A, Taube C, Park JW, *et al.* (2004). Contribution of antigen-primed CD8+ T cells to the development of airway hyperresponsiveness and inflammation is associated with IL-13. *J Immunol* **172**(4)**:** 2549-2558.

Mocchegiani E, Giacconi R, Costarelli L (2011). Metalloproteases/anti-metalloproteases imbalance in chronic obstructive pulmonary disease: genetic factors and treatment implications. *Current opinion in pulmonary medicine* **17 Suppl 1:** S11-19.

Montuschi P, Collins JV, Ciabattoni G, Lazzeri N, Corradi M, Kharitonov SA, *et al.* (2000). Exhaled 8-isoprostane as an in vivo biomarker of lung oxidative stress in patients with COPD

and healthy smokers. *American journal of respiratory and critical care medicine* **162**(3 Pt 1)**:** 1175-1177.

Montuschi P, Kharitonov SA, Barnes PJ (2001). Exhaled carbon monoxide and nitric oxide in COPD. *Chest* **120**(2)**:** 496-501.

Montuschi P, Peters-Golden ML (2010). Leukotriene modifiers for asthma treatment. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* **40**(12)**:** 1732-1741.

Moore WC, Bleecker ER, Curran-Everett D, Erzurum SC, Ameredes BT, Bacharier L, *et al.* (2007). Characterization of the severe asthma phenotype by the National Heart, Lung, and Blood Institute's Severe Asthma Research Program. *The Journal of allergy and clinical immunology* **119**(2)**:** 405-413.

Morcillo EJ, Cortijo J (2006). Mucus and MUC in asthma. *Curr Opin Pulm Med* **12**(1)**:** 1-6.

Moreira AP, Cavassani KA, Hullinger R, Rosada RS, Fong DJ, Murray L, *et al.* (2010). Serum amyloid P attenuates M2 macrophage activation and protects against fungal spore-induced allergic airway disease. *J Allergy Clin Immunol* **126**(4)**:** 712-721 e717.

Moreira AP, Hogaboam CM (2011). Macrophages in allergic asthma: fine-tuning their pro- and anti-inflammatory actions for disease resolution. *J Interferon Cytokine Res* **31**(6)**:** 485-491.

Morissette MC, Parent J, Milot J (2009). Alveolar epithelial and endothelial cell apoptosis in emphysema: what we know and what we need to know. *International journal of chronic obstructive pulmonary disease* **4:** 19-31.

Morjaria JB, Malerba M, Polosa R (2010). Biologic and pharmacologic therapies in clinical development for the inflammatory response in COPD. *Drug Discov Today* **15**(9-10)**:** 396-405.

Morris A, Kinnear G, Wan WY, Wyss D, Bahra P, Stevenson CS (2008). Comparison of cigarette smoke-induced acute inflammation in multiple strains of mice and the effect of a matrix metalloproteinase inhibitor on these responses. *J Pharmacol Exp Ther* **327**(3)**:** 851-862.

Moss ML, Jin SL, Milla ME, Bickett DM, Burkhart W, Carter HL, *et al.* (1997). Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-alpha. *Nature* **385**(6618)**:** 733-736.

Mukherjee AB, Zhang Z (2011). Allergic asthma: influence of genetic and environmental factors. *J Biol Chem* **286**(38)**:** 32883-32889.

Mulcahy RT, Bailey HH, Gipp JJ (1995). Transfection of complementary DNAs for the heavy and light subunits of human gamma-glutamylcysteine synthetase results in an elevation of intracellular glutathione and resistance to melphalan. *Cancer Res* **55**(21)**:** 4771-4775.

Muller T, Gebel S (1998). The cellular stress response induced by aqueous extracts of cigarette smoke is critically dependent on the intracellular glutathione concentration. *Carcinogenesis* **19**(5)**:** 797-801.

Murata M, Kawanishi S (2004). Oxidative DNA damage induced by nitrotyrosine, a biomarker of inflammation. *Biochem Biophys Res Commun* **316**(1)**:** 123-128.

Murphy DM, O'Byrne PM (2010). Recent advances in the pathophysiology of asthma. *Chest* **137**(6)**:** 1417-1426.

Murphy KM, Reiner SL (2002). The lineage decisions of helper T cells. *Nat Rev Immunol* **2**(12)**:** 933-944.

Myou S, Leff AR, Myo S, Boetticher E, Tong J, Meliton AY, *et al.* (2003). Blockade of inflammation and airway hyperresponsiveness in immune-sensitized mice by dominant-negative phosphoinositide 3-kinase-TAT. *J Exp Med* **198**(10)**:** 1573-1582.

Naeije R (2005). Pulmonary hypertension and right heart failure in chronic obstructive pulmonary disease. *Proc Am Thorac Soc* **2**(1)**:** 20-22.

Nair MG, Gallagher IJ, Taylor MD, Loke P, Coulson PS, Wilson RA, *et al.* (2005). Chitinase and Fizz family members are a generalized feature of nematode infection with selective upregulation of Ym1 and Fizz1 by antigen-presenting cells. *Infection and immunity* **73**(1)**:** 385-394.

Nair P, Pizzichini MM, Kjarsgaard M, Inman MD, Efthimiadis A, Pizzichini E, *et al.* (2009). Mepolizumab for prednisone-dependent asthma with sputum eosinophilia. *The New England journal of medicine* **360**(10)**:** 985-993.

Nanduri S, Nyavanandi VK, Thunuguntla SS, Kasu S, Pallerla MK, Ram PS, *et al.* (2004). Synthesis and structure-activity relationships of andrographolide analogues as novel cytotoxic agents. *Bioorg Med Chem Lett* **14**(18)**:** 4711-4717.

Negi AS, Kumar JK, Luqman S, Shanker K, Gupta MM, Khanuja SP (2008). Recent advances in plant hepatoprotectives: a chemical and biological profile of some important leads. *Med Res Rev* **28**(5)**:** 746-772.

Nembrini C, Marsland BJ, Kopf M (2009). IL-17-producing T cells in lung immunity and inflammation. *J Allergy Clin Immunol* **123**(5)**:** 986-994; quiz 995-986.

Neogy S, Das S, Mahapatra SK, Mandal N, Roy S (2008). Amelioratory effect of Andrographis paniculata Nees on liver, kidney, heart, lung and spleen during nicotine induced oxidative stress. *Environ Toxicol Pharmacol* **25**(3)**:** 321-328.

Newton R, Holden NS, Catley MC, Oyelusi W, Leigh R, Proud D, *et al.* (2007). Repression of inflammatory gene expression in human pulmonary epithelial cells by small-molecule IkappaB kinase inhibitors. *J Pharmacol Exp Ther* **321**(2)**:** 734-742.

Nograles KE, Zaba LC, Guttman-Yassky E, Fuentes-Duculan J, Suarez-Farinas M, Cardinale I, *et al.* (2008). Th17 cytokines interleukin (IL)-17 and IL-22 modulate distinct inflammatory and keratinocyte-response pathways. *The British journal of dermatology* **159**(5)**:** 1092-1102.

O'Byrne PM, Gauvreau GM, Brannan JD (2009). Provoked models of asthma: what have we learnt? *Clin Exp Allergy* **39**(2)**:** 181-192.

O'Byrne PM, Inman MD (2003). Airway hyperresponsiveness. *Chest* **123**(3 Suppl)**:** 411S-416S.

O'Donnell R, Breen D, Wilson S, Djukanovic R (2006). Inflammatory cells in the airways in COPD. *Thorax* **61**(5)**:** 448-454.

Obase Y, Shimoda T, Mitsuta K, Matsuo N, Matsuse H, Kohno S (2001). Correlation between airway hyperresponsiveness and airway inflammation in a young adult population: eosinophil, ECP, and cytokine levels in induced sputum. *Ann Allergy Asthma Immunol* **86**(3)**:** 304-310.

Oberley-Deegan RE, Regan EA, Kinnula VL, Crapo JD (2009). Extracellular superoxide dismutase and risk of COPD. *Copd* **6**(4)**:** 307-312.

Oettgen HC (2000). Regulation of the IgE isotype switch: new insights on cytokine signals and the functions of epsilon germline transcripts. *Current opinion in immunology* **12**(6)**:** 618-623.

Ohmori Y, Wyner L, Narumi S, Armstrong D, Stoler M, Hamilton TA (1993). Tumor necrosis factor-alpha induces cell type and tissue-specific expression of chemoattractant cytokines in vivo. *Am J Pathol* **142**(3)**:** 861-870.

Ostroukhova M, Seguin-Devaux C, Oriss TB, Dixon-McCarthy B, Yang L, Ameredes BT, *et al.* (2004). Tolerance induced by inhaled antigen involves CD4(+) T cells expressing membrane-bound TGF-beta and FOXP3. *J Clin Invest* **114**(1)**:** 28-38.

Otterbein LE, Bach FH, Alam J, Soares M, Tao Lu H, Wysk M, *et al.* (2000). Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. *Nature medicine* **6**(4)**:** 422-428.

Page CP, Spina D (2012). Selective PDE inhibitors as novel treatments for respiratory diseases. *Curr Opin Pharmacol* **12**(3)**:** 275-286.

Palmqvist C, Wardlaw AJ, Bradding P (2007). Chemokines and their receptors as potential targets for the treatment of asthma. *Br J Pharmacol* **151**(6)**:** 725-736.

Pantano C, Ather JL, Alcorn JF, Poynter ME, Brown AL, Guala AS, *et al.* (2008). Nuclear factor-kappaB activation in airway epithelium induces inflammation and hyperresponsiveness. *Am J Respir Crit Care Med* **177**(9)**:** 959-969.

Pappu BP, Angkasekwinai P, Dong C (2008). Regulatory mechanisms of helper T cell differentiation: new lessons learned from interleukin 17 family cytokines. *Pharmacol Ther* **117**(3)**:** 374-384.

Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, *et al.* (2005). A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* **6**(11)**:** 1133-1141.

Park SJ, Lee YC (2010). Interleukin-17 regulation: an attractive therapeutic approach for asthma. *Respir Res* **11:** 78.

Pauwels RA, Buist AS, Calverley PM, Jenkins CR, Hurd SS (2001). Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) Workshop summary. *Am J Respir Crit Care Med* **163**(5)**:** 1256-1276.

Pauwels RA, Rabe KF (2004). Burden and clinical features of chronic obstructive pulmonary disease (COPD). *Lancet* **364**(9434)**:** 613-620.

Pearce N, Ait-Khaled N, Beasley R, Mallol J, Keil U, Mitchell E, *et al.* (2007). Worldwide trends in the prevalence of asthma symptoms: phase III of the International Study of Asthma and Allergies in Childhood (ISAAC). *Thorax* **62**(9)**:** 758-766.

Pene J, Chevalier S, Preisser L, Venereau E, Guilleux MH, Ghannam S, *et al.* (2008). Chronically inflamed human tissues are infiltrated by highly differentiated Th17 lymphocytes. *J Immunol* **180**(11)**:** 7423-7430.

Pesci A, Balbi B, Majori M, Cacciani G, Bertacco S, Alciato P, *et al.* (1998a). Inflammatory cells and mediators in bronchial lavage of patients with chronic obstructive pulmonary disease. *Eur Respir J* **12**(2)**:** 380-386.

Pesci A, Majori M, Cuomo A, Borciani N, Bertacco S, Cacciani G, *et al.* (1998b). Neutrophils infiltrating bronchial epithelium in chronic obstructive pulmonary disease. *Respir Med* **92**(6)**:** 863-870.

Pholphana N, Rangkadilok N, Thongnest S, Ruchirawat S, Ruchirawat M, Satayavivad J (2004). Determination and variation of three active diterpenoids in Andrographis paniculata (Burm.f.) Nees. *Phytochem Anal* **15**(6)**:** 365-371.

Platz J, Pinkenburg O, Beisswenger C, Puchner A, Damm T, Bals R (2005). Application of small interfering RNA (siRNA) for modulation of airway epithelial gene expression. *Oligonucleotides* **15**(2)**:** 132-138.

Polosa R (2007). Critical appraisal of antileukotriene use in asthma management. *Current opinion in pulmonary medicine* **13**(1)**:** 24-30.

Poolsup N, Suthisisang C, Prathanturarug S, Asawamekin A, Chanchareon U (2004). Andrographis paniculata in the symptomatic treatment of uncomplicated upper respiratory tract infection: systematic review of randomized controlled trials. *J Clin Pharm Ther* **29**(1)**:** 37-45.

Poynter ME, Irvin CG, Janssen-Heininger YM (2002). Rapid activation of nuclear factor-kappaB in airway epithelium in a murine model of allergic airway inflammation. *Am J Pathol* **160**(4)**:** 1325-1334.

Prefontaine D, Lajoie-Kadoch S, Foley S, Audusseau S, Olivenstein R, Halayko AJ, *et al.* (2009). Increased expression of IL-33 in severe asthma: evidence of expression by airway smooth muscle cells. *J Immunol* **183**(8)**:** 5094-5103.

Prefontaine D, Nadigel J, Chouiali F, Audusseau S, Semlali A, Chakir J, *et al.* (2010). Increased IL-33 expression by epithelial cells in bronchial asthma. *J Allergy Clin Immunol* **125**(3)**:** 752-754.

Profita M, Sala A, Bonanno A, Riccobono L, Siena L, Melis MR, *et al.* (2003). Increased prostaglandin E2 concentrations and cyclooxygenase-2 expression in asthmatic subjects with sputum eosinophilia. *J Allergy Clin Immunol* **112**(4)**:** 709-716.

Pryor WA (1992). Biological effects of cigarette smoke, wood smoke, and the smoke from plastics: the use of electron spin resonance. *Free radical biology & medicine* **13**(6)**:** 659-676.

Pulendran B, Smith JL, Caspary G, Brasel K, Pettit D, Maraskovsky E, *et al.* (1999). Distinct dendritic cell subsets differentially regulate the class of immune response in vivo. *Proceedings of the National Academy of Sciences of the United States of America* **96**(3)**:** 1036-1041.

Pushparaj PN, Tay HK, H'Ng S C, Pitman N, Xu D, McKenzie A, *et al.* (2009). The cytokine interleukin-33 mediates anaphylactic shock. *Proc Natl Acad Sci U S A* **106**(24)**:** 9773-9778.

Qiu Y, Zhu J, Bandi V, Atmar RL, Hattotuwa K, Guntupalli KK, *et al.* (2003). Biopsy neutrophilia, neutrophil chemokine and receptor gene expression in severe exacerbations of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* **168**(8)**:** 968-975.

Quint JK, Wedzicha JA (2007). The neutrophil in chronic obstructive pulmonary disease. *J Allergy Clin Immunol* **119**(5)**:** 1065-1071.

Rabe KF, Hurd S, Anzueto A, Barnes PJ, Buist SA, Calverley P, *et al.* (2007). Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. *Am J Respir Crit Care Med* **176**(6)**:** 532-555.

Radomski MW, Vallance P, Whitley G, Foxwell N, Moncada S (1993). Platelet adhesion to human vascular endothelium is modulated by constitutive and cytokine induced nitric oxide. *Cardiovasc Res* **27**(7)**:** 1380-1382.

Rahman I, Adcock IM (2006a). Oxidative stress and redox regulation of lung inflammation in COPD. *Eur Respir J* **28**(1)**:** 219-242.

Rahman I, Biswas SK, Kode A (2006b). Oxidant and antioxidant balance in the airways and airway diseases. *Eur J Pharmacol* **533**(1-3)**:** 222-239.

Rahman I, MacNee W (1999). Lung glutathione and oxidative stress: implications in cigarette smoke-induced airway disease. *Am J Physiol* **277**(6 Pt 1)**:** L1067-1088.

Rahman I, MacNee W (2000). Regulation of redox glutathione levels and gene transcription in lung inflammation: therapeutic approaches. *Free Radic Biol Med* **28**(9)**:** 1405-1420.

Rahman I, Morrison D, Donaldson K, MacNee W (1996). Systemic oxidative stress in asthma, COPD, and smokers. *American journal of respiratory and critical care medicine* **154**(4 Pt 1)**:** 1055-1060.

Rahman I, van Schadewijk AA, Crowther AJ, Hiemstra PS, Stolk J, MacNee W, *et al.* (2002). 4-Hydroxy-2-nonenal, a specific lipid peroxidation product, is elevated in lungs of patients with chronic obstructive pulmonary disease. *American journal of respiratory and critical care medicine* **166**(4)**:** 490-495.

Rahman MS, Yamasaki A, Yang J, Shan L, Halayko AJ, Gounni AS (2006c). IL-17A induces eotaxin-1/CC chemokine ligand 11 expression in human airway smooth muscle cells: role of MAPK (Erk1/2, JNK, and p38) pathways. *J Immunol* **177**(6)**:** 4064-4071.

Rajendrasozhan S, Chung S, Sundar IK, Yao H, Rahman I (2010). Targeted disruption of NF-{kappa}B1 (p50) augments cigarette smoke-induced lung inflammation and emphysema in mice: a critical role of p50 in chromatin remodeling. *American journal of physiology. Lung cellular and molecular physiology* **298**(2)**:** L197-209.

Ramsay CF, Pearson D, Mildenhall S, Wilson AM (2011). Oral montelukast in acute asthma exacerbations: a randomised, double-blind, placebo-controlled trial. *Thorax* **66**(1)**:** 7-11.

Rangasamy T, Cho CY, Thimmulappa RK, Zhen L, Srisuma SS, Kensler TW, *et al.* (2004). Genetic ablation of Nrf2 enhances susceptibility to cigarette smoke-induced emphysema in mice. *The Journal of clinical investigation* **114**(9)**:** 1248-1259.

Reiss TF, Chervinsky P, Dockhorn RJ, Shingo S, Seidenberg B, Edwards TB (1998). Montelukast, a once-daily leukotriene receptor antagonist, in the treatment of chronic asthma: a multicenter, randomized, double-blind trial. Montelukast Clinical Research Study Group. *Arch Intern Med* **158**(11)**:** 1213-1220.

Rennard SI, Fogarty C, Kelsen S, Long W, Ramsdell J, Allison J, *et al.* (2007). The safety and efficacy of infliximab in moderate to severe chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* **175**(9)**:** 926-934.

Robbins CS, Bauer CM, Vujicic N, Gaschler GJ, Lichty BD, Brown EG, *et al.* (2006). Cigarette smoke impacts immune inflammatory responses to influenza in mice. *Am J Respir Crit Care Med* **174**(12)**:** 1342-1351.

Robinson DS (2009). Regulatory T cells and asthma. *Clin Exp Allergy* **39**(9)**:** 1314-1323.

Robinson DS (2010). The role of the T cell in asthma. *The Journal of allergy and clinical immunology* **126**(6)**:** 1081-1091; quiz 1092-1083.

Rose CE, Jr., Sung SS, Fu SM (2003). Significant involvement of CCL2 (MCP-1) in inflammatory disorders of the lung. *Microcirculation* **10**(3-4)**:** 273-288.

Roth K, Chen WM, Lin TJ (2008). Positive and negative regulatory mechanisms in high-affinity IgE receptor-mediated mast cell activation. *Archivum immunologiae et therapiae experimentalis* **56**(6)**:** 385-399.

Russell RE, Culpitt SV, DeMatos C, Donnelly L, Smith M, Wiggins J, *et al.* (2002a). Release and activity of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 by

alveolar macrophages from patients with chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol* **26**(5)**:** 602-609.

Russell RE, Thorley A, Culpitt SV, Dodd S, Donnelly LE, Demattos C, *et al.* (2002b). Alveolar macrophage-mediated elastolysis: roles of matrix metalloproteinases, cysteine, and serine proteases. *Am J Physiol Lung Cell Mol Physiol* **283**(4)**:** L867-873.

Ryan JJ, Kashyap M, Bailey D, Kennedy S, Speiran K, Brenzovich J, *et al.* (2007). Mast cell homeostasis: a fundamental aspect of allergic disease. *Critical reviews in immunology* **27**(1)**:** 15-32.

Ryder MI, Saghizadeh M, Ding Y, Nguyen N, Soskolne A (2002). Effects of tobacco smoke on the secretion of interleukin-1beta, tumor necrosis factor-alpha, and transforming growth factor-beta from peripheral blood mononuclear cells. *Oral Microbiol Immunol* **17**(6)**:** 331-336.

Ryter SW, Morse D, Choi AM (2007). Carbon monoxide and bilirubin: potential therapies for pulmonary/vascular injury and disease. *American journal of respiratory cell and molecular biology* **36**(2)**:** 175-182.

Ryu JH, Scanlon PD (2001). Obstructive lung diseases: COPD, asthma, and many imitators. *Mayo Clin Proc* **76**(11)**:** 1144-1153.

Saha S, Doe C, Mistry V, Siddiqui S, Parker D, Sleeman M, *et al.* (2009). Granulocyte-macrophage colony-stimulating factor expression in induced sputum and bronchial mucosa in asthma and COPD. *Thorax* **64**(8)**:** 671-676.

Santos MC, Oliveira AL, Viegas-Crespo AM, Vicente L, Barreiros A, Monteiro P, *et al.* (2004). Systemic markers of the redox balance in chronic obstructive pulmonary disease. *Biomarkers* **9**(6)**:** 461-469.

Sarady JK, Otterbein SL, Liu F, Otterbein LE, Choi AM (2002). Carbon monoxide modulates endotoxin-induced production of granulocyte macrophage colony-stimulating factor in macrophages. *American journal of respiratory cell and molecular biology* **27**(6)**:** 739-745.

Sauty A, Dziejman M, Taha RA, Iarossi AS, Neote K, Garcia-Zepeda EA, *et al.* (1999). The T cell-specific CXC chemokines IP-10, Mig, and I-TAC are expressed by activated human bronchial epithelial cells. *Journal of immunology* **162**(6)**:** 3549-3558.

Sawicka E, Noble A, Walker C, Kemeny DM (2004). Tc2 cells respond to soluble antigen in the respiratory tract and induce lung eosinophilia and bronchial hyperresponsiveness. *European journal of immunology* **34**(9)**:** 2599-2608.

Scapini P, Lapinet-Vera JA, Gasperini S, Calzetti F, Bazzoni F, Cassatella MA (2000). The neutrophil as a cellular source of chemokines. *Immunological reviews* **177:** 195-203.

Schermer T, Chavannes N, Dekhuijzen R, Wouters E, Muris J, Akkermans R, *et al.* (2009). Fluticasone and N-acetylcysteine in primary care patients with COPD or chronic bronchitis. *Respir Med* **103**(4)**:** 542-551.

Schmitz J, Owyang A, Oldham E, Song Y, Murphy E, McClanahan TK, *et al.* (2005). IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* **23**(5)**:** 479-490.

Schuh JM, Blease K, Bruhl H, Mack M, Hogaboam CM (2003). Intrapulmonary targeting of RANTES/CCL5-responsive cells prevents chronic fungal asthma. *European journal of immunology* **33**(11)**:** 3080-3090.

Schulz C, Kratzel K, Wolf K, Schroll S, Kohler M, Pfeifer M (2004). Activation of bronchial epithelial cells in smokers without airway obstruction and patients with COPD. *Chest* **125**(5)**:** 1706-1713.

Schulze-Luehrmann J, Ghosh S (2006). Antigen-receptor signaling to nuclear factor kappa B. *Immunity* **25**(5)**:** 701-715.

Scott DA, Palmer RM (2002). The influence of tobacco smoking on adhesion molecule profiles. *Tobacco induced diseases* **1**(1)**:** 7-25.

Scott DA, Todd DH, Coward PY, Wilson RF, Odell EW, Poston RN, *et al.* (2000). The acute influence of tobacco smoking on adhesion molecule expression on monocytes and neutrophils and on circulating adhesion molecule levels in vivo. *Addiction biology* **5**(2)**:** 195-205.

Seemungal T, Harrinarine R, Rios M, Abiraj V, Ali A, Lacki N, *et al.* (2008). Obstructive lung disease in acute medical patients. *West Indian Med J* **57**(1)**:** 7-13.

Sethi S, Mallia P, Johnston SL (2009). New paradigms in the pathogenesis of chronic obstructive pulmonary disease II. *Proc Am Thorac Soc* **6**(6)**:** 532-534.

Sha Q, Truong-Tran AQ, Plitt JR, Beck LA, Schleimer RP (2004). Activation of airway epithelial cells by toll-like receptor agonists. *Am J Respir Cell Mol Biol* **31**(3)**:** 358-364.

Shapiro SD (2002). Proteinases in chronic obstructive pulmonary disease. *Biochem Soc Trans* **30**(2)**:** 98-102.

Sheeja K, Shihab PK, Kuttan G (2006). Antioxidant and anti-inflammatory activities of the plant Andrographis paniculata Nees. *Immunopharmacol Immunotoxicol* **28**(1)**:** 129-140.

Sheller JR, Polosukhin VV, Mitchell D, Cheng DS, Peebles RS, Blackwell TS (2009). Nuclear factor kappa B induction in airway epithelium increases lung inflammation in allergen-challenged mice. *Exp Lung Res* **35**(10)**:** 883-895.

Shinohara H, Kurosaki T (2009). Comprehending the complex connection between PKCbeta, TAK1, and IKK in BCR signaling. *Immunol Rev* **232**(1)**:** 300-318.

Shinohara T, Kaneko T, Nagashima Y, Ueda A, Tagawa A, Ishigatsubo Y (2005). Adenovirus-mediated transfer and overexpression of heme oxygenase 1 cDNA in lungs attenuates elastase-induced pulmonary emphysema in mice. *Hum Gene Ther* **16**(3)**:** 318-327.

Shiraishi Y, Asano K, Niimi K, Fukunaga K, Wakaki M, Kagyo J, *et al.* (2008). Cyclooxygenase-2/prostaglandin D2/CRTH2 pathway mediates double-stranded RNA-induced enhancement of allergic airway inflammation. *J Immunol* **180**(1)**:** 541-549.

Shuhui L, Mok YK, Wong WS (2009). Role of mammalian chitinases in asthma. *Int Arch Allergy Immunol* **149**(4)**:** 369-377.

Siebenlist U, Brown K, Claudio E (2005). Control of lymphocyte development by nuclear factor-kappaB. *Nat Rev Immunol* **5**(6)**:** 435-445.

Sies H, Sharov VS, Klotz LO, Briviba K (1997). Glutathione peroxidase protects against peroxynitrite-mediated oxidations. A new function for selenoproteins as peroxynitrite reductase. *The Journal of biological chemistry* **272**(44)**:** 27812-27817.

Sin DD, Man SF (2005). Chronic obstructive pulmonary disease as a risk factor for cardiovascular morbidity and mortality. *Proc Am Thorac Soc* **2**(1)**:** 8-11.

Singh A, Misra V, Thimmulappa RK, Lee H, Ames S, Hoque MO, *et al.* (2006a). Dysfunctional KEAP1-NRF2 interaction in non-small-cell lung cancer. *PLoS Med* **3**(10)**:** e420.

Singh A, Rangasamy T, Thimmulappa RK, Lee H, Osburn WO, Brigelius-Flohe R, *et al.* (2006b). Glutathione peroxidase 2, the major cigarette smoke-inducible isoform of GPX in lungs, is regulated by Nrf2. *American journal of respiratory cell and molecular biology* **35**(6)**:** 639-650.

Skrepnek GH, Skrepnek SV (2004). Epidemiology, clinical and economic burden, and natural history of chronic obstructive pulmonary disease and asthma. *The American journal of managed care* **10**(5 Suppl)**:** S129-138.

Smirnova NA, Haskew-Layton RE, Basso M, Hushpulian DM, Payappilly JB, Speer RE, *et al.* (2011). Development of Neh2-luciferase reporter and its application for high throughput screening and real-time monitoring of Nrf2 activators. *Chemistry & biology* **18**(6)**:** 752-765.

Soroosh P, Doherty TA (2009). Th9 and allergic disease. *Immunology* **127**(4)**:** 450-458.

Souwer Y, Szegedi K, Kapsenberg ML, de Jong EC (2010). IL-17 and IL-22 in atopic allergic disease. *Curr Opin Immunol* **22**(6)**:** 821-826.

Standiford TJ, Kunkel SL, Phan SH, Rollins BJ, Strieter RM (1991). Alveolar macrophage-derived cytokines induce monocyte chemoattractant protein-1 expression from human pulmonary type II-like epithelial cells. *J Biol Chem* **266**(15)**:** 9912-9918.

Stav D, Raz M (2009). Effect of N-acetylcysteine on air trapping in COPD: a randomized placebo-controlled study. *Chest* **136**(2)**:** 381-386.

Steenwinckel V, Louahed J, Orabona C, Huaux F, Warnier G, McKenzie A, *et al.* (2007). IL-13 mediates in vivo IL-9 activities on lung epithelial cells but not on hematopoietic cells. *J Immunol* **178**(5)**:** 3244-3251.

Stevenson CS, Birrell MA (2011). Moving towards a new generation of animal models for asthma and COPD with improved clinical relevance. *Pharmacology & therapeutics* **130**(2)**:** 93-105.

Stirling RG, van Rensen EL, Barnes PJ, Chung KF (2001). Interleukin-5 induces CD34(+) eosinophil progenitor mobilization and eosinophil CCR3 expression in asthma. *Am J Respir Crit Care Med* **164**(8 Pt 1)**:** 1403-1409.

Stockley RA (1999). Neutrophils and protease/antiprotease imbalance. *Am J Respir Crit Care Med* **160**(5 Pt 2)**:** S49-52.

Strickland DH, Stumbles PA, Zosky GR, Subrata LS, Thomas JA, Turner DJ, *et al.* (2006). Reversal of airway hyperresponsiveness by induction of airway mucosal CD4+CD25+ regulatory T cells. *J Exp Med* **203**(12)**:** 2649-2660.

Suebsasana S, Pongnaratorn P, Sattayasai J, Arkaravichien T, Tiamkao S, Aromdee C (2009). Analgesic, antipyretic, anti-inflammatory and toxic effects of andrographolide derivatives in experimental animals. *Arch Pharm Res* **32**(9)**:** 1191-1200.

Sugiura H, Ichinose M (2011). Nitrative stress in inflammatory lung diseases. *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society* **25**(2)**:** 138-144.

Sussan TE, Rangasamy T, Blake DJ, Malhotra D, El-Haddad H, Bedja D, *et al.* (2009). Targeting Nrf2 with the triterpenoid CDDO-imidazolide attenuates cigarette smoke-induced emphysema and cardiac dysfunction in mice. *Proceedings of the National Academy of Sciences of the United States of America* **106**(1)**:** 250-255.

Sutcliffe AM, Clarke DL, Bradbury DA, Corbett LM, Patel JA, Knox AJ (2009). Transcriptional regulation of monocyte chemotactic protein-1 release by endothelin-1 in human airway smooth muscle cells involves NF-kappaB and AP-1. *Br J Pharmacol* **157**(3)**:** 436-450.

Sutherland TE, Maizels RM, Allen JE (2009). Chitinases and chitinase-like proteins: potential therapeutic targets for the treatment of T-helper type 2 allergies. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* **39**(7)**:** 943-955.

Suzuki M, Taha R, Ihaku D, Hamid Q, Martin JG (1999). CD8+ T cells modulate late allergic airway responses in Brown Norway rats. *J Immunol* **163**(10)**:** 5574-5581.

Svoboda P, Ko SH, Cho B, Yoo SH, Choi SW, Ye SK, *et al.* (2008). Neopterin, a marker of immune response, and 8-hydroxy-2'-deoxyguanosine, a marker of oxidative stress, correlate at high age as determined by automated simultaneous high-performance liquid chromatography analysis of human urine. *Analytical biochemistry* **383**(2)**:** 236-242.

Taguchi K, Motohashi H, Yamamoto M (2011). Molecular mechanisms of the Keap1-Nrf2 pathway in stress response and cancer evolution. *Genes Cells* **16**(2)**:** 123-140.

Takatsu K, Nakajima H (2008). IL-5 and eosinophilia. *Curr Opin Immunol* **20**(3)**:** 288-294.

Tan ML, Kuroyanagi M, Sulaiman SF, Najimudin N, Muhammad TST (2005). Cytotoxic activities of major diterpenoid constituents of Andrographis paniculata in a panel of human tumor cell lines. *Pharm Biol* **43**(6)**:** 501-508.

Tang Y, Guan SP, Chua BY, Zhou Q, Ho AW, Wong KH, *et al.* (2012). Antigen-specific effector CD8 T cells regulate allergic responses via IFN-gamma and dendritic cell function. *J Allergy Clin Immunol* **129**(6)**:** 1611-1620 e1614.

Tanino M, Betsuyaku T, Takeyabu K, Tanino Y, Yamaguchi E, Miyamoto K, *et al.* (2002). Increased levels of interleukin-8 in BAL fluid from smokers susceptible to pulmonary emphysema. *Thorax* **57**(5)**:** 405-411.

Tashkin DP, Cooper CB (2004). The role of long-acting bronchodilators in the management of stable COPD. *Chest* **125**(1)**:** 249-259.

Taylor JD (2010). COPD and the response of the lung to tobacco smoke exposure. *Pulm Pharmacol Ther* **23**(5)**:** 376-383.

Tharanathan RN, Kittur FS (2003). Chitin--the undisputed biomolecule of great potential. *Crit Rev Food Sci Nutr* **43**(1)**:** 61-87.

Thomas PS, Yates DH, Barnes PJ (1995). Tumor necrosis factor-alpha increases airway responsiveness and sputum neutrophilia in normal human subjects. *American journal of respiratory and critical care medicine* **152**(1)**:** 76-80.

Tipnis SR, Blake DG, Shepherd AG, McLellan LI (1999). Overexpression of the regulatory subunit of gamma-glutamylcysteine synthetase in HeLa cells increases gamma-glutamylcysteine synthetase activity and confers drug resistance. *Biochem J* **337 (Pt 3):** 559-566.

Traves SL, Culpitt SV, Russell RE, Barnes PJ, Donnelly LE (2002). Increased levels of the chemokines GROalpha and MCP-1 in sputum samples from patients with COPD. *Thorax* **57**(7)**:** 590-595.

Trivedi NP, Rawal UM, Patel BP (2007). Hepatoprotective effect of andrographolide against hexachlorocyclohexane-induced oxidative injury. *Integr Cancer Ther* **6**(3)**:** 271-280.

Truyen E, Coteur L, Dilissen E, Overbergh L, Dupont LJ, Ceuppens JL, *et al.* (2006). Evaluation of airway inflammation by quantitative Th1/Th2 cytokine mRNA measurement in sputum of asthma patients. *Thorax* **61**(3)**:** 202-208.

Tsoumakidou M, Tzanakis N, Chrysofakis G, Siafakas NM (2005). Nitrosative stress, heme oxygenase-1 expression and airway inflammation during severe exacerbations of COPD. *Chest* **127**(6)**:** 1911-1918.

Tsuchiya K, Isogai S, Tamaoka M, Inase N, Akashi T, Martin JG, *et al.* (2009). Depletion of CD8+ T cells enhances airway remodelling in a rodent model of asthma. *Immunology* **126**(1)**:** 45-54.

Tzeng YM, Lee YC, Cheng WT, Shih HN, Wang HC, Rao YK, *et al.* (2012). Effects of andrographolide and 14-deoxy-11,12-didehydroandrographolide on cultured primary astrocytes and PC12 cells. *Life Sci* **90**(7-8)**:** 257-266.

Tzortzaki EG, Dimakou K, Neofytou E, Tsikritsaki K, Samara K, Avgousti M, *et al.* (2012). Oxidative DNA damage and somatic mutations: a link to the molecular pathogenesis of chronic inflammatory airway diseases. *Chest* **141**(5)**:** 1243-1250.

Tzortzaki EG, Dimakou K, Neofytou E, Tsikritsaki K, Samara K, Avgousti M, *et al.* (2011). Oxidative DNA Damage and Somatic Mutations: a link to the molecular pathogenesis of Chronic Inflammatory Airway Diseases. *Chest*.

Valdiani A, Kadir MA, Tan SG, Talei D, Abdullah MP, Nikzad S (2012). Nain-e Havandi Andrographis paniculata present yesterday, absent today: a plenary review on underutilized herb of Iran's pharmaceutical plants. *Molecular biology reports* **39**(5)**:** 5409-5424.

van der Toorn M, Smit-de Vries MP, Slebos DJ, de Bruin HG, Abello N, van Oosterhout AJ, *et al.* (2007). Cigarette smoke irreversibly modifies glutathione in airway epithelial cells. *American journal of physiology. Lung cellular and molecular physiology* **293**(5)**:** L1156-1162.

van Durme YM, Verhamme KM, Stijnen T, van Rooij FJ, Van Pottelberge GR, Hofman A, *et al.* (2009). Prevalence, incidence, and lifetime risk for the development of COPD in the elderly: the Rotterdam study. *Chest* **135**(2)**:** 368-377.

van Overveld FJ, Jorens PG, Rampart M, de Backer W, Vermeire PA (1991). Tumour necrosis factor stimulates human skin mast cells to release histamine and tryptase. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* **21**(6)**:** 711-714.

Varin A, Mukhopadhyay S, Herbein G, Gordon S (2010). Alternative activation of macrophages by IL-4 impairs phagocytosis of pathogens but potentiates microbial-induced signalling and cytokine secretion. *Blood* **115**(2)**:** 353-362.

Varma A, Padh H, Shrivastava N (2009). Andrographolide: A New Plant-Derived Antineoplastic Entity on Horizon. *Evid Based Complement Alternat Med*.

Velazquez JR, Teran LM (2011). Chemokines and their receptors in the allergic airway inflammatory process. *Clinical reviews in allergy & immunology* **41**(1)**:** 76-88.

Veldhoen M, Uyttenhove C, van Snick J, Helmby H, Westendorf A, Buer J, *et al.* (2008). Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat Immunol* **9**(12)**:** 1341-1346.

Verri WA, Jr., Guerrero AT, Fukada SY, Valerio DA, Cunha TM, Xu D, *et al.* (2008). IL-33 mediates antigen-induced cutaneous and articular hypernociception in mice. *Proc Natl Acad Sci U S A* **105**(7)**:** 2723-2728.

Vestbo J, Sorensen T, Lange P, Brix A, Torre P, Viskum K (1999). Long-term effect of inhaled budesonide in mild and moderate chronic obstructive pulmonary disease: a randomised controlled trial. *Lancet* **353**(9167)**:** 1819-1823.

Vibhuti A, Arif E, Deepak D, Singh B, Qadar Pasha MA (2007). Correlation of oxidative status with BMI and lung function in COPD. *Clin Biochem* **40**(13-14)**:** 958-963.

Vlahos R, Bozinovski S, Chan SP, Ivanov S, Linden A, Hamilton JA, *et al.* (2010). Neutralizing granulocyte/macrophage colony-stimulating factor inhibits cigarette smoke-induced lung inflammation. *Am J Respir Crit Care Med* **182**(1)**:** 34-40.

Vlahos R, Bozinovski S, Hamilton JA, Anderson GP (2006). Therapeutic potential of treating chronic obstructive pulmonary disease (COPD) by neutralising granulocyte macrophage-colony stimulating factor (GM-CSF). *Pharmacol Ther* **112**(1)**:** 106-115.

Vroling AB, Duinsbergen D, Fokkens WJ, van Drunen CM (2007). Allergen induced gene expression of airway epithelial cells shows a possible role for TNF-alpha. *Allergy* **62**(11)**:** 1310-1319.

Wang XS, Tan TN, Shek LP, Chng SY, Hia CP, Ong NB, *et al.* (2004). The prevalence of asthma and allergies in Singapore; data from two ISAAC surveys seven years apart. *Arch Dis Child* **89**(5)**:** 423-426.

Wang YH, Liu YJ (2008). The IL-17 cytokine family and their role in allergic inflammation. *Current opinion in immunology* **20**(6)**:** 697-702.

Wang YH, Wills-Karp M (2011). The potential role of interleukin-17 in severe asthma. *Current allergy and asthma reports* **11**(5)**:** 388-394.

Wang Z, Yu P, Zhang G, Xu L, Wang D, Wang L, *et al.* (2010). Design, synthesis and antibacterial activity of novel andrographolide derivatives. *Bioorg Med Chem* **18**(12)**:** 4269-4274.

Wang Z, Zheng T, Zhu Z, Homer RJ, Riese RJ, Chapman HA, Jr., *et al.* (2000). Interferon gamma induction of pulmonary emphysema in the adult murine lung. *J Exp Med* **192**(11)**:** 1587-1600.

Weathington NM, van Houwelingen AH, Noerager BD, Jackson PL, Kraneveld AD, Galin FS, *et al.* (2006). A novel peptide CXCR ligand derived from extracellular matrix degradation during airway inflammation. *Nat Med* **12**(3)**:** 317-323.

Webb DC, McKenzie AN, Foster PS (2001). Expression of the Ym2 lectin-binding protein is dependent on interleukin (IL)-4 and IL-13 signal transduction: identification of a novel allergy-associated protein. *J Biol Chem* **276**(45)**:** 41969-41976.

Wegmann M (2011). Targeting eosinophil biology in asthma therapy. *Am J Respir Cell Mol Biol* **45**(4)**:** 667-674.

Wenzel SE (2012). Asthma phenotypes: the evolution from clinical to molecular approaches. *Nature medicine* **18**(5)**:** 716-725.

Wiart C, Kumar K, Yusof MY, Hamimah H, Fauzi ZM, Sulaiman M (2005). Antiviral properties of ent-labdene diterpenes of Andrographis paniculata nees, inhibitors of herpes simplex virus type 1. *Phytotherapy research : PTR* **19**(12)**:** 1069-1070.

Wills-Karp M (2004). Interleukin-13 in asthma pathogenesis. *Immunol Rev* **202:** 175-190.

Wong WS, Zhao J (2008). Proteome analysis of chronically inflamed lungs in a mouse chronic asthma model. *Int Arch Allergy Imm* **147**(3)**:** 179-189.

Woo AY, Waye MM, Tsui SK, Yeung ST, Cheng CH (2008). Andrographolide up-regulates cellular-reduced glutathione level and protects cardiomyocytes against hypoxia/reoxygenation injury. *The Journal of pharmacology and experimental therapeutics* **325**(1)**:** 226-235.

Woodruff PG, Modrek B, Choy DF, Jia G, Abbas AR, Ellwanger A, *et al.* (2009). T-helper type 2-driven inflammation defines major subphenotypes of asthma. *American journal of respiratory and critical care medicine* **180**(5)**:** 388-395.

Woolhouse IS, Bayley DL, Lalor P, Adams DH, Stockley RA (2005). Endothelial interactions of neutrophils under flow in chronic obstructive pulmonary disease. *Eur Respir J* **25**(4)**:** 612-617.

World Health Organization. (2005). *Global tobacco treaty enters into force with 57 countries already committed*. edn. World Health Organization: Geneva.

World Health Organization. (2003). *The World health report : 2003 : shaping the future*. edn. World Health Organization: Geneva.

Wright JL, Cosio M, Churg A (2008). Animal models of chronic obstructive pulmonary disease. *American journal of physiology. Lung cellular and molecular physiology* **295**(1)**:** L1-15.

Wuthrich M, Warner T, Klein BS (2005). CD28 is required for optimal induction, but not maintenance, of vaccine-induced immunity to Blastomyces dermatitidis. *Infection and immunity* **73**(11)**:** 7436-7441.

Wynn TA (2003). IL-13 effector functions. *Annu Rev Immunol* **21:** 425-456.

Xia YF, Ye BQ, Li YD, Wang JG, He XJ, Lin X, *et al.* (2004). Andrographolide attenuates inflammation by inhibition of NF-kappa B activation through covalent modification of reduced cysteine 62 of p50. *J Immunol* **173**(6)**:** 4207-4217.

Xiao C, Puddicombe SM, Field S, Haywood J, Broughton-Head V, Puxeddu I, *et al.* (2011). Defective epithelial barrier function in asthma. *J Allergy Clin Immunol* **128**(3)**:** 549-556 e541-512.

Xing J, Wu Y, Ni B (2011). Th9: a new player in asthma pathogenesis? *The Journal of asthma : official journal of the Association for the Care of Asthma* **48**(2)**:** 115-125.

Xing Z, Ohkawara Y, Jordana M, Graham F, Gauldie J (1996). Transfer of granulocyte-macrophage colony-stimulating factor gene to rat lung induces eosinophilia, monocytosis, and fibrotic reactions. *J Clin Invest* **97**(4)**:** 1102-1110.

Yamaguchi Y, Nasu F, Harada A, Kunitomo M (2007). Oxidants in the gas phase of cigarette smoke pass through the lung alveolar wall and raise systemic oxidative stress. *J Pharmacol Sci* **103**(3)**:** 275-282.

Yang KS, Ng TP, Kwang YP, Thilagaratnam S, Wong CS, Chia F (2007). Prevalence of childhood asthma and control in children assessed in a pilot school-based intervention programme in Singapore. *J Paediatr Child Health* **43**(5)**:** 353-358.

Yang L, Cohn L, Zhang DH, Homer R, Ray A, Ray P (1998). Essential role of nuclear factor kappaB in the induction of eosinophilia in allergic airway inflammation. *J Exp Med* **188**(9)**:** 1739-1750.

Yang M, Kumar RK, Hansbro PM, Foster PS (2012). Emerging roles of pulmonary macrophages in driving the development of severe asthma. *Journal of leukocyte biology* **91**(4)**:** 557-569.

Yao H, Rahman I (2011). Current concepts on oxidative/carbonyl stress, inflammation and epigenetics in pathogenesis of chronic obstructive pulmonary disease. *Toxicol Appl Pharmacol* **254**(2)**:** 72-85.

Yao H, Rahman I (2009). Current concepts on the role of inflammation in COPD and lung cancer. *Curr Opin Pharmacol* **9**(4)**:** 375-383.

Ying S, Humbert M, Barkans J, Corrigan CJ, Pfister R, Menz G, *et al.* (1997). Expression of IL-4 and IL-5 mRNA and protein product by CD4+ and CD8+ T cells, eosinophils, and mast cells in bronchial biopsies obtained from atopic and nonatopic (intrinsic) asthmatics. *J Immunol* **158**(7)**:** 3539-3544.

Ying S, Robinson DS, Varney V, Meng Q, Tsicopoulos A, Moqbel R, *et al.* (1991). TNF alpha mRNA expression in allergic inflammation. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* **21**(6)**:** 745-750.

Yoshida T, Ikuta K, Sugaya H, Maki K, Takagi M, Kanazawa H, *et al.* (1996). Defective B-1 cell development and impaired immunity against Angiostrongylus cantonensis in IL-5R alpha-deficient mice. *Immunity* **4**(5)**:** 483-494.

Young RP, Hopkins R, Black PN, Eddy C, Wu L, Gamble GD, *et al.* (2006). Functional variants of antioxidant genes in smokers with COPD and in those with normal lung function. *Thorax* **61**(5)**:** 394-399.

Yu AL, Lu CY, Wang TS, Tsai CW, Liu KL, Cheng YP, *et al.* (2010). Induction of heme oxygenase 1 and inhibition of tumor necrosis factor alpha-induced intercellular adhesion molecule expression by andrographolide in EA.hy926 cells. *Journal of agricultural and food chemistry* **58**(13)**:** 7641-7648.

Zhang C, Kuroyangi M, Tan BK (1998). Cardiovascular activity of 14-deoxy-11,12-didehydroandrographolide in the anaesthetised rat and isolated right atria. *Pharmacol Res* **38**(6)**:** 413-417.

Zhang YG, Huang J, Zhang J, Li XB, He C, Xiao YL, *et al.* (2010). RANTES gene polymorphisms and asthma risk: A meta-analysis. *Archives of medical research* **41**(1)**:** 50-58.

Zhao J, Zhu H, Wong CH, Leung KY, Wong WS (2005). Increased lungkine and chitinase levels in allergic airway inflammation: a proteomics approach. *Proteomics* **5**(11)**:** 2799-2807.

Zhao S, Qi Y, Liu X, Jiang Q, Liu S, Jiang Y, *et al.* (2001). Activation of NF-kappa B in bronchial epithelial cells from children with asthma. *Chin Med J (Engl)* **114**(9)**:** 909-911.

Zheng T, Kang MJ, Crothers K, Zhu Z, Liu W, Lee CG, *et al.* (2005). Role of cathepsin S-dependent epithelial cell apoptosis in IFN-gamma-induced alveolar remodeling and pulmonary emphysema. *J Immunol* **174**(12)**:** 8106-8115.

Zheng T, Zhu Z, Wang Z, Homer RJ, Ma B, Riese RJ, Jr., *et al.* (2000). Inducible targeting of IL-13 to the adult lung causes matrix metalloproteinase- and cathepsin-dependent emphysema. *The Journal of clinical investigation* **106**(9)**:** 1081-1093.

Zhou L, Chong MM, Littman DR (2009). Plasticity of CD4+ T cell lineage differentiation. *Immunity* **30**(5)**:** 646-655.

Zhou Y, McLane M, Levitt RC (2001). Th2 cytokines and asthma. Interleukin-9 as a therapeutic target for asthma. *Respir Res* **2**(2)**:** 80-84.

Zhu Z, Ma B, Zheng T, Homer RJ, Lee CG, Charo IF, *et al.* (2002). IL-13-induced chemokine responses in the lung: role of CCR2 in the pathogenesis of IL-13-induced inflammation and remodeling. *J Immunol* **168**(6)**:** 2953-2962.

Zhu Z, Zheng T, Homer RJ, Kim YK, Chen NY, Cohn L, *et al.* (2004). Acidic mammalian chitinase in asthmatic Th2 inflammation and IL-13 pathway activation. *Science* **304**(5677)**:** 1678-1682.