

Effect of Simvastatin on Airway Inflammation, Remodelling and Hyperreactivity
in a House Dust Mite Challenged Murine Model of Allergic Asthma

By

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Dedication

This thesis is dedicated to my lovely daughters, my wife and my Parents: without their support it would have been impossible to complete this journey.

Abstract

Asthma is a chronic lung disease characterized by airway inflammation, remodelling and hyperresponsiveness. Many patients remain symptomatic despite use of current therapies. Awareness of pleiotropic benefits of statins positions them as a candidate for alternate asthma therapy. Though high dose simvastatin treatment is effective in disease models, this has not been replicated in clinical trials. My project tested the hypothesis that lung-targeted delivery of simvastatin alleviates pathophysiologic hallmarks of allergen-induced ‘asthma’ in mice.

Using a clinically relevant murine model of allergic asthma, in Chapter Four, we show that though high-dose systemically delivered simvastatin does not prevent house dust mite (HDM) challenge-induced airway hyperreactivity; it does blunt airway inflammation (immune cell infiltration and cytokine release), and even more so using a prophylactic treatment regime. Notably, Ultra High Performance Liquid Chromatography-tandem Mass Spectrometry (UHPLC-MS/MS) showed that pharmacologically active simvastatin does not accumulate in the lungs. In Chapter Five, we delivered low dose simvastatin by intranasal instillation, and in addition to ablating lung inflammation; it also prevented HDM challenge-induced airway hyperreactivity. This effect was at least equivalent to that for standard fluticasone therapy. To understand the mechanism of action of intranasally instilled simvastatin, in Chapter Six, using HPLC-MS/MS lipidomics we revealed that allergen challenge induces lung accumulation of pro-inflammatory oxidised phosphatidylcholine (OxPCs) in mice as well as in humans. Moreover, intranasally delivered simvastatin concomitantly prevented OxPC accumulation, and allergen challenge-induced lung inflammation and dysfunction. In Chapter Seven, we reveal that intranasally instilled simvastatin can reverse established airway inflammation, goblet cell hyperplasia and hyperreactivity.

Our results reveal potential therapeutic benefits of simvastatin to treat human asthma and need for clinical trials to test the effects of inhaled simvastatin delivery. We show that intranasally instilled simvastatin therapy uniquely mitigates the accumulation of lung OxPCs. Oxidative stress is primarily responsible for the generation these bioactive compounds; however, they are controlled by several mechanisms, and our data suggest new asthma therapies that target such pathways may be clinically effective. Our findings also suggest that OxPCs may play a heretofore-unappreciated role in asthma pathogenesis, and they also have a potential for use as biomarkers for asthma sub-phenotypes and disease control.

Acknowledgement,

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A PhD is a long journey where the supervisor is the guiding "Force" for the trainee. I would consider myself lucky to be the "one with the Force, and the Force was with me" during my entire PhD journey. For this, I must thank Dr Andrew J Halayko for constant support, encouragement, guidance, and above all his patience with which he listened to my ideas.

I must thank my committee members Drs Pawan Singal, Ed Kroeger, Aaron Marshall and Richard Keijzer for their efforts to keep me focused on my path. Their constant encouragement and critique were very vital in the success of my PhD journey. At this point, I would also like to highlight the name of Dr Adrian West, who was a mentor and a friend to me. I would also like to acknowledge my collaborators Dr Charles Wong and Dr Amir Ravandi, who brought valuable expertise to the success of my project.

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List of Abbreviations

ACQ	Asthma Control Questionnaire
ACT	Asthma Control Test
AERD	Aspirin Exacerbated Respiratory Disease
AHC	Agglomerative Hierarchical Clustering
AHR	Airway Hyperresponsiveness
AQLQ	Asthma Quality of Life Questionnaire
ARRIVE	Animal Research: Reporting of In Vivo Experiments
ASM	Airway Smooth Muscle
BALF	Bronco-alveolar lavage fluid
BHT	Butylated hydroxytoluene
BSM	Bronchial Smooth Muscle
BT	Bronco Thermoplasty
CCL	Chemokine ligands
CHD	Coronary Heart Disease
DNA	Deoxyribonucleic acid
DPPC	Dipalmitoyl-PC
EAR	Early Asthma Response
ECM	Extra cellular matrix
ED	Emergency Department
EIA	Exercise Induced Asthma
EOA	Early Onset Asthma
ESI	Electro Spray Ionization
FA	Fatty acid
FAK	Focal adhesion kinase
FDA	Federal Drug Agency
FEV1	Forced expiratory volume in 1 second
FGF	Fibroblast growth factor
FOXO	Forkhead box class O

FPP	Farnesyl pyrophosphate
FT	Farnesyl transferase
FVC	Forced vital capacity
G	Tissue Resistance
GABA	Gamma amino butyric acid
GGPP	Geranylgeranyl pyrophosphate
GGT	Geranylgeranyl transferase
GINA	Global Initiative for Asthma
GM-CSF	Granulocyte-monocyte colony-stimulating factor
GRE	Glucocorticoid Response Element
H	Tissue Elastance
H&E	Hematoxylene and Eosin
HB-EGF	Heparin-binding epidermal growth factor-like growth factor
HDL	High-density lipoprotein
HDM	House dust mite
HLB	Hydrophilic-lipophilic balance
HMG-CoA	Hydroxy methyl glutaryl co-enzyme A
HO	Hemoxygenase
HO-1	Hemoxygenase -1
HODA-PC	9-Hydroxy-12-oxo-10-dodecenoic acid ester of PC
HPLC	High-performance liquid chromatography
HSD	Honest significant difference
i.n.	Intranasal
ICS	Inhaled corticosteroid
IFN γ	Interferon gamma
IGF	Insulin-like growth factor
IL	Interleukins
IsoP	Isoprostane
IVC	Individually ventilated cage
JAM	Junctional adhesion molecules
JUPITER	The Justification for the Use of Statins in Prevention:

an Intervention Trial Evaluating Rosuvastatin

KC/Gro	Keratinocyte chemoattractant/ Human growth-regulated oncogene
KDiA-PC	9-Keto-10-dodecendioic acid of PC
KODA-PC	9-Keto-12-oxo-10-dodecenoic acid ester of PC
KOHA-PC	1- <i>O</i> -Hexadecyl-2-(4-oxo-7-oxohept-5-enoyl)- <i>sn</i> -glycero-3-phosphatidylcholine
KOOA-PC	5-Keto-8-oxo-6-octenoic acid esters of PC
LABA	Long-acting beta-2-adrenoceptor agonist
LAR	Late asthma response
LDL	Low-density lipoprotein
LOA	Late onset asthma
LPCAT	Lysophosphatidylcholine acyltransferase
LPS	Lipopolysaccharide
MANOVA	Multivariate analysis of variance
MCP	Monocyte chemotactic protein
MHC	Major histocompatibility complex
MLCK	Myosin light chain kinase
MMP	Matrix metalloproteinase
MRM	Multiple reaction monitoring
NA	Neutrophilic asthma
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NOS	Nitric oxide synthase
OR	Odds ratio
OVA	Ovalbumin
OxPC	Oxidized phosphatidylcholine
PAF-AH	Platelet-activating factor acetylhydrolase
PAPC	1-Palmitoyl-2-arachidonoyl- <i>sn</i> -glycero-3-phosphocholine
PAS	Periodic acid schiff
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PC ₂₀	Provocative concentration of MCh required to increase R _N by 43.5%

PCA	Principal component analysis
PDGF	Platelet derived growth factor
PEEP	Positive end expiratory pressure
PEIPC	1-Palmitoyl-2-epoxyisoprostane-sn-glycero-3-phosphocholine
PFT	Pulmonary function test
PG	Prostaglandins
PGPC	1-Palmytoyl-2(5-glutaryl)-sn-glycero-3-phosphocholine
PLC	Phospholipase-C
PLPC	1-Palmitoyl-2-linoleyl-sn-glycero-3-phosphocholine
PLSDA	Partial least square discriminant analysis
PONPC	1-Palmitoyl-2-(9-oxononanoyl)-sn-glycero-3-phosphocholine
POPC	1-Palmitoyl-2-oleyl-sn-glycero-3-phosphocholine
POVPC	1-Palmytoyl-2(5-oxovaleryl)-sn-glycero-3-phosphocholine
PPAR	Peroxisome proliferation activator receptor
PUFA	Polyunsaturated fatty acid
R _N	Newtonian Resistance
ROCK	Rho-associated protein kinase
ROS	Reactive oxygen species
RP-HPLC	Reverse phase high performance liquid chromatography
s.c..	Subcutaneous
SABA	Short-acting Beta-2-adrenoceptor agonist
SAPC	1-Stearoyl-2-arachidonoyl- <i>sn</i> -glycero-3-phosphocholine
SLPC	1-Stearoyl-2-linoleyl-sn-glycero-3-phosphocholine
SPE	Solid phase extraction
TFPI	Tissue factor pathway inhibitor
TG	Triglycerides
TGFβ1	Transforming growth factor β1
TLR	Toll-like receptor
TNFα	Tumour necrosis factor alpha
UHPLC/MS/MS	Ultrahigh-performance liquid chromatography-tandem mass spectrometry
VCAM	Vascular cell adhesion molecule

VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VIP	Variance importance in projection
VLDL	Very low-density lipoprotein
WHO	World Health Organization
ZO	Zonula occludens
Zrs	Respiratory mechanical input impedance

Chapter 1: Literature Review

1.1 Asthma:

1.1.1 Epidemiology

Asthma is a chronic disease of the lung that is highly prevalent among people of all ages and all sexes (1, 2). The prevalence of asthma has increased significantly: according to the Global Initiative for Asthma (GINA) guidelines 2014, over 300 million people worldwide have asthma, an estimate that has jumped almost 42% from the previous survey published in 2011 in The Global Asthma Report. Unfortunately, the number of people who have asthma includes 14% of the world's children. Children approaching adolescence and elderly are at the greatest risk of asthma-related disability and mortality (2, 3) (Figure 1.1), leading to hospitalisation and school absenteeism among children, and hospitalisation among the elderly (4). Asthma-related hospitalisation, physician or emergency department visits and prescription medicines represent the direct cost for asthma management. Hidden beneath this direct cost is a huge indirect economic burden resulting from the loss of productivity in patients with asthma and their parents (Figure 1.2). These direct and indirect asthma management cost can amount to billions of dollars that have to be born by the society (3-7). A better understanding of the disease pathology and a better treatment approach is required to improve the quality of life of the asthma patients and reduce the economic burden on society.

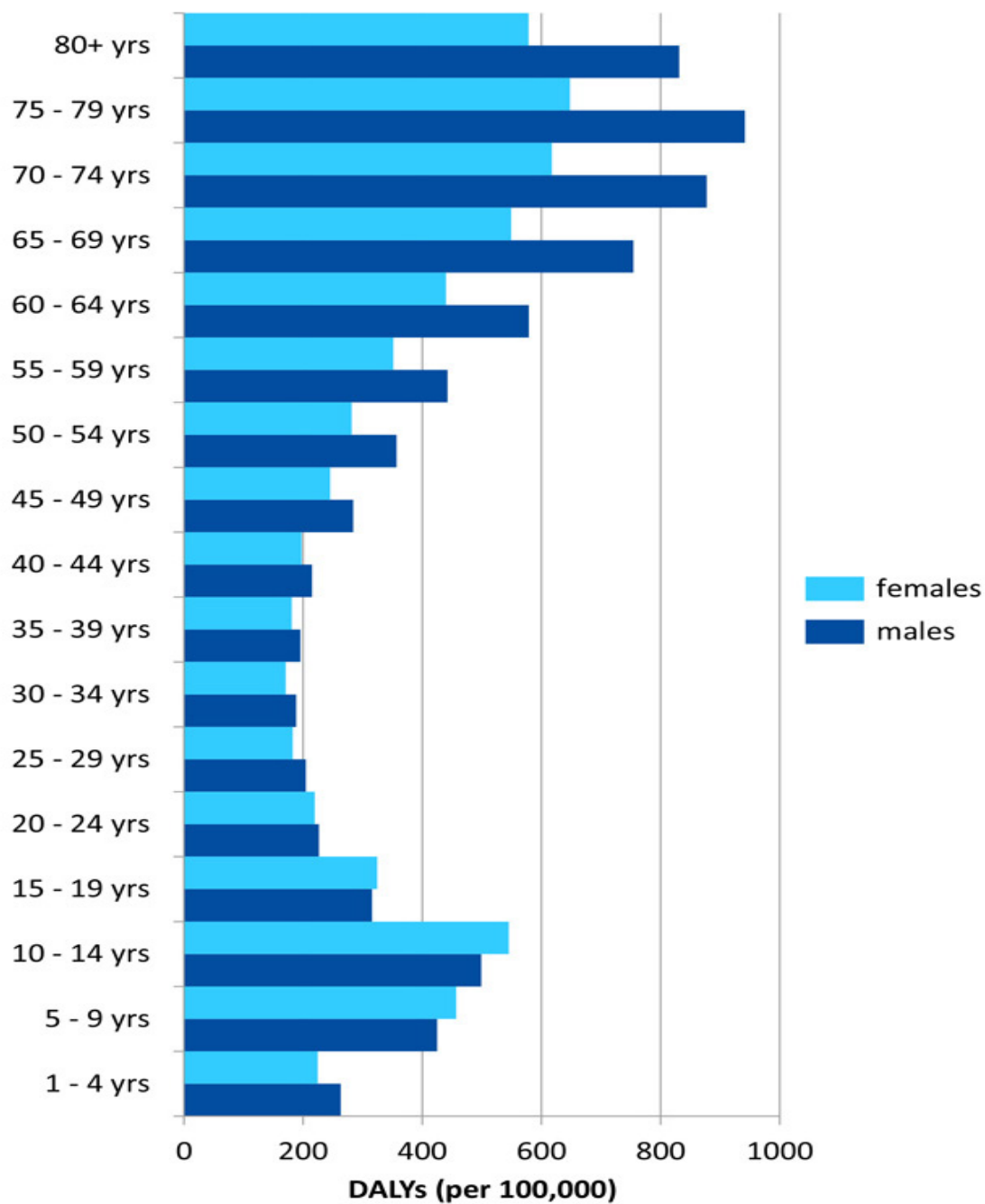


Figure 1.1: Asthma Disease Burden by Disability.

Adjusted Life Years (DALYs) per 100,000 populations attributed to asthma by age group and sex. From The Global Asthma Report 2014 (3). Reproduced with permission.

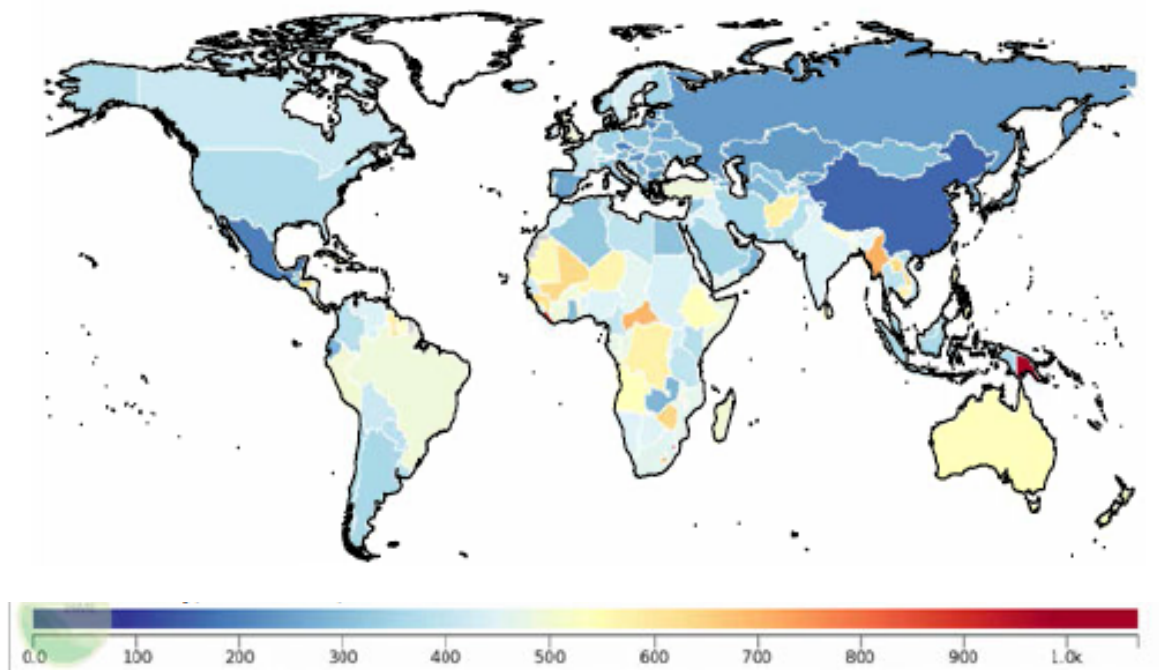


Figure 1.2: Asthma Disease Burden By Country.

Disability adjusted life years (DALYs) per 100,000 populations attributed to asthma by country, both sexes. From The Global Asthma Report 2014 (3). Reproduced with permission.

1.1.2 Pathophysiology

The word "Asthma" is Greek in origin and means difficulty in breathing. During the 19th century, physicians began to appreciate the distinct pathophysiology of asthma from other causes of breathlessness, mainly due to the exceptional work of Dr Henry Hyde Salter, who described asthma as "paroxysmal dyspnea of peculiar character with intervals of healthy respiration in between attacks." Dr Salter explained many characteristic features of the disease such as bronchial hyperresponsiveness to various environmental, physical and chemical cues (1, 8). Later in the 19th century, Sir William Osler described other characteristic features of asthma such as airway smooth muscle spasm (ASM), swelling of the bronchial mucous membrane, distinct sputum and typical inflammation of smaller bronchioles, in his first edition of the textbook *Principals and Practice of Medicine*. Both Salter and Osler noticed that the airway inflammation and structural changes (remodelling) accompanied bronchial hyperresponsiveness. The features of remodelled (thickened) airway wall include increased smooth muscle mass, deposition of matrix proteins (8). By the early 20th century, it became a known fact that all asthma-related death was associated with severe lung inflammation and remodelling. However, at that time asthma was mainly regarded as an acute disorder of episodic bronchospasm, driven mainly by the smooth muscle contraction, and the role of inflammation remained under-appreciated (1), until the discovery of IgE that explained the allergic inflammation induced asthma exacerbation (9, 10). Since then inflammation became the pivotal point in asthma pathophysiology.

Currently, GINA defines asthma as "a heterogeneous disease, usually characterized by chronic airway inflammation. It is defined by the history of respiratory symptoms such as

wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation”(11) This definition is based on the common characteristics of asthma including episodic airflow limitation with symptoms of chest tightness, wheeze or a cough. The key pathologies behind these symptoms of asthma and airflow limitations are airway inflammation; tissue remodelling and hyperresponsiveness, which together lead to the airflow limitation (Figure 1.3). In subsequent sections, we will discuss individual pathological features.

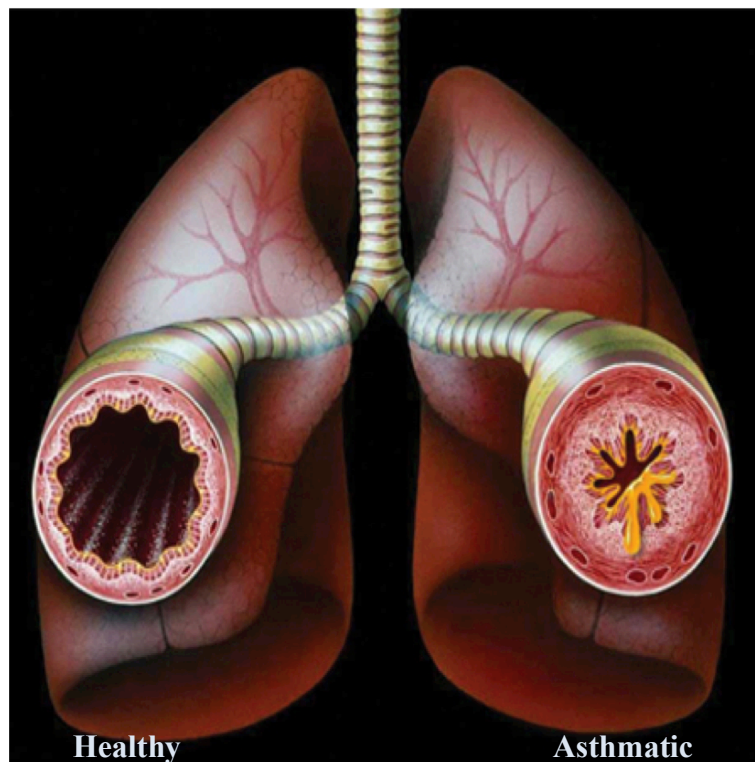


Figure 1.3: Healthy Vs. Asthmatic Airway.

Pathological changes in asthmatic airways when compared to a healthy airway. The asthmatic airway shows excessive airway remodelling: smooth muscle thickening, thickening of the basal membrane and epithelial lining, mucus plug, and inflammation. From Jeffery PK, Haahtela T. BMC Pulm Med. 2006 (12). Reproduced with permission.

1.1.2.1 Inflammation

Inflammation plays an important role in the pathophysiology of asthma. However, the nature of inflammation in asthma is quite complex and heterogeneous (13). Airway inflammation in asthma is mainly limited to the conducting airways, and depending on the severity of the disease it can spread up to the alveoli (14). Under the classic paradigm, inflammation in asthma is a biphasic process, characterized by an early- and late- phase. The early phase of inflammation begins with the interaction of IgE with FcεR1 surface receptors on mast cells in the presence of an antigen. This interaction results in the degranulation of mast cells that release stored inflammatory mediators such as histamine, prostaglandin D2, leukotriene C4, and mast cell-specific tryptase, all of which are capable of driving both early phase inflammation and bronchial hyperresponsiveness. At the same time, the activation of mast cells initiate the synthesis of cytokines such as IL-4, 5 and 13 and thereby skews the immune system towards the Th2 phenotype, a process that marks the beginning of the late phase of the inflammatory response. In this phase proinflammatory cells such as eosinophils, neutrophils, macrophages and lymphocytes are recruited (15). These immune cells in-turn induce a cycle of cytokine release and recruitment of proinflammatory cells (16) that further aggravates the inflammation, at the same time inducing oxidative stress, which is known to exacerbate local inflammation and contribute to tissue damage and remodelling (17).

1.1.2.2 Oxidative Stress

Human lungs have an enormous surface area almost equivalent to a tennis court, which constantly remains in contact with environmental as well as circulatory oxygen (18) that makes oxidative stress unavoidable in the lung. In fact, a low level of oxidative stress is essential

for the normal cell functions such as signalling and proliferation (17). In a healthy person, antioxidant machinery maintains strict homeostasis in redox balance (19). However, in some asthmatics the oxidative stress produced by the environmental oxidants as well as ongoing inflammation (Figure 1.4) (20) may overwhelm the body's defense mechanism against oxidative stress. This results in the excessive free radical generation such as reactive oxygen species and reactive nitrogen species that attack the phospholipid-rich plasma membrane and the fluid lining of the lungs (21, 22).

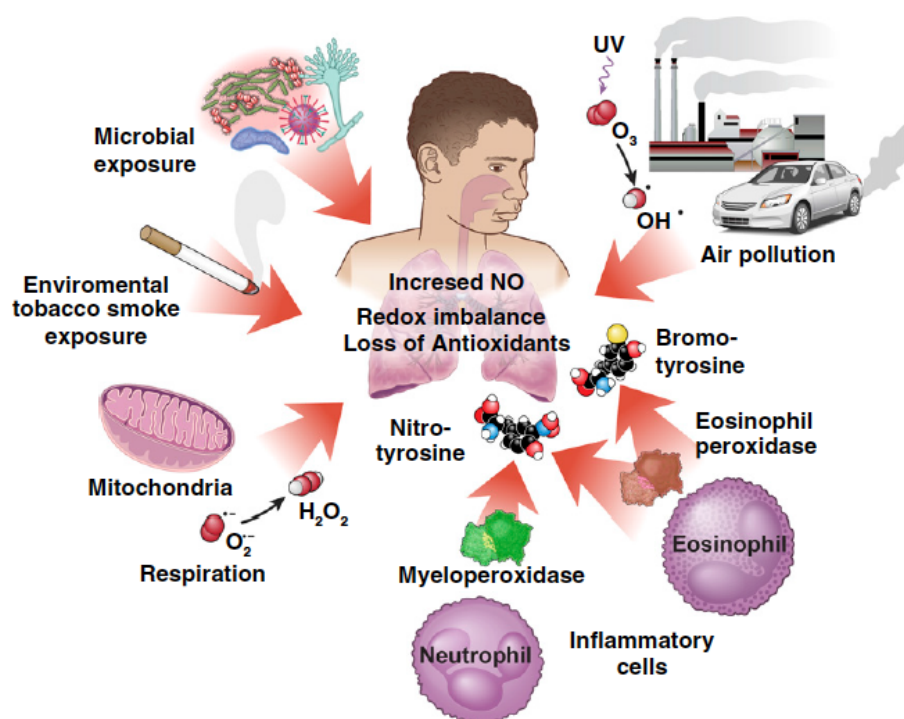


Figure 1.4: Oxidative stress in asthma.

The redox imbalance and loss of antioxidant machinery triggers excessive generation of free radicals and excessive oxidative stress, in response to various environmental cues such as pollution, cigarette smoke, and even mitochondrial function. The chronic inflammatory state further aggravates oxidative stress in asthma. From Erzurum SC. Ann Am Thorac Soc.2016 (20). Reproduced with permission.

Phosphatidylcholine (PC) is a major phospholipid in lung plasma membrane, as well as lung surfactant. It consists of a phosphorylated choline that forms the polar head group, and

two fatty acid chains at sn-1 and sn-2 positions. Normally the sn-1 position is occupied by a saturated fatty acid, whereas, the sn-2 position contains an oxidation-prone, polyunsaturated fatty acid (PUFA) (Figure 1.5). PUFAs in mammalian plasma membrane phospholipids consist of fatty acids such as arachidonic acid, linoleic acid, and their endogenous derivatives. The oxidation of PUFAs produces hundreds of oxidized products of PCs (OxPCs). For example, 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (PAPC) is one of such naturally occurring PCs that contains a saturated palmitate (16:0) moiety at sn-1 position and a polyunsaturated arachidonate moiety (20:4) at sn-2 position (23). Oxidative stress converts arachidonate moiety into peroxyl radical, which undergoes either cyclization or fragmentation to generate a variety of biologically active OxPAPCs (Figure 1.5) (22, 24).

The PCs in lung surfactants are unique as they mainly contain saturated fatty acids at both sn-1 and -2 positions. For example, the main component of lung surfactant Dipalmitoyl-PC (DPPC) has two saturated palmitoyl (16:0) moieties attached to choline head group (25, 26). De-novo PC synthesis is the major source of DPPC production: the unsaturated fatty acid in PC undergoes hydrolysis followed by reacylation in the presence of enzymes such as phospholipase A2 (PLA2) and Acyl-CoA: lysophosphatidylcholine acyltransferase (LPCAT) to form DPPC; a process that produces up to 75% of DPPC (21, 25, 27). However, under excessive oxidative stress, the PUFA chain may undergo an oxidation reaction similar the PUFA chain in the plasma membrane, compromising the surfactant quality and generation of bioactive OxPCs such as 1-palmitoyl-2-(9-oxononanoyl)-sn-glycero-3-phosphocholine (PONPC) and 1-palmitoyl-2-azetyl-sn-glycero-3-phosphocholine (PAzPC) (28). The parent compounds for PONPC and PAzPC are 1-palmitoyl-2-linoleyl-sn-glycero-3-phosphocholine (PLPC) and 1-palmitoyl-2-oleyl-sn-glycero-3-phosphocholine (POPC) (Figure 1.6) (24).

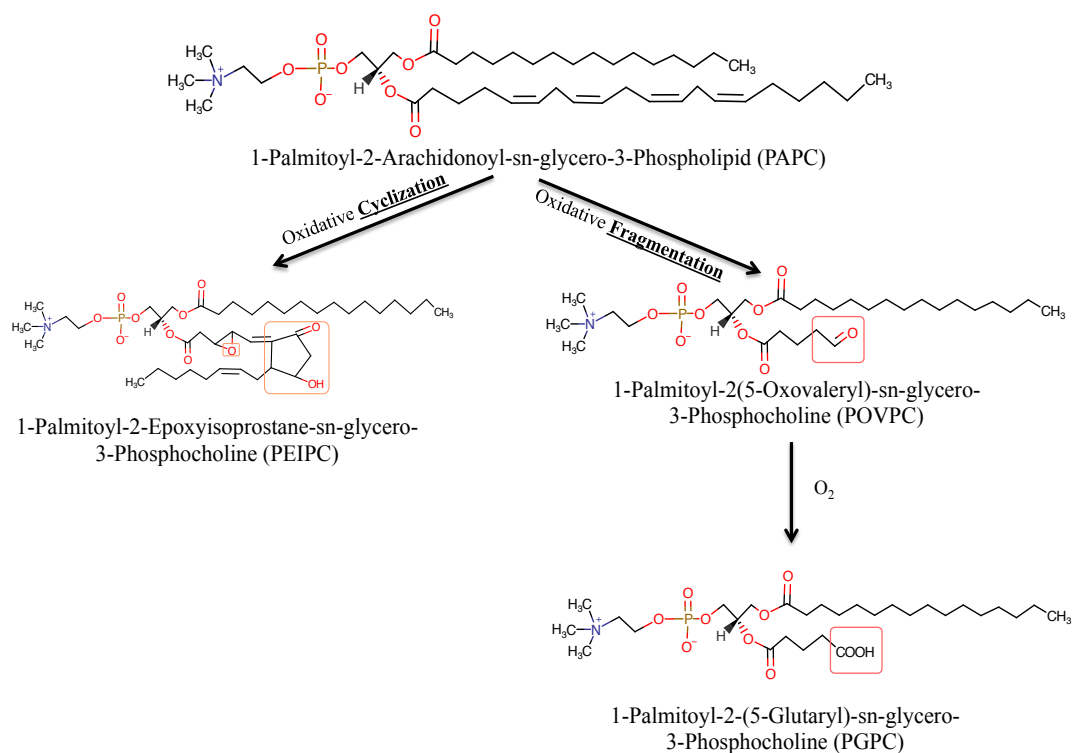


Figure 1.5: Schematic of OxPAPC synthesis.

The parent phosphatidylcholine compound, 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (PAPC) undergoes oxidation to produce cyclized and fragmented OxPCs. PEIPC is a prototype of un-fragmented OxPCs while POVPC and PGPC are examples of fragmented products.

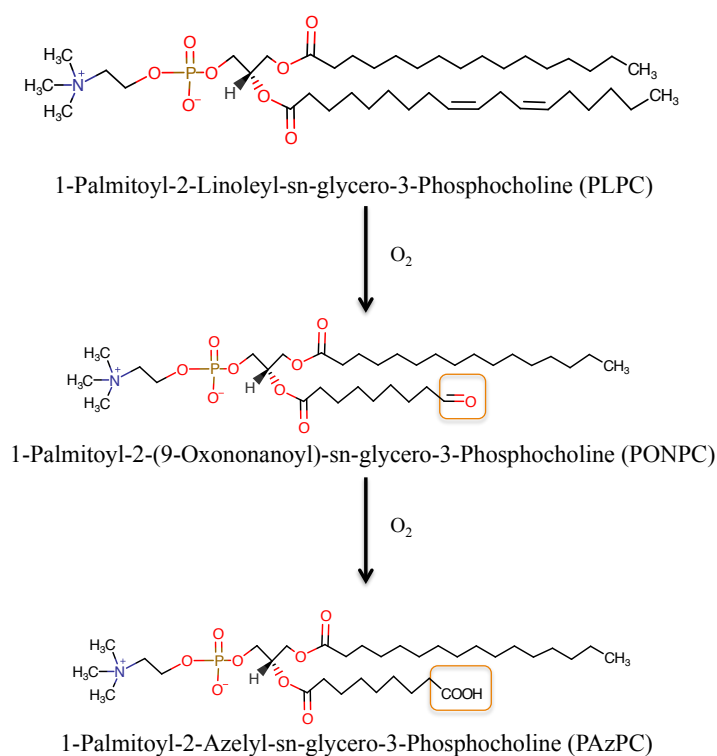


Figure 1.6: Schematic of OxPLPC synthesis.

1-palmitoyl-2-linoleyl-sn-glycero-3-phosphocholine (PLPC), naturally occurring in plasma membrane as well as the precursor of lung surfactant DPPC, undergoes oxidation to produce highly bioactive PONPC and PAzPC.

OxPCs are like a double-edged sword, as they are required at low doses for healthy endothelial barrier function, while at higher dosages they disrupt it. They are known to induce inflammation in various diseases (Figure 1.7).

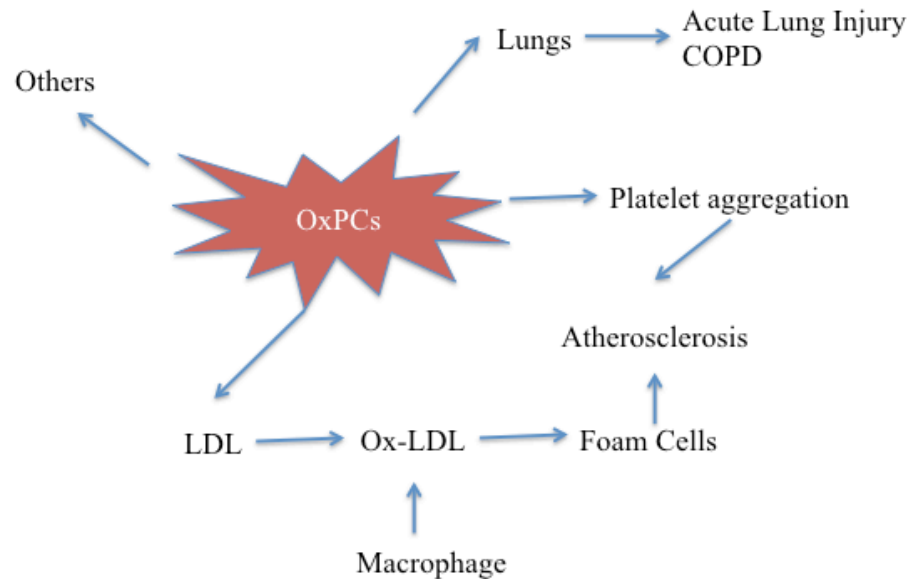


Figure 1.7: Schematic of OxPCs affecting various diseases.

From Ashraf, Lipoproteins 2012 (29). Reproduced with permission.

The proinflammatory effects of OxPCs are mainly receptor-mediated via the platelet-activating factor (PAF) receptor and toll-like receptor (TLR)-4 (30, 31). They can directly induce the vascular smooth muscle phenotype switch that makes them hypersecretory and hyperproliferative (32, 33).

Apart from their proinflammatory effects, OxPCs are also known to induce cytoskeletal remodelling in endothelial cells; however, the mechanism is debatable. In one school of thought, OxPCs induce cytoskeletal remodelling by binding to auto-inhibitory c-terminus of Rho Kinase and thereby activating it (34), which leads to phosphorylation of myosin light chain kinase (MLCK) (35). Another school of thought claims that OxPCs phosphorylate certain regulatory proteins such as cofilin, paxillin and focal adhesion kinase (FAK), thereby

inducing cytoskeletal remodelling in endothelial cells (36). However, the bottom-line is that both schools of thought support the concept that OxPCs can induce cytoskeletal remodelling in endothelial cells.

Apart from the collective pathological effects of hundreds of OxPCs, the biological activities of only a handful of individual OxPCs are well known, and they vary considerably in spite of sharing structural homology (37). For example, the cyclized form of OxPAPC, i.e., PEIPC improves epithelial barrier function *in-vitro* (38). However, it also drives the inflammation process via enhanced tethering of monocytes to the endothelial wall and production of chemo-attractants such as monocyte chemotactic protein (MCP)-1 and IL-8 required for the chemotaxis of monocytes (39). PEIPC can also dampen the innate immune response to pathogens by blunting TLR2/1 response and production of IL-12; consequently, it promotes the survival of infected cells (40). Similarly POVPC, a fragmented oxidized product of PAPC, enhances monocyte binding to the endothelial wall by enhancing the surface expression of the connecting segment (CS)-1 domain of fibronectin; however, it does not promote the binding of neutrophils to the endothelial wall (37). Interestingly, PGPC, a downstream oxidized product of POVPC, not only enhances monocyte binding but also enhances binding of neutrophils to the endothelial wall, as well as the expression of E-selectin and vascular cell adhesion molecule (VCAM)-1 (37). Both PEIPC and POVPC lack this activity (41). PGPC also plays a role in the maturation of monocyte expression of CD-36 via activation of peroxisome proliferation activator receptor (PPAR)- γ in the nucleus of monocytes. The matured monocytes take up oxidized lipids and transform into the “Foam cells” - a key pathogenic feature of atherosclerosis (30). Apart from their proinflammatory role both POVPC and PGPC are proapoptotic as well (23, 42). PONPC and PAzPC are not as well characterized as their family

members that belong to PAPC. However, they have been associated with blood coagulation via inhibition of the tissue factor pathway inhibitor (TFPI) (24). PAzPC is also a driver of apoptosis; it translocates apoptosis-inducing factor from the inner mitochondrial membrane to the nucleus, which results in leakage of cytochrome-c from mitochondria to the cytoplasm (43). Combining evidence of the effects of OxPCs on vascular endothelial and smooth muscle cell remodelling makes them an interesting but understudied target for airway diseases like asthma, where oxidative stress and airway remodelling play a pivotal role in disease pathogenesis (38, 44).

OxPCs are neutralized by the hydrolysis of the epoxy group or by the phospholipolysis (cleaves the oxidized sn-2 chain), mediated via different PLA2 such as calcium-independent intracellular PLA2 (iPLA2), calcium-dependent PLA2 (cPLA2) and calcium-dependent secretory PLA2 (sPLA2) (45). Platelet-activating factor acetylhydrolase (PAF-AH), also known as lipoprotein associated phospholipase A2 (LP-PLA2) hydrolyzes the short acyl chain present at the SN2 position of OxPCs such as POVPC and PGPC (46). Therefore, these two enzymes play an important role in preventing the accumulation of OxPCs in the body. PAF-AH is a major constituent of high-density lipoprotein (HDL). Apart from the PAF-AH enzyme HDL also contains enzymes such as paraoxonases (PON-1, -2 & -3) and lecithin cholesterol acyltransferase (LCAT) that hydrolyze short-chain OxPCs, in combination with apolipoproteins (Apo), especially Apo-A1. Apo-A1 not only plays an important role in converting the bioactive OxPCs into their inactive hydroxide forms, but it is also important for the functioning of HDL associated enzymes mentioned above. Thus, a functional HDL plays an important role in mitigating the oxidative stress induced accumulation of OxPCs and imparts its antioxidant effects (47). Since statins are also known to enhance HDL production apart from

their impact on low-density lipoprotein (LDL) (48), they are a potential therapeutic option against oxidative stress induced accumulation of OxPCs.

1.1.2.3 Airway Remodelling

Airway remodelling refers to progressive structural changes in the lungs. The exact mechanism of remodelling is elusive. A probable mechanism involves chronic wound repair and inflammation in the lungs, leading to structural changes in epithelial cells along with goblet cell hyperplasia, angiogenesis, and airway smooth muscle (ASM) phenotype switching and hypertrophy (49, 50).

As the lung is the largest internal organ that is in constant contact with the outside environment, the airway epithelium forms the first line of defense against an environmental insult on the lungs. Under normal conditions lung epithelium consists of tightly regulated ciliated columnar, goblet, and Clara cells that form a tight, impermeable barrier, which is a result of cell-cell and cell-extracellular matrix (ECM) interactions via tight junctions and cell adhesion molecules such as desmosomes and hemidesmosomes (21, 51-53). The tight junction normally consists of various proteins such as ZO 1-3, occludins, claudins 1-5, β -catenin, E-cadherin and JAM (54, 55). It is a well known that in established asthma the airway epithelium is stressed, damaged and lacks tight junctions. *In-vitro* observations show that epithelial cells from asthmatics have inherent defective tight junctions that keep them leaky even when fully differentiated (56, 57). At the same time, the exogenous insults can more easily disrupt the epithelial barrier in asthmatics compared to non-asthmatics (56). Apart from this in asthma epithelial desquamation/ epithelial shedding and metaplasia are also observed. While epithelial cell metaplasia could be the result of stress due to ongoing immune reaction, inflammation and oxidative burden (58, 59), the exact mechanism is relatively unknown for "epithelial

desquamation." Evidence suggests that a lack of desmosomes in asthmatics is responsible for this as desmosomes help in cell-cell adhesion (52, 59). Apart from damage to structural integrity, goblet cells, (the chief mucus-producing cells) also undergo hyperplasia in asthma. Studies from animal models of allergic asthma have shown that although there is no change in the total number of epithelial cells, the number of Clara cells and ciliated cells were significantly reduced: up to 75% and 25% respectively. This suggests that both cell types trans-differentiate into goblet cells, thereby increasing the number of goblet cells per unit of basement membrane (60). Under normal conditions, trans-differentiated goblet cells would undergo apoptosis, and ciliated and Clara cells populating the normal epithelium would replace them, reinforcing the epithelial barrier via cell migration and proliferation to heal this wound-like condition. However, these processes are impaired in asthmatics (61-63), resulting in a chronic wound scenario (56).

Chronic epithelial wound in conjunction with mechanical stress, environmental insults and ongoing inflammation induces the epithelium to secrete growth factors (50, 64-70), such as TGF- β , EGF, HB_EGF, amphiregulin, FGF, PDGF, IGF, and VEGF, in asthmatics (71-73). These growth factors are capable of interacting with the structural cells to induce proliferation and differentiation (74) resulting in the pseudo thickening of the basal membrane (75), which is considered a compensatory response to the lack of epithelial barrier function. However, this has little benefit against allergic insults (allergens/ pollutants/ pathogens) and is known to aggravate the immune reaction instead (76). Changes in ECM elements are also known to influence ASM mass, phenotype, and function: a marker of lung remodelling in asthma (77). In asthma, ASM cells hyper-proliferate both in size (hypertrophy) and in number (hyperplasia) (78). Proliferating ASM cells have less contractile protein (79) and acquire a hypersecretory phenotype, secreting proinflammatory mediators including arrays of cytokines as well as

profibrotic mediators such as collagen laminin and fibronectin (80, 81). Therefore, they not only drive the vicious cycle of inflammation, but also act as an accomplice in airway remodelling by adding more ECM proteins to the already remodelled subepithelial space. At the same time, mature smooth muscle cells acquire a more pro-contractile phenotype that aids to airway hypercontractility (82, 83). The presence of such a heterogeneous population of ASM cells significantly alters the response of the airway to different stimuli.

Evidence of lung remodelling have been observed in children as young as 1-3 years who wheeze. Although they show significantly elevated eosinophils, it is not clear if the structural changes observed in the lung ECM is in response to inflammation, or if it is inherent in children who go on to develop asthma (84, 85). However, a safe interpretation would be that both processes are part of the initiation of asthma and not secondary to each other and lead to another classic feature of asthma: Airway Hyperresponsiveness (AHR).

1.1.2.4 Airway Hyperresponsiveness

“Hyperresponsiveness” is defined as an exaggerated response to a given stimulus. Asthmatics airways are hyperresponsive because they show an exaggerated constriction response to otherwise non-noxious stimuli, a phenomenon called Airway Hyperresponsiveness (AHR). AHR is measured through a bronchial provocation test using a variety of physiologically relevant stimuli (11). Such stimuli could either directly stimulate the muscarinic or the histaminic receptors on smooth muscle cells and make them constrict (for example acetylcholine (ACh), Methacholine (MCh) or histamine) or indirectly elicit the release of the mediators leading to the bronchoconstriction (86).

In the laboratory, it is easier to measure constrictive responses *ex-vivo* in isolated tracheal rings or muscle strips. It is much more complex to measure the bronchoconstriction

directly *in-vivo*. Thus, the response is measured indirectly as the capacity of lungs to expire forcefully in one second, i.e., forced expiratory volume in one second (FEV1). Such a test is commonly known as pulmonary/ lung function test (PFT). A PFT exploits the concept that in an obstructive disease like asthma, the FEV1 is compromised during an exacerbation and bronchial provocation, and the decrease in FEV1 is drastic in comparison to a healthy individual. This concept was demonstrated in the classic work by Ann Woolcock in 1984 (Figure 1.8), showing that when a normal individual is exposed to a direct contractile agonist, a sigmoidal response curve is obtained, where FEV1 decreases rapidly with an increasing concentration of agonist. However, it quickly attains a plateau. In an asthmatic, the slope of the linear part of this curve shifts to the left (becomes steeper), a phenomenon known as “hypersensitive”, and upward, a phenomenon known as “hyperreactive”. If these two phenomena are observed in an airway together, the airway is termed *hyperresponsive*. Interestingly, with increasing severity of the disease the slope becomes steeper, and the plateau effect is lost (87). This work by Woolcock et al. proved that asthmatics lack the mechanism to counter airway narrowing in response to an agonist.

The mechanism behind the observed difference in response of the airway between asthmatics and non-asthmatics is still unknown. However, it is known that the remodelled structural cells in the lungs of an asthmatic can influence AHR. Out of all the components of airway remodelling, the increase in ASM mass is the most influential factor for airway contractility (78, 88). This could be intuitively explained based on the basic principles of biophysics, since increasing the mass of the ASM increases the contractile force generating capacity proportionally, when the change in velocity of contraction over a unit time is kept constant. In asthmatics, an increased velocity of shortening accompanies the increase in ASM

mass, and together they increase the force of contraction and the extent of shortening (89, 90). Apart from the thickened ASM, the remodelled airway as a whole plays an important role in AHR. Also, Lung parenchyma is normally tethered to the airway and acts like a spring, thereby preventing the collapse of the airway and helping constricted airways to reopen. Furthermore, the dynamic strain produced by normal breathing impedes airway narrowing (78, 91). However, remodelling in established asthma not only impairs the dynamic strain produced by breathing, but also decouples the airway from the lung parenchyma leading to the AHR (89). Also, the thickened airway wall inherently leads to increased airway resistance, as resistance is inversely proportional to the 4th power of radius. Thus, a small decrease in luminal radius of an airway will substantially increase its resistance (78). Thus, remodelling of the airway, combined with ASM contractility can thoroughly explain AHR in a murine model of allergic asthma (92). In parallel, a remodelled asthmatic airway has a leaky epithelium, and a secretory ASM phenotype that constantly exposes the mature contractile ASM to the variety of spasmogens leading to overall increased tone in ASM. As a result, the ASM becomes hypersensitive to otherwise non-noxious stimuli (82, 83, 93-96). All of these factors interact to cause variable levels of AHR among different patients with asthma.

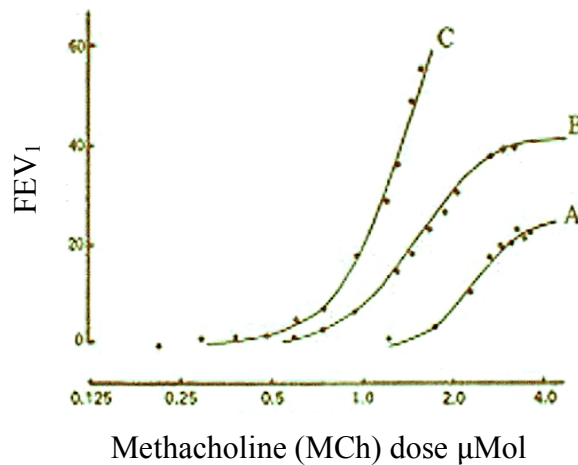


Figure 1.8: Airway responsiveness to MCh challenge.

A: normal individual, B: mild-moderate asthmatic. The curve shifts to the left and up, compared to normal. C: Severe asthmatic, where the FEV1 curve has no plateau. From Woolcock AJ Am Rev Respir Dis. 1984 (87). Reproduced with permission.

1.2 Asthma Treatment

The pathologies and airflow limitations that come with asthma severely compromise quality of life. Thus, the long-term goal of therapy is control of symptoms and minimization of the risk of exacerbation, without affecting normal daily activity, while at the same time reducing drug-related side effects (97). GINA 2016 guidelines classify asthma medications in three broad categories (11).

- Controller medications
- Reliever medications
- Add-on therapies

Controller medications are used to reduce asthma-related inflammation and control the symptoms. Inhaled corticosteroids (ICS) such as fluticasone, budesonide, beclomethasone, ciclesonide, mometasone and triamcinolone (97), are the pharmacological agents from this class. GINA strongly recommends the introduction of ICS as the first step in asthma treatment. Studies

have shown that an early start of ICS therapy has been linked to a better control of asthma symptoms and improve lung function, whereas a delay in ICS therapy results in a decline in asthma control (98). All inhaled corticosteroids are equally effective; however, they mainly differ in their pharmacokinetics. For example, budesonide, fluticasone, ciclesonide, and mometasone have lower bioavailability than beclomethasone or triamcinolone. Thus, they produce fewer side effects and hence are preferred in severe asthmatics where higher doses are required. Both beclomethasone and ciclesonide are pro-drugs and produce active corticosteroid only when the esterase enzymes in the lungs break their ester groups. Potency is another important parameter that affects the choice of drug especially when a higher dose of ICS is required to control asthma symptoms. In this regard generally, the potency of all steroids are measured against the potency of beclomethasone. Fluticasone is twice as potent as beclomethasone, budesonide is of equal potency, while triamcinolone is the least potent (97).

The corticosteroids bind to the cytoplasmic glucocorticoid receptor (NR3C1), which then changes conformation to translocate to the nucleus, followed by dimerization and binding to the glucocorticoid response element (GRE) that produces glucocorticoid-dependent antiinflammatory responses (99). The antiinflammatory response includes reduced expression of the inflammatory genes, inhibition of inflammatory gene transcription (transrepression). At the same time glucocorticoids also enhance the expression and transcription of antiinflammatory genes (100) such as dual specificity phosphatase-1 (DUSP1) (also known as the mitogen-associated protein kinase (MAPK) phosphatase 1) (101, 102), ZFP36 (103), TSC22D3 (104) and CDKN1C (105). DUSP1 is an inhibitor of MAPK activation required for stable mRNA expression and translation of inflammatory genes along with NF- κ B and AP-1 dependent transcription of inflammatory protein. ZFP36 gene prevents expression of inflammatory genes

via mRNA destabilising protein tristetraprolin. TSC22D3 repress both NF- κ B and AP-1 dependent transcription, CDKN1C inhibits JNK MAPK pathway(101-105).

Corticosteroid therapy, particularly by oral routes and at high doses, has several adverse effects, such as adrenal and growth suppression, dermal thinning and skin capillary fragility, as well as osteoporosis. An inhaled dose of $\leq 400\mu\text{g/day}$ is devoid of these adverse events, although the use of ICS leads to vocal chord atrophy and oropharyngeal candidiasis due to deposition of the drug, which can be controlled by proper oral hygiene. Nevertheless, the benefits of ICS therapy outweigh the risks, and it is the gold standard in current asthma therapy (97).

Other controller medications include leukotriene receptor antagonists (montelukast, zafirlukast, and pranlukast) and the 5-lipoxygenase (key enzyme for leukotriene production) inhibitor zileuton. However, they are less effective than ICS and are generally prescribed for patients who do not tolerate ICS, or for patients with concomitant allergic rhinitis (11, 106, 107).

Reliever medications for asthma are mainly comprised of bronchodilators. Based on pharmacology, they are classified as β_2 adrenergic receptor agonists (β ARA), anticholinergics, and methylxanthines (11, 97). β ARAs are the most commonly used reliever medicines in asthma. As the name suggests they act on the β_2 adrenergic receptor and activate receptor-coupled G_s protein to reduce intracellular Ca^{2+} and promote repolarization of the ASM, causing relaxation of bronchial constriction (108). Based on pharmacokinetics β ARAs are grouped as short-acting (SABA) or long-acting (LABA). The most common SABAs available for asthma treatment are albuterol and salbutamol. SABAs are highly effective for the quick relief of asthma symptoms (109) however its use should be restricted to manage symptoms only (110). LABAs such as formoterol and salmeterol are considered one of the more significant advances in asthma therapy

as their broncho-protective effects last over 12 hours and are associated with significantly better asthma control when compared to SABAs (111). Currently, they are used in combination with ICS as their synergistic effects result in better control of asthma symptoms. Also, reports show that ICS decreases the risk of developing tolerance of β ARs therapy (112, 113). Adverse events associated with β ARs are generally dose related and due to extrapulmonary stimulation of β ARs. Some common ones are muscle tremors, hypokalemia, and ventilation-perfusion mismatch. These adverse events are uncommon with the inhaled drugs and more common with oral/systemic β ARs (97).

Other reliever medications include long-acting cholinergic receptor antagonists tiotropium and ipratropium. These agents are antagonists of the M3 cholinergic receptor, thereby preventing activation of the PLC-IP3 pathway responsible for Ca^{2+} release from intracellular sources. Clinically they are not as effective as SABA or LABA. Thus, their use is limited to patients with fixed airway obstruction or who are not well controlled with a LABA. These medications are contraindicated in children below 12 years of age (97).

Theophylline (a class of methylxanthine) has been in use as an asthma therapy for over 80 years. Its mechanism is not entirely understood, although it has been ascribed various effects, from phosphodiesterase inhibition to immune modulation to an agent that controls gene transcription and epigenetic modification (114-117). However, clinically its efficacy is weak when compared to β ARs, and its use is no longer advisable especially with β ARs as it may increase their side effects (97).

Other therapies for asthma include biologics and bronchial-thermoplasty (BT). Currently, the approved biologics for the treatment of asthma are monoclonal antibodies for IgE (omalizumab) (118) and IL-5 (mepolizumab and reslizumab) (119, 120). While omalizumab

binds directly to IgE and reduces free IgE that could interact with high-affinity IgE receptors (FcεRI) on mast cells and basophils, mepolizumab and reslizumab block bioactivity of IL-5 by sequestering it and preventing its binding to the α -chain of the IL-5 receptor. These are effective medications; however, their efficacy is limited to severe asthmatics with high eosinophils. Mepolizumab and reslizumab, but not omalizumab, are contraindicated in children below 12 years of age (121-123). The astronomical cost of these new drugs, along with their limited efficacy, has prevented them from becoming a drug of choice for asthma (124-126).

Bronchial-thermoplasty is an invasive technique that uses radio waves to heat the airways tissues in a controlled manner to reduce the airway smooth muscles. Danek et al. 2004 from Dr Jim Hogg's group tested BT in dogs. Their results showed significantly diminished airway responsiveness to the methacholine challenge post BT treatment (127). In 2005 Miller et al. demonstrated the feasibility of this technique in humans and showed that it is safe for human use (128). Further, Cox et al. in 2006 demonstrated that BT is not only safe for humans, but it reduces the airway hyperresponsiveness and improves symptoms free days in asthmatics for up to 2 years (129). However, this technique does not appear to improve lung function. However, the numbers of studies to date are small, and use a very selective cohort that by no means represents the diversity in asthma population. Thus, the long-term safety and efficacy of BT needs to be assessed in a wider population base (130).

In summary, ICS alone or in combination with LABAs are the first line of treatment for asthma, with other complementing therapies as and when needed. These medications only control symptoms, and do not treat the underlying disease pathology that stems from oxidative stress and lipid pathobiology. As explained earlier these pathologies are sufficient to drive both the inflammation and the remodelling, and likely the bronchial hyperresponsiveness, associated

with asthma. In spite of the regular use of controller medications, many patients remain symptomatic due to intrinsic refractoriness or become progressively desensitized to current controller and/or reliever therapeutics (106, 131, 132). Research in the molecular biology of individual pathology has given little in the past several decades regarding new asthma therapies. Thus, it is important to more clearly define the relationship between symptoms and pathophysiology with respect to lung function, inflammation, hyperresponsiveness, and remodelling. Such understanding will help us define asthma phenotypes and will lead to the development of better therapies, and/or will enable the use of currently available medications more effectively.

1.3 Asthma Phenotype

Despite sharing a broad common pathology, asthmatics exhibit remarkable heterogeneity for atopy (a type of inflammation) and remodelling not only with the severity of the disease but even within mild to moderate conditions (133, 134). Thus, asthma has become an umbrella term that encompasses different subgroups of the disease (134).

Different consortiums have defined asthma by combining the various symptoms of the disease such as inflammation, remodelling, and AHR. At the same time, experts have always questioned if it is a single disease or symptoms of asthma are merely manifestations of various diseases. For example, fever in the 19th century was considered a single disease rather than a manifestation of the underlying pathology of the disease (134, 135). Consensus with respect to asthma being a heterogeneous disease is building, and efforts are emerging to phenotype and even endotype asthma.

The term phenotype defines the observable properties that arise due to the interactions between genetics and environment (136), whereas endotype defines the underlying pathobiology responsible for the specific traits observed with a particular phenotype (137). A correct phenotype definition would contain consistent history, clinical, and physiological features. Although current subgroups of asthma do not meet the definition of phenotype and/or endotype, however efforts have begun to classify asthma phenotypes (134).

Initial classification of asthma distinguished extrinsic (allergic/ atopic) from intrinsic (non-allergic) asthma. Extrinsic asthma develops early in life generally in response to an allergic insult to the lungs. Such asthmatics have a well-characterized inflammatory pattern in comparison to people with intrinsic asthma. The onset of intrinsic asthma takes place later in life. As the name suggests, the intrinsic asthma was considered non-allergic, but with symptoms more

severe than allergic asthma (138). However, these differences fell apart when studies showed that the inflammatory signature was same among the two suspected phenotypes and they even responded equally to the same treatment strategy to an equivalent extent (134).

Currently, more structured efforts are in play to phenotype asthma that involves biased and unbiased approaches. The biased approach utilizes a single clinical variable such as patterns of airway obstruction, triggers, and severity of the disease. The unbiased approach utilizes cluster analyses on critical asthma-related parameters like exacerbations, inflammatory markers, treatments, emergency department visits, and hospitalisations. Based on these grouped variables clusters of different phenotypes of asthma are identified within a large cohort of asthmatics. Currently, both biased and unbiased approaches for asthma classification accept two broad categories: Th2-associated and non-Th2-associated asthma (134).

Th2-associated asthma is characterized by the presence of atopy (or allergy), type-I hypersensitivity reaction, eosinophilic inflammation, and response to corticosteroid treatment (136, 139). Within this category, clinically separate asthma sub-phenotypes such as Early onset asthma, Late onset asthma and Exercise induced asthma (EIA) have been identified (140, 141). Both Early and Late onset asthma are among the most well-characterized asthma sub-phenotypes, distinguished by the age of onset. Early onset asthma starts in pre-adolescence, characterized by increased total allergen-specific IgE. Comorbid conditions associated with Early onset asthma are other atopic diseases such as allergic rhinitis and atopic dermatitis. Patients with such asthma phenotype respond well to the corticosteroid therapy. In contrast, late onset asthma appears after age 20. It is mostly non-atopic and associated with eosinophilia ($>2\%$ of total sputum inflammatory cells). It is almost always severe from the onset and patients with

this phenotype do not respond well to the corticosteroid therapy. Comorbid conditions associated with Late onset asthma include sinusitis and nasal polyps (134).

Despite the fact that an almost equivalent number of people might be suffering from "Non-Th2 asthma", its features are not as well characterized as Th2 associated asthma. As the name suggests, this phenotype does not show typical markers of Th2 immunity. Compared to their counterparts, these asthmatics suffer from less airway obstruction and hyperresponsiveness. However, their symptoms are more persistent and non-responsive to steroid treatment (142). Based on these characteristics Obesity-related asthma and Neutrophilic asthma can be categorized as subtypes of Non-Th2 Asthma.

The strong association of obesity with asthma severity stems from obesity-related increased levels of corticosteroid-refractory inflammatory mediators including IL-6, TNF- α , and oxidative stress. However, eosinophilia is not observed in this phenotype, suggesting the inflammation is non-Th2 in nature. Thus, it is not surprising that corticosteroids are ineffective in Obesity-related asthma (143). However, this phenotype is different from asthmatics that are obese with Th2 type inflammation: Obesity-related asthma often resolves with weight loss, whereas obese asthmatics with Th2 inflammation actually show a worsened inflammatory signature with weight loss (144). Therefore, care must be taken during diagnosis as sometimes obesity-associated shortness of breath and gastro-oesophageal reflux related coughing and chest tightness might lead to misdiagnosis. A proper physiological test to measure airway obstruction must therefore be carried out for appropriate management of different asthma phenotypes, and to prevent misdiagnosis (145, 146).

Neutrophilic asthma is associated with reduced airflow and increased air trapping in the lungs along with increased lung tissue remodelling and neutrophil elastase activity (147,

148). Treatment with a corticosteroid aggravates the neutrophilic inflammation in this subtype, as steroidal treatment increases neutrophil survivability (149). This phenotype is associated with late-onset asthma, irreversible airway obstruction, and accounts for highest health care cost among all phenotypes (136). Even with these identified characteristics, more studies are required to identify a cut-off point for neutrophilia for asthmatics to qualify in this phenotype of asthma.

To date, there has been limited consensus in defining the phenotypes of asthma, but as our understanding of the disease and the complex interaction of various pathological features evolves, more structured phenotypes of asthma will likely emerge in the near future. Until then, the GINA classification of asthma will remain the gold standard. According to GINA, asthma can be divided into mild, moderate and severe asthma. As the names suggest, mild and moderate asthmatics respond well to controller medications (to be discussed in the next section), whereas severe asthmatics do not respond to standard treatments for asthma (11). The terms most commonly associated with severe asthma are "uncontrolled asthma," "severe refractory asthma." It is important to distinguish these two conditions here, as the latter is a genuinely severe form of the disease associated with severe pathologies, whereas uncontrolled asthma is mainly a result of treatment non-adherence, co-morbidities, and other psychosocial factors. In low-resource countries where inhaled corticosteroids are not available, the World Health Organization (WHO) has identified uncontrolled asthma as "untreated severe asthma" (133).

1.4 Preclinical Animal Models to Study Asthma

Animal models of a disease play a vital role in understanding the disease pathologies and are essential for the development of new drugs before their testing in humans. In this pursuit animal models recapitulate certain classic features of the disease: however, by no means can they represent any human disease in its entirety (150, 151). In part, this is because of the differences in physiology and anatomy among humans and animals; however, limitations of animal models also stem from a lack of understanding and even bias or assumptions about the pathologies of a disease. Thus, animal models are really only as good as our understanding of the disease (151).

Animal models of asthma are no exception. As our knowledge about asthma is increasing, so is the refinement of animal models. There is currently no globally accepted model for asthma, because no animal spontaneously develops asthma except horses (heaves) and cats (allergic lung inflammation) (152, 153). Initially, when asthma was considered primarily a disease of excessive bronchoconstriction driven by smooth muscle, the animal models utilized various physiological bronchoconstrictor agents such as histamine, acetylcholine, and methacholine to study airway smooth muscle contraction (154). These animal models were vital in the development of bronchodilators now used as reliever medications against asthma induced bronchoconstriction. However, with an increasing understanding of the immunopathology of the disease, the concept of challenging lab animals with a substance that could elicit an allergic response (allergen) in them has emerged. Use of such animal models highlighted the altered immune response and recruitment of proinflammatory molecules in the lungs, and led to the development and use of antiinflammatory corticosteroid in asthma (152). However, asthma is more complex than either of these two schools of thought. Thus, current animal models incorporate the use of allergens to induce allergic asthma-like conditions followed by

bronchoprovocative agents to measure the airway hyperresponsiveness. This two-step process is a step closer to the real-life condition in humans (155).

Different laboratory animals are utilized in the laboratory to study asthma; however, the mouse model of allergic asthma predominates, mostly because of the immunological and molecular tools available to study them, in addition to a relatively low maintenance cost and ease of handling (156). The allergic asthma-like condition is induced in mice using viruses or allergens such as ovalbumin (OVA), house dust mite (HDM), ragweed (157), cockroach (158) and fungal allergen. Of these, OVA and HDM are the two most commonly used.

OVA-induced allergic asthma is the oldest, most well studied, and most common murine model of allergic asthma. OVA, the protein in egg whites, is used as an antigen/allergen to elicit the immune response in mice. This model consists of 2 phases: a sensitization phase and an allergen challenge phase. In the sensitization phase, OVA adsorbed to an adjuvant (aluminum hydroxide, *Bordetella pertussis*, or ricin) is administered intraperitoneally (i.p.) to steer the immune system towards Th2 type immunity. This phase lasts for three weeks, during which one i.p. injection of allergen+adjuvant is administered per week. The allergen challenge phase follows, where animals receive OVA through inhalation. This model replicates many features of human allergic asthma, such as Th2 skewed immune response, goblet cell metaplasia, antigen-specific IgE secretion, early and late phase responses, and airway hyperresponsiveness (150, 153, 159). However, there are certain major shortcomings of this model. First of all, OVA is not a physiologically relevant allergen, as it does not trigger any form of human asthma. Secondly, it requires the sensitization phase in conjunction with adjuvants to skew the immune system towards Th2 type, as an intranasal challenge on its own does not elicit the appropriate immune response in mice. A third and most critical shortcoming of this model is the development of

tolerance with continuous exposure, due to up-regulated T-regulatory cells. In fact, studies have shown that with chronic exposure to OVA, inflammation and AHR actually resolves itself instead of worsening (150, 160-163).

The limitations of the OVA model have led to the use of other more relevant allergens to induce more realistic features of asthma in mice. Among these, house dust mite (HDM) is a popular choice. Mites belong to the taxonomical subclass Acari, which consists of over 50,000 species. *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*, are the most commonly used as HDM in the murine model of allergic asthma (160). In 1967, Stevenson et al. established the potential of HDM to induce various allergic diseases (159). Since then HDM has been extensively studied, and its allergens are classified into 19 groups; the first two groups, consisting of proteases Der p1/f1 and Der p2/f2, evoke the strongest allergic reaction (160). Apart from these, HDM also contain microbial compounds such as lipopolysaccharides (LPS) and β -glucans that could activate pattern recognition receptors and elicit the innate immune response. The chitinous exoskeleton of the mite also acts as an antigen and can stimulate the immune system. Overall HDM is a complex system of allergens in itself. Thus, it is not surprising that up to 85% asthmatics are allergic to it (160, 164), which makes it clinically relevant.

A common acute model of HDM-induced allergic asthma in mice involves a 2-week (5days/week) intranasal challenge with HDM (25 μ g/day), invoking asthma-like symptoms in mice such as robust airway inflammation that includes infiltration of eosinophils, neutrophils, macrophages, and lymphocytes into the airways along with Th2 specific cytokines. A study by Cambaris et al. (2013) showed that HDM is more potent than any other allergen to prime the murine immune system towards Th2 (165). It also increases the HDM-specific IgE and IgG1

levels, as well as inducing AHR to methacholine challenge. Unlike the OVA model, long-term use of HDM does not induce tolerance in mice especially for AHR (166). A study by Johnson et al. (2004) evaluated whether mice could develop tolerance to chronic HDM-challenge. Mice were challenged with HDM for 5 weeks (5-days/week). Within two weeks of the end of the challenge period, the inflammatory cellular infiltrates returned to baseline. However, animals remained hyperresponsive to methacholine challenge, and the airway remodelling did not resolve up to 9 weeks after the HDM challenge seizure (167-169). Martin Stampfli's group from McMaster showed that this failure to develop tolerance is due to the local production of granulocyte monocyte-colony stimulating factor (GM-CSF) (170). Later, Hammad et al. (2009) showed that HDM-induced production of GM-CSF is dependent upon Toll-Like Receptor (TLR)-4 activation (171). Thus, resistance to tolerance development is a significant advantage of this model over the OVA model of allergic asthma. Another advantage of the HDM model over the OVA model of allergic asthma is that it replicates certain aspects of severe persistent asthma, such as excessive infiltration of both eosinophils and neutrophils, and more importantly the presence of AHR and airway remodelling that remained unaffected by the standard treatment, i.e. fluticasone and formoterol combination (168). HDM at 5µg/day, in combination with ragweed and *Aspergillus sp.*, induces a much more robust inflammatory response that persisted even in the absence of the allergen challenge (172).

Despite these distinct advantages, the HDM model of allergic asthma has certain limitations. Since HDM is a complex allergen, it is challenging to delineate the impact of an individual component on allergic inflammation and AHR. The other criticism comes from the fact that HDM-challenge in mice is unable to replicate the limitless AHR of a severe asthmatic.

However, the HDM-challenge-induced murine model of allergic asthma has brought clinical relevance to animal models (159).

Despite the advances and advantages of the murine model of allergic asthma, their utility and relevance have constantly been put through the scrutiny chiefly because of the failure to translate preclinical findings into clinical therapies. However, we need to appreciate the fact that asthma in humans is heterogeneous, without an established pathophysiological mechanism, and in animals only an aspect of it is reproduced, and that aspect is mainly governed by the research hypothesis and question (153). Moreover, most animal studies involving the evaluation of a potential drug candidate use an astronomical dose that while it may produce an effect in the model, would be impossible to translate to the clinical setting due to drug toxicity. A perfect example would be the evaluation of statins for their potential benefits in asthma-like conditions in mice, where scientists have shown the benefit at a dose that is at least three-fold higher than the maximum recommended dose for humans (173). Differences in physiology between mouse and human make the dose selection even more complicated. Furthermore, preclinical studies are not as tightly regulated as clinical trials are in terms of protocol, evaluation of endpoints, and data analysis. Recently, Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines have tried to streamline the process, but a more stringent implementation of such guidelines are required to optimize the translation of preclinical works into clinical settings (174).

1.5 Statins

1.5.1 Overview

1.5.1.1 Discovery

Most mammalian cells produce cholesterol. The lipid hypothesis linked low-density lipid (LDL: 70% constituent of cholesterol) with coronary heart disease (CHD) and opened the floor for the discovery of various drugs that targeted the *de-novo* synthetic pathway of lipids. The first in this class was Triparanol, which inhibits the enzyme 24-dihydrocholesterol reductase and thereby prevents the conversion of desmosterol into cholesterol (the final step in cholesterol biosynthesis). However, the drug was discontinued due to dermal and ocular toxicity (175, 176). Around the same time in 1970's, Akira Endo a microbiologist working for Sankyo Pharmaceuticals in Japan was in charge of screening thousands of compounds, obtained from microbial cultures, for their ability to prevent cholesterol biosynthesis by blocking hydroxy methyl glutaryl co-enzyme- (HMG-Co) A reductase. In this pursuit, he found an active compound produced by *Penicillium citrinum*, a mold that infects Japanese oranges. The compound was later known as mevastatin or compactin, the first lead compound in the statin class (175). Japanese clinical trials proved that mevastatin was the most potent compound discovered for reducing the plasma cholesterol in humans to date (177). Despite such efficacy, Sankyo dumped their project fearing the toxicity of mevastatin (lymphoma and cataract at higher dosages in dogs and other animals). Meanwhile, in 1978 scientists at Merck isolated another potent inhibitor of HMG-CoA reductase from the fermentation broth of the *Aspergillus terreus*, which they named mevinolin or lovastatin. Akira had also identified this compound independently. However, the fate of mevastatin affected the development of lovastatin, until

Merck investigated the toxicity of lovastatin. They also clarified that the Sankyo's lymphoma concern was a misinterpretation of subcellular processing of an excessive amount of the drug. With batteries of toxicity tests, the FDA finally gave approval to Merck for clinical trials, and later in 1987 approved the drug for clinical use (176, 178).

The efficacy of statins in reducing cardiovascular events due to reduction in LDL cholesterol was established after the result of a landmark trial was made public in 1994 (176, 178). This initial work stimulated pharmaceutical companies to produce more potent semisynthetic and synthetic inhibitors of HMG-CoA reductase, now collectively known as statins.

1.5.1.2 Classification of statins

Statins can be naturally derived from the fungal fermentation broth or semi synthetically from a natural analogue or synthesized as an organic molecule in the lab. As described in the previous section lovastatin and mevastatin are the two naturally occurring statins. Simvastatin and pravastatin are semisynthetic derivatives of lovastatin and mevastatin, respectively. All other statins, including atorvastatin, rosuvastatin, cerivastatin, pitavastatin, and fluvastatin are synthetically derived statins (176, 178).

All statins are either hydrophilic or hydrophobic based on their solubility in an aqueous solvent. Atorvastatin, simvastatin, fluvastatin, lovastatin, and cerivastatin are hydrophobic, while rosuvastatin and pravastatin are hydrophilic (179).

Table 1. 1: Classification of different statins based on origin and water solubility

Classification	Hydrophilic	Hydrophobic
Natural		Mevastatin, Lovastatin
Semisynthetic	Pravastatin	Simvastatin
Synthetic	Rosuvastatin	Atorvastatin, Cerivastatin, Fluvastatin

Based on their chemical structure statins can be classified as Type I and Type II statins. The commonality and differences in structure are highlighted in Figure 1.9.

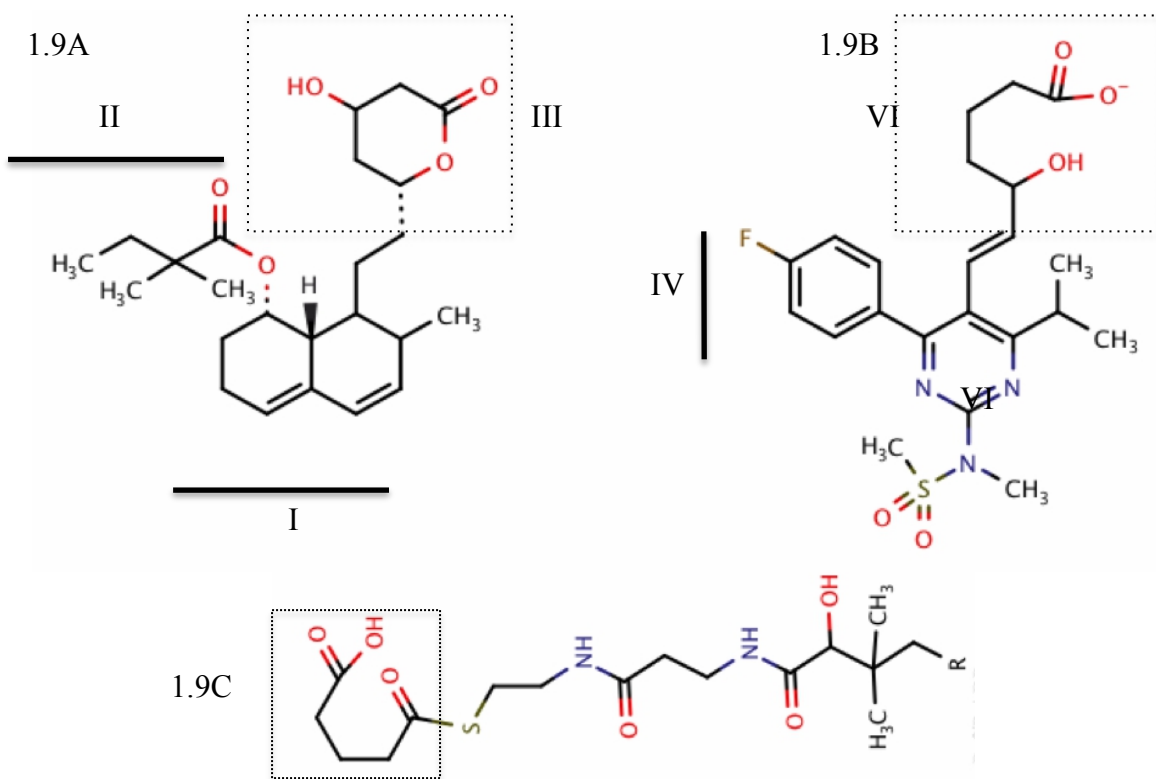


Figure 1.9: Structural difference between Type I and Type II statins.

A) Prototype of Type 1 statins (Compactin, pravastatin, simvastatin) where (I) is the decaline ring structure while (II) is the butyryl group. B) Prototype structure of Type II statins (atorvastatin, fluvastatin, rosuvastatin) containing a pyrimidinyl group in the centre (VI), attached to a signatory fluorophenyl group (IV). C) Chemical structure of hydroxy methyl

glutaryl Co-A. Both Type I and II statins have an HMG-like moiety (III and V). From Istvan Atherosclerosis 2003, Science 2003. Reproduced with permission (179, 180).

1.5.1.3 Pharmacology of statins

As illustrated in Figure 1.9 all statins contain a moiety very similar to the HMG portion of HMG-CoA, the substrate for the enzyme HMG-CoA reductase (the rate-limiting enzyme in cholesterol biosynthesis), but the hydrocarbon chain of statins is bulkier than HMG-CoA. The HMG-like moiety in statins binds to the active site in the HMG-CoA reductase; this induces a conformational change in the enzyme structure to expose hydrophobic binding site for the bulky hydrocarbon chain of statins. Thus, statins competitively inhibit HMG-CoA from binding to HMG-CoA reductase. The HMG moiety of both type-I and -II statins interact similarly to the active site, by forming a polar ionic bond. The conformational change results in an increase in the number of hydrophobic bonds between the statin and the shallow hydrophobic binding site of the enzyme. This hydrophobic interaction determines the affinity of statins for the enzyme HMG-CoA reductase illustrated in Table 1.2 (180, 181).

Table 1.2: IC₅₀ of HMG-CoA reductase inhibitors

Compound	IC₅₀ (nM)
Compactin	23
Simvastatin	9
Cerivastatin	10
Atorvastatin	10
Rosuvastatin	2
Fluvastatin	18

In humans, statins are administered orally. Following ingestion, statins undergo extensive hepatic first-pass metabolism, which limits their bioavailability to <5% for simvastatin, up to 20% maximum for rosuvastatin. Most statins reach their peak plasma concentration within 1-4h. Simvastatin is a pro-drug that is readily hydrolyzed to its β -hydroxy acid form, which is a potent inhibitor of HMG-CoA reductase. All other statins act as HMG-CoA reductase inhibitors by themselves, and upon metabolism, they lose their activity. The exception is atorvastatin, whose primary metabolites possess an enzyme inhibitory activity equal to the parent compound (48, 182, 183). Cyp3A4 belonging to the cytochrome P450 superfamily are responsible for the hepatic metabolism of hydrophobic statins, while Cyp2A9 metabolizes hydrophilic statins. Statins are primarily excreted out of the body through feces (179).

1.5.1.4 Clinical use of statins

Currently, statins are approved for the following clinical indications:

- To reduce the risk of total mortality associated with congestive heart disease.
- To reduce the risk of non-fatal myocardial infarction, stroke, and a need for revascularization procedures in patients at high risk of coronary events.
- To reduce elevated total cholesterol, low-density lipoprotein (LDL) cholesterol, Apo B, triglycerides (TG), and increase high-density lipoprotein (HDL)- cholesterol in patients with familial and non-familial hypercholesterolemia (includes boys and girls of age 10-17yrs), and in patients with mixed dyslipidemia.
- To reduce elevated TG in patients with hypertriglyceridemia.
- To reduce TG and VLDL-cholesterol in patients with primary diabetolipoproteinemia.

Statins are available in oral tablet form. The recommended starting dose of statins ranges from 10 to 20 mg/day, and for uncontrolled conditions the dose can be increased up to 80 mg for most statins, except for rosuvastatin, which has a highest recommended dose of 40 mg/day. However, continued high dose statin therapy should be restricted to patients who have been taking statins chronically with no signs of muscle toxicity. Indeed, 120 mg tablets of statins of all kinds have been discontinued, due to muscle toxicity side effects (48, 182, 183).

1.5.1.5 Statins Adverse Events

Statins are well-tolerated drugs where the risk to benefit ratio is hugely in favour of statins (178); however, like every drug they do have some adverse effects. The most common adverse events associated with statins include upper respiratory tract infection, headache, abdominal pain, constipation, and nausea. The most serious side effects of statins include myopathy and rhabdomyolysis (48, 182, 183). Several reports have proposed the mechanism behind statin-induced myopathy, although there is no consensus on this issue. One proposed mechanism is via the reduction in mitochondrial coenzyme Q10, which is one of the end products of the mevalonate pathway. Absence of Co-Q10 was thought to impair mitochondrial function. However, both animal and human studies suggest that Co-Q10 in skeletal muscle remains unchanged with statin use (184, 185). Furthermore, supplementation with Co-Q10 currently does not show evidence of efficacy against statins induced myopathy(186). Another theory attributes statin use to T-tubule damage in skeletal muscle, due to cholesterol depletion in the sarcolemma and/or in the T-tubule itself (187). Other evidences suggest that statin-induced inhibition of prenylation of small GTPases leads to inhibition of phosphorylation of Akt (188), which allows the forkhead box class O (FOXO) protein group to enter the nucleus unphosphorylated, and activate the muscle atrophy genes such as atrogen-1, muscle-specific ring

finger (MuRF)-1, and muscle atrophy F box (MAFbx) resulting into myopathy (188, 189). In support of this theory, a study by Mallinson et al. showed that statin users with myalgia had impaired muscle function evidenced by a delay in peak power output(190). At the same time, they showed that 6 six genes that were differentially expressed in statin users, were associated with mitochondrial damage and apoptosis. In contrast, they also reported that statin users with myalgia were similar to the statin non-users in lean muscle mass and muscle strength (190).

Overall the evidence suggests that statin-induced myopathy is due to its ability, through the blocking of HMG-CoA reductase, to inhibit generation of the cholesterol intermediates geranylgeranyl (GG) and farnesyl (F) pyrophosphate (PP), responsible for prenylation of small GTPases in the presence of prenyl-transferase enzymes (GGT and FT). In support of the theory of statin-induced myopathy, Cao et al. and Hanai et al. showed that knockdown of HMG-CoA reductase in zebra fish induced muscle damage similar to that observed with statin treatment (188, 191). Further, geranylgeranol – a cell-permeable alcoholic precursor of geranylgeranyl pyrophosphate prevented statin-induced myopathy. At the same time, inhibition of GGT induced the myopathy, while the other intermediate FP did not affect statin induced myopathy (174), suggesting that the backbone of statins' mechanism of action is also responsible for its most feared side effects. However, statin-induced myopathy is very rare even in the elderly (192) and mostly observed only at the highest doses (48, 182, 183). Hence the benefits of statin therapy outweigh the risks.

1.5.1.6 Pleiotropic effects of statin

Statins provide health benefits that are independent of its cholesterol-lowering capacity. The pleiotropic effects of statins include induction of endothelial nitric oxide (eNOS), antioxidant, and anti-inflammatory effects. Although the mechanism behind the pleiotropic

benefits of statins is diverse and not fully understood, however, several mechanisms have been proposed (Figure 1.10). Interestingly, most of these benefits are linked to inhibition of the mevalonate cascade via inhibition of HMG-CoA reductase, thus inhibiting the synthesis of the cholesterol precursors, 15- and 20-carbon farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP). These isoprenoids are substrates for farnesyl transferase (FT) and geranylgeranyl transferase 1 (GGT1) that conjugate FPP or GGPP to monomeric G-proteins (eg. RhoA, an upstream effector of Rho kinase (ROCK)), allowing G-proteins to be anchored in the cell membrane and activated. Inhibition of the mevalonate cascade by simvastatin prevents the prenylation of G-proteins, which is linked to the inhibition of inflammation, preventing release of inflammatory cell mediators (eg. TGF- β 1, CTGF, and MMPs) (71, 173, 193, 194).

Figure 1.10

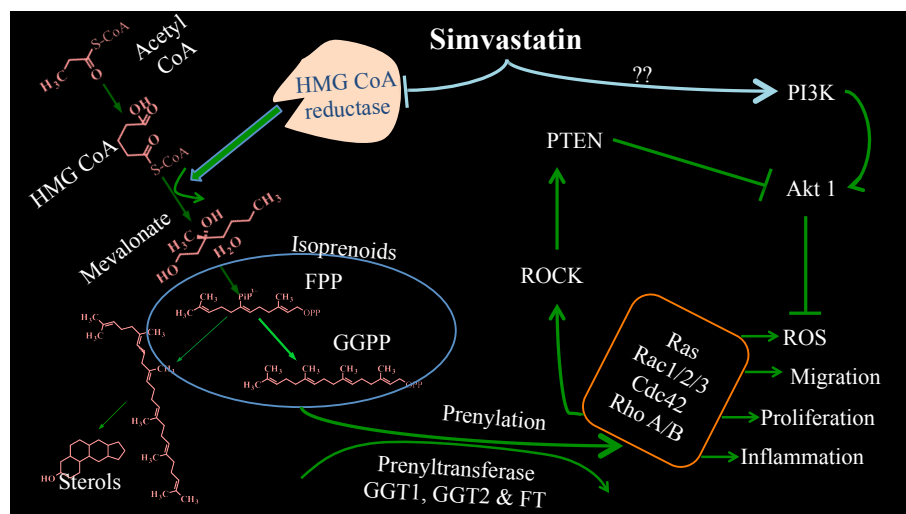


Figure 1.10: Possible mechanism for simvastatin's pleiotropic effects.

Details included in the text. Arrow: activation, line with blunt head: inhibition, green lines: canonical pathway, blue lines: possible impact of simvastatin.

HMG-CoA: β -Hydroxy β -methylglutaryl-CoA, FPP: Farnesyl pyrophosphate, GGPP: Geranylgeranyl pyrophosphate, GGT: Geranylgeranyl transferase, FT: Farnesyl transferase, ROCK: Rho associated kinase, PTEN: Phosphatase and tensin homolog, PI3K: Phosphatidylinositol-4, 5-bisphosphate 3-kinase.

Statins also directly impact chemotaxis via inhibition of interstitial cell adhesion molecules (ICAMs), vascular cell adhesion molecules (VCAMs) and E-selectin, while blunting p-selectin expression via induction of eNOS (195). In vascular endothelial cells statins also inhibit Major Histocompatibility Complex Class II (MHC-II), and CD-40 expression required for activation of T-lymphocytes, which play an important role in atherosclerosis (196). The landmark clinical trial, JUPITER, proved that statins are beneficial in the primary prevention of cardiovascular diseases due to their lipid-lowering property as well as their antiinflammatory effect, where they significantly reduced the high sensitivity C-reactive protein concentration in systemic circulation when compared to the placebo group (197, 198).

Statin-mediated inhibition of mevalonate cascade is also linked to antioxidant effects that include eNOS activation (199), as well as prevention of ROS generation via prenylation of Rac1. Statins also prevent proliferation and migration of vascular smooth muscle cells via the mevalonate cascade, as these effects were reversed by GGPP replenishment (200, 201).

Apart from their effects on the mevalonate cascade, statins also impart their antioxidant property via induction of the heme-oxygenase (HO)-1 gene through activation of nuclear factor-erythroid-2 related factor (Nrf-2) (202). Interestingly statins also induce eNOS via activation of the PI3K/Akt pathway activation (203). Such an effect of statins is counterintuitive, considering they inhibit PI3K mediated Akt and FOXO phosphorylation in myopathies. However, the impact of statins on the PI3K/Akt pathway is biphasic, i.e. at lower dosages statins are known to activate PI3K pathway and produce beneficial pleiotropic effects, while at higher dosages they produce the opposite effect (204, 205). The other mechanism involved in statin-induced eNOS induction is depletion of caveolin-1 protein that binds to eNOS in caveolae and prevents NO synthesis(206).

In addition, as explained earlier statins also increase the HDL that is associated with various biological effects including antiinflammatory and antioxidant activities (47). Thus, not only do statins reduce total and LDL cholesterol, they also have other beneficial effects that include anti-inflammatory and antioxidant effects, as well as preventing cardiac muscle remodelling by interfering with hypertrophy. Since inflammation, oxidative stress, and remodelling are key pathologies of asthma, it is of interest to evaluate the potential benefits of statins in asthma.

1.5.2 Statins in asthma

The potential benefits of statins in asthma have been a subject of research for over a decade. While retrospective and preclinical evidence support the benefit of statins in asthma, results from clinical trials are not compelling. This section will review and highlight the literature available so far on statin use in asthma and will identify the disconnect between clinical trials and other research platforms so that future studies can address this disconnect for bench to bedside transition of statin use in asthma.

Retrospective pharmaco-epidemiological studies are perfect ground to build a case for the efficacy profile of a drug, already in clinical use, for indications different from the approved ones. Similarly, in the case of statins, there are several pharmaco-epidemiological studies that evaluate their benefits in asthma patients who have been taking statins for cardiovascular complications. . These studies evaluated statins for steroid-sparing effects, as well as hospitalisation and emergency room visits due to asthma, which in turn are markers for asthma control and exacerbation frequency (207, 208). The Table.1.3 highlights research methodologies employed and the key take-home point from these studies.

Table 1.3: Pharmacoepidemiology studies to evaluate efficacy of statins in asthma

Authors; Year; Journal	Study Design & End Point	Results	Conclusion
Huang CC, Chan WL, Chen YC, Chen TJ, Chou KT, Lin SJ, Chen JW, Leu HB; 2011; <u>Eur J Clin Invest.</u>	Retrospective nationwide population-based study to investigate the possible influence of statin use on patients with asthma using the Taiwan National Health Insurance Database. 3965 asthma patients who were receiving statins were matched for age, sex and asthma medications with 7843 asthma patients who were not on statins.	Statin use was associated with decreased risk for hospitalisation for asthma (hazard ratio: 0.82; p=0.006)	Statin use reduces the risk for hospitalisation due to asthma.
Lokhandwala T, West-Strum D, Banahan BF, Bentley JP, Yang Y; 2012; BMJ Open	Retrospective cohort study, included 1,473 asthmatics of age 19yrs or older that were on corticosteroid treatment. Of these 479 were exposed to statins (exposed group with at least 80% prescription adherence), while 958 were unexposed to statins (Unexposed group). End Point: Hospitalisation and emergency room (ER) visit due to asthma Follow-up period: 1yr	The statin exposed asthmatics had significantly reduced odds of getting hospitalized (0.55; p=0.0059) and ER visit (0.48; p=0.0069) due to asthma related reasons.	Statin in combination with corticosteroid is protective against asthma related exacerbations leading to hospitalisation and emergency room visit.
Tse SM, Li L, Butler MG, Fung V, Kharbanda EO, Larkin EK, Vollmer WM, Miroshnik I, Rusinak D, Weiss ST, Lieu T, Wu AC; 2013; Am J Respir Crit Care Med.	Retrospective Cohort study on large population of asthmatics. Study included asthmatics with age >30 Yrs. From PEAL cohort 8,348 statin users (with at least 80% prescription adherence) were matched with 8,348 non-users for age, baseline asthma therapy, site of enrolment, season at baseline, and propensity score. End Point: Asthma exacerbations defined by two or more oral corticosteroid dispensing, asthma-related emergency department visits, or asthma-related hospitalisations. Follow up period: 2 yrs.	Statin users had reduced odds of having asthma related emergency department visit (OR=0.64; p<0.0001) and 2 or more oral corticosteroid dispensing (OR= 0.90; p=0.04) however there were no difference in asthma related hospitalisation.	Statin use significantly reduced asthma related emergency department visit and oral corticosteroid dispensing.

Authors; Year; Journal	Study Design & End Point	Results	Conclusion
Zeki AA1, Oldham J, Wilson M, Fortenko O, Goyal V, Last M, Last A, Patel A, Last JA, Kenyon NJ; 2013; BMJ Open	Retrospective cohort study included 165 severe asthma patients 31 of them were on statins and rests were non-statin user. Primary endpoint: asthma control as measured by the Asthma Control Test (ACT). Secondary endpoints: lung function, symptoms and the need for corticosteroid and peripheral eosinophil count	Statin users had better asthma symptoms control than non-users although they were associated with cardiovascular comorbidities and higher age group and lower lung function at baseline.	Patients with severe asthma who were on statins had better asthma symptom control.
Tse SM, Li L, Butler MG, Fung V, Kharbanda EO, Larkin EK, Vollmer WM, Miroshnik I, Rusinak D, Weiss ST, Lieu T, Wu AC; 2014; Am J Respir Crit Care Med.	Retrospective cohort study included 6,652 asthmatics 18yrs or older from Medco Health Solutions Administrative database. Cohort was categorized into inhaled corticosteroid (ICS) users (3747) or non-users (2905). End Point: Asthma related hospitalisations and emergency department (ED) visits. Follow up period: 12 months	Among ICS users 21% were on statins and showed significant reduction in odds of asthma related ED visits (OR=0.77, p=0.008) in comparison to ICS users only.	Statins showed synergistic affect with ICS in reducing ED visit.

Unfortunately, population-based retrospective studies cannot establish the causal relationships between statin use and benefits in asthma, but they do fuel hypothesis-driven research in the laboratory.

The first laboratory research to evaluate the role of statins in asthma comes from McKay et al. 2004; in this study, they highlighted the antiinflammatory potential of statins (40mg/kg) in a mouse model of allergic asthma using OVA as an allergen (173). Another study by Yeh et al. showed that pravastatin suppressed the OVA-induced airway inflammation augmented by the dietary cholesterol, showing that cholesterol plays an important role in mediating inflammation and that limiting cholesterol biosynthesis is an important step in mitigating its effects on inflammation (194). There are several other reports that demonstrate the antiinflammatory role of statins both in *in vitro* and *in vivo*, and also indicate its potential to inhibit airway remodelling in lungs (71, 193, 200, 209). A summary of these studies is in Table 1. 4.

Table 1.4: Preclinical studies highlighting the effect of statins on classic features of asthma in cell and animals

Authors; Year; Journal	Study Details	Key Findings
McKay A, Leung BP, McInnes IB, Thomson NC, Liew FY; 2004; J Immunol.	<i>In-vivo</i> ; Balb/c mice (female) Allergen: OVA Statin used: Simvastatin Dose: 4 & 40mg/kg; Route of administration: Oral and Intraperitoneal (i.p)	Simvastatin @ 40mg/kg blunted the influx of total inflammatory cells and eosinophilia in bronchoalveolar lavage fluid (BALF). Simvastatin delivered through i.p route had more potent effect than orally delivered simvastatin. Simvastatin i.p also blunted cytokine IL-4 and 5 in BALF and thoracic lymph nodes. In Thoracic lymph nodes IL-6 and IFN γ were also decreased, however there was no effect on Ova specific IgE or IgG.
Yeh YF, Huang SL; 2004; J Biomed Sci.	<i>In-vivo</i> ; C57BL6 mice (female) Allergen: OVA with or without high cholesterol diet Statin used: Pravastatin (drinking water) Dose: 0.08mg/ml (considering 5ml drinking vol/mouse of weight 20g $\approx$ 0.4mg/20g $\approx$ 20mg/kg)	High cholesterol diet enhanced Ova induced influx of inflammatory cells and cytokines such as IL-5, Prostaglandin (PG)E-2, MCP-1 in the BALF while IL-4 and IFN γ were elevated in lung tissue. Oral pravastatin blunted the cholesterol and Ova induced inflammation and cytokine signalling in the lungs as well as cells and in the BALF.
Takeda N1, Kondo M, Ito S, Ito Y, Shimokata K, Kume H; 2006; Am J Respir Cell Mol Biol.	<i>In-vitro</i> ; Primary cultured normal human bronchial smooth muscle (BSM) cells Statins used: Simvastatin (0.1-1.0 μ mol)	Simvastatin dose dependently blunted fetal bovine serum induced BSM cell proliferation and DNA synthesis and the effect was reversed by both mevalonate and GGPP but not by squalene or FPP other intermediates of mevalonate cascade. GGT, Rho and ROCK inhibitors mimicked the anti-proliferative effect of simvastatin. Suggesting the anti-proliferative effect of simvastatin is via inhibition of RhoA-ROCK activity.
Chiba Y, Sato S, Misawa M; 2008; Smooth Muscle Res.	<i>Ex-vivo</i> ; Balb/c mice (female) Allergen: OVA Statin used: lovastatin Dose: 4mg/kg/day, 1 day prior to inhaled allergen challenge till the duration of allergen challenge every day. Route: i.p Bronchial smooth muscle contractility was measured to ACh and high-K $^{+}$	OVA challenge significantly augmented the bronchial smooth muscle contractility to ACh but not to high K $^{+}$, which was significantly blunted by lovastatin treatment.

Authors; Year; Journal	Study Details	Key Findings
Chiba Y, Arima J, Sakai H, Misawa M; 2008; Am J Physiol Lung Cell Mol Physiol	<i>In-vivo</i> ; Wistar Rats (male) Allergen: 2,4-dinitrophenylated <i>Ascaris sum</i> Statin used: Lovastatin Dose: 4mg/kg/day Route: i.p	The antigen challenge induced BSM hyperresponsiveness to ACh challenge with increased expression and membrane translocation of Rho-A. It also induced serum IgE, influx of inflammatory cells and cytokines (IL-4, 6, 13) in the BALF and lung tissue inflammation. Treatment with lovastatin blunted the allergen induced BSM hyperresponsiveness; lung inflammation and Rho-A membrane translocation but had no impact on IgE and cytokines induced by the allergen.
Imamura M, Okunishi K, Ohtsu H, Nakagome K, Harada H, Tanaka R, Yamamoto K, Dohi M; 2009; Thorax	<i>In-vivo</i> ; Balb/c mice (male) Allergen: Ova Statins used: Pravastatin Dose: 10mg/kg Route: i.p	Pravastatin attenuated allergen induced The cytokine production and proliferation in spleen cells. It also prevented IL-17 production in thoracic lymph nodes antigen presenting capacity of CD11c ⁺ cells. Consistent with these effects it blunted the influx of inflammatory cells in the BALF.
Zeki AA1, Franzi L, Last J, Kenyon NJ; 2009; Am J Respir Crit Care Med.	<i>In-vivo</i> ; Balb/c mice (female) Allergen: Ova Statins: Simvastatin Dose: 20mg/kg Route: i.p	Simvastatin improved overall lung health by blunting ova induced influx of inflammatory cells including eosinophils and macrophages along with proinflammatory cytokines IL-4 & 13 in the lung lavage at the same time it also improved lung compliance and reduced hyperreactivity to MCh challenge. The exogenous mevalonate mitigated antiinflammatory effects of simvastatin but could not reverse the impact of simvastatin on improved lung function. This indicates that impact of simvastatin on lung function is independent of its primary mechanism of action i.e inhibition of mevalonate pathway.
Zeki AA1, Bratt JM, Rabowsky M, Last JA, Kenyon NJ; 2010; Transl Res.	<i>In-vivo</i> ; Balb/c mice (female) Allergen: Ova Statins used: Simvastatin Dose: 40mg/kg Route: i.p	Simvastatin prevented the ova induced goblet cell hyperplasia and increased expression of arginase-1 protein and total arginase enzyme activity in the lungs that could be responsible for airway remodelling. The effect was mevalonate dependent.

Authors; Year; Journal	Study Details	Key Findings
Schaafsma D1, Dueck G, Ghavami S, Kroeker A, Mutawe MM, Hauff K, Xu FY, McNeill KD, Unruh H, Hatch GM, Halayko AJ; 2011; Am J Respir Cell Mol Biol.	<i>In-vitro</i> ; Primary Cultured Human Airway Smooth Muscle Cells Statins used: Simvastatin Concentration: 1, 10 and 15 μ mol.	Simvastatin prevented the TGF β -1 induced collagen-1 and fibronectin expression, collagen-1 secretion was also blunted, a key step in airway wall remodelling. Replenishing the media with mevalonate/ GGPP/ FPP mitigated the effect. Interestingly inhibition of GGT and not FT mimicked the results obtained with simvastatin. Both GGT inhibitor and simvastatin also prevented TGF β -1 induced membrane association of Rho-A.
Ahmad T1, Mabalirajan U, Sharma A, Aich J, Makhija L, Ghosh B, Agrawal A; 2011; Am J Respir Cell Mol Biol.	<i>In-vivo/ in-vitro</i> ; Balb/c mice male, A549 cells (epithelial cells) Allergen: Ova Statins used: Simvastatin Dose: 40mg/kg Route: i.p	<i>In-vivo</i> simvastatin blunted allergen induced airway inflammation, hyperreactivity and remodelling which correlated to its inhibition of inducible (i)-NOS, and asymmetric dimethyl-arginine and up regulation of e-NOS. Simvastatin also blunted oxidative stress evident from the reduction in nitrotyrosine apart from this simvastatin also blunted various pro-apoptotic markers such as caspase 3, 9, cytosolic cytochrome-c and apoptotic protease activating factor (apaf)-1. <i>In-vitro</i> simvastatin induced e-NOS expression.
Zhu T1, Zhang W, Wang DX, Huang NW, Bo H, Deng W, Deng J.	<i>In-vivo</i> ; Balb/c mice (female) Allergen: Ova Statins used: Rosuvastatin Dose: 10 & 30mg/kg/day Route: subcutaneous (s.c.)	Simvastatin blunted the influx of inflammatory cells in the lung lavage along with proinflammatory cytokines such as IL-4, 4, 13 TNF- α and histological mucus index and they linked these effects to the ability of rosuvastatin to inhibit gamma amino butyric acid (GABA) receptors in lungs.
Xu L1, Dong XW, Shen LL, Li FF, Jiang JX, Cao R, Yao HY, Shen HJ, Sun Y, Xie QM; 2012; Int Immunopharmacol.	<i>In-vivo</i> ; Balb/c mice (female) Allergen: Ova Statins used: Simvastatin Dose and Route: Inhalation: 1, 5 and 20mg/ml for 10 min (dose can not be calculated as flow rate is not mentioned) Intratracheal (i.t): 2mg/kg i.p & oral: 40mg/kg	Inhaled simvastatin dose dependently blunted the airway inflammation and hyperresponsiveness to MCh challenge and the effect was more potent than the i.p or oral administration of the drug.

Authors; Year; Journal	Study Details	Key Findings
Huang CF1, Peng HJ, Wu CC, Lo WT, Shih YL, Wu TC; 2013; Ann Allergy Asthma Immunol.	<i>In-vivo</i> ; Balb/c mice (female) Allergen: Ova Statins used: Pravastatin and Atorvastatin Dose: 4mg/kg/day Route: Oral	Both pravastatin and atorvastatin blunted the ova induced Th1 and Th2 cytokine production in mice as well as reduced the airway hyperresponsiveness compared to ova challenged mice.
Michalik M1, Soczek E, Kosińska M, Rak M, Wójcik KA, Lasota S, Pierzchalska M, Czyż J, Madeja Z; 2013 Eur J Pharmacol.	<i>In-vitro</i> ; Human bronchial fibroblasts derived from asthmatic patients Statin used: Lovastatin Concentration: 2.5-20 μ M	Lovastatin blunted the TGF β induced fibroblast to myofibroblast (important step in tissue remodelling in asthma) transition dose dependently. This effect was independent of statin induced inhibition of prenylation instead this effect was directly associated to statins ability to inhibit cholesterol synthesis as squalene synthase inhibitor zaragozic acid had similar effect as that of lovastatin.
Capra V1, Rovati GE2; 2014; Pulm Pharmacol Ther.	<i>In-vitro</i> ; Human ASMs Statin used: Rosuvastatin Concentration: 0.1, 1, 50 μ M	Rosuvastatin dose dependently blunted the mitogenic response of serum, epidermal growth factor, thromboxane A2 analogue (U46619) and leucotriene D4 on ASM cells and prevented the growth and the response was dependent on mevalonate pathway induced prenylation using both GGPP and FPP.
Zeki AA1, Bratt JM2, Chang KY3, Franz LM2, Ott S2, Silveria M4, Fiehn O5, Last JA2, Kenyon NJ2; 2015; Physiol Rep.	<i>In-vivo</i> ; Balb/c mice (male) Allergen: Ova Statins used: Pravastatin Dose: 15mg/kg twice daily (30 min before and after Ova challenge)	Pravastatin blunted the ova induced goblet cell hyperplasia and induction cytokines TNF- α and KC however it did not have any beneficial effect on the inflammatory cell influx in the BALF. It also did not reduce the hyperresponsiveness of the airways to the MCh challenge however it did reduce the hypersensitivity.

The common finding from these studies is that the most efficacious dose of statins in *in vivo* studies was 40mg/kg/day, which is equivalent to an enormous human dose of 240mg/day, which far exceeds then dose that can induce statin mediated toxicity including myopathy. Use of more clinically relevant systemic doses, i.e., 4mg/kg/day had only equivocal efficacy that varied from study to study. Another important point is that though most studies have evaluated the anti-inflammatory benefits of statins, they have not demonstrated a significant impact on airway hyperresponsiveness in OVA-exposed animals. Furthermore, there is no direct evidence or assessment of the efficacy of statins on airway remodelling in preclinical models of allergic asthma. There are data from *in vitro* studies with cultured cells showing that statins can dampen ECM protein expression; however, it is unclear whether this will also occur *in vivo*.

Several clinical trials have been conducted to evaluate the applicability of statins in human asthma. However, unlike most preclinical studies, these clinical trials assessed the efficacy of statins at their clinical dose in mild to moderate asthmatics. The results are ambiguous as there is equivocal support for the antiinflammatory effects of statins at the same time there is no evidence of efficacy of statins improving lung function in asthmatics (210-214). A summary of results from these clinical trials is in Table 1.5.

Table 1.5: Summary of clinical trials conducted to evaluate efficacy of statins in asthma

Authors; Year; Journal	Study Design	Key results
Samson KT, Minoguchi K, Tanaka A, Oda N, Yokoe T, Yamamoto Y, Yamamoto M, Ohta S, Adachi M; 2006; Clin Exp Allergy	Clinical/ <i>Ex-vivo</i> study: 7 asthmatics (age 28±3.4 yrs) that were allergic to HDM with FEV₁ %predicted>70% and were on β₂ agonist only for at least 3 months prior to inception, were recruited. Statin used: Fluvastatin (0.1, 1, 10μM) Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of patients recruited and these were exposed to <i>Dermatophagoides ferinae</i> extract and phytohaemagglutinin in presence or absence of fluvastatin. PBMCs proliferation and cytokine and chemokine production from cytokines were studied.	Fluvastatin blunted the PBMCs proliferation and production of cytokines and chemokines from the PBMCs in response to the allergen
Menzies D, Nair A, Meldrum KT, Fleming D, Barnes M, Lipworth BJ; 2007; J Allergy Clin Immunol.	Double blind cross over randomized clinical trial: Statin used: Simvastatin Dose: 20mg/day (oral; 2wks) + 40mg/day (oral; 2-wks) Comparator: Placebo Outcomes: Asthma related inflammation (exhaled and alveolar NO, sputum and peripheral eosinophils, c-reactive protein and salivary eosinophilic cationic protein), MCh hyperresponsiveness. Note: All antiinflammatory medications were withdrawn before commencement of study and only rescue medications were allowed. 16 patients completed the protocol.	Statins did not show any benefits against any parameters measured in the study in comparison to placebo.

Authors; Year; Journal	Study Design	Key results
Hothersall EJ, Chaudhuri R, McSharry C, Donnelly I, Lafferty J, McMahon AD, Weir CJ, Meiklejohn J, Sattar N, McInnes I, Wood S, Thomson NC; 2008; Thorax	Double blind randomized controlled cross-over trial: Statin used: Atorvastatin Dose: 40mg/day for 8 weeks Comparator: Placebo Outcomes: Primary: Morning peak expiratory flow rate; secondary: FEV ₁ , Asthma control questionnaire, MCh-challenge airway hyperresponsiveness, inflammatory signature	There was no benefit of atorvastatin over placebo in primary as well as most of secondary end points however atorvastatin did reduce sputum macrophage and leucotriene B4 levels significantly.
Ostroukhova M, Kouides RW, Friedman E; 2009; Ann Allergy Asthma Immunol.	Quasi Study Design: Asthmatics that started using statins (n=24) after their initial asthma evaluation were compared against asthmatics that never used statins (n=26), for FEV ₁ at baseline, 3, 6, 12 and 24 months after the inception date (primary end point). Secondary end points were patient reported increase in use of rescue medication, hospital and emergency department visits due to asthma related reasons and use of oral corticosteroid.	Statin group had worsening of FEV ₁ and required more day-time use of rescue medication and were associated with higher clinician office visit due to asthma.
Maneechotesuwan K, Ekjitrakul W, Kasetsinsombat K, Wongkajornsilp A, Barnes PJ; 2010; J Allergy Clin Immunol.	Randomized double blind clinical trial; Statin used: 10mg/day for 8 weeks 50 asthmatics received 200µg budesonide and they were randomized to receive 10mg of simvastatin or placebo post 2 weeks of wash out period for all previous drugs. Outcomes: Inflammation: eosinophil counts, secretory signalling molecules, and immunocytochemistry of macrophages in sputum	The combined therapy of budesonide and simvastatin blunted the eosinophil percentage in the sputum of asthmatic significantly compared to budesonide alone and enhanced the effect of corticosteroid on IL-10 release.

Authors; Year; Journal	Study Design	Key results
Cowan DC1, Cowan JO, Palmay R, Williamson A, Taylor DR; 2010; Thorax	Randomized placebo controlled cross over trial; Statin used: Simvastatin 40mg/day Outcome: the dose of fluticasone was step wise decreased till the loss of symptom control appeared and then dose of fluticasone was step wise increased till the asthma symptoms were under control. 51 asthma patients were recruited with steroid free sputum eosinophils >2%.	45 patients completed the trial. The dose of fluticasone at which control on asthma symptoms were lost was not significantly different among statin and placebo group. However in patients where fluticasone dose was reduced to 0µg/day (n=18), the asthma control was better and FEV ₁ was higher than placebo and overall in statin treated group sputum eosinophils were significantly less than the placebo group.
Moini A1, Azimi G, Farivar A; 2012; Allergy Asthma Immunol Res.	Double blind randomized placebo controlled trial Statin used: Atorvastatin Dose: 40mg/day for 8 weeks Outcomes: Standardized asthma control test: FEV ₁ %, forced vital capacity (FVC)% and peripheral blood eosinophils	Standardized asthma control test yielded similar results in both placebo and atorvastatin treated groups.
Thomson NC1, Charron CE2, Chaudhuri R3, Spears M4, Ito K5, McSharry C3; 2015; Pulm Pharmacol Ther	Placebo controlled randomized trial Statin used: Atorvastatin Dose: 40mg/kg 39 smokers with mild to moderate asthma received atorvastatin 40mg/day for first 4 weeks alone and then in combination with 400µg beclometasone/day for additional 4 weeks. Outcome: Asthma Control Questionnaire (ACQ), Asthma Quality of life Questionnaire (AQLQ), Inflammation (cytokine and chemokine)	Simvastatin treatment alone blunted sputum concentrations of CCL7, IL-12p70, sCD40L, FGF-2, CCL4, TGF-α and MMP-8 in comparison to placebo group. The combination of atorvastatin and beclometasone was significantly better than beclometasone alone in reducing sputum MMP-8, IL-1β, IL-10, MMP-9, sCD40L, FGF-2, IL-7, G-CSF and CCL7. Both ACQ and AQLQ were improved with statin alone or in combination.

Thus, it is clear that although preclinical studies complement results from observational, retrospective studies of statin use, and their beneficial effects on asthma symptoms and control, RCTs do not support any significant benefit of statin therapy in asthmatics. This thesis is a step to address some of the gaps between preclinical studies and clinical trials, so that results may be translated more efficiently into the clinics for the benefit of asthmatics.

Chapter 2: Study Rationale

2.1 Study Rationale:

The projects in this thesis were designed to (i) evaluate the effects of simvastatin therapy in a clinically relevant murine model of allergic asthma, (ii) evaluate if lung-specific delivery of simvastatin could provide optimal benefits on both inflammation and lung function at a reduced dose, and (iii) begin to unravel the mechanism of action. These are intended to add clarity to the apparent disparities between retrospective epidemiological, *in vivo* and *in vitro* studies and data from statin clinical trials for asthma. The significance of this work is that it will inform potential efforts to bring statins to clinical use so as to be of benefit to people with asthma, perhaps specifically for those with steroid refractory asthma. Based on the known pleiotropic effects of statins, **the main hypothesis for this thesis is that simvastatin will blunt the allergen-induced airway inflammation, remodelling and hyperresponsiveness in mice, moreover a lung-targeted delivery will be more effective than systemically delivered simvastatin.** To test this hypothesis, I developed three major objectives:

Objective 1: To assess if simvastatin can prevent airway inflammation and hyperresponsiveness in an acute murine model of allergic asthma

Objective 2: To assess the impact of house dust mite on phosphatidylcholine peroxidation, and the effects of intranasal simvastatin treatment on oxidized phosphatidylcholine formation

Objective 3: To assess if simvastatin can reverse airway inflammation, remodelling and hyperresponsiveness in a murine model of allergic asthma

Chapter 3: General Methodology

3.0 Materials & Methods

3.1 Chemicals and reagents

House dust mite extract was obtained as a lyophilized powder from GREER labs (Lenoir, NC 28645, USA). Isoflorane (Fornae) was obtained from Baxter USA. Simvastatin, simvastatin-6 hydroxy acid, and fluticasone were obtained from Toronto Research Chemicals (North York, ON M3J 2J8, Canada). 1-Palmitoyl, 2-arachidinoyl sn-phosphatidylcholine (PAPC) was obtained from AvantiPolar Lipids (Alabaster, AL 35007, USA). Isoflurane was obtained from Baxter (Deerfield, IL 60015, USA).

3.2 Animals

Female Balb/c mice (6-8 weeks old) were obtained from the Central Animal Care Facility at the University of Manitoba and maintained in 12-hour light/dark cycles in an individually ventilated cage (IVC) system (obtained from Techniplast, Italy), with access to standard chow food and water *ad libitum*. Each mouse was assigned an identity number (to be accessed by the study coordinator only) and was randomized based on body weight to different groups, so that all experiments could be carried out in a blinded and bias-free manner. The sample size was estimated based on the power calculation G*Power 3.1, with power set at 0.95 and alpha at 0.05. Animals were sacrificed at the end of the experiments with an intraperitoneal (i.p) overdose of pentobarbital sodium. All procedures were carried out as per protocols approved by the institutional ethics committee.

3.3 HDM formulation for intranasal (i.n.) challenge

HDM stock was prepared by dissolving lyophilized HDM in saline to obtain a protein concentration of 2.5mg/mL. For each dose administered during HDM challenge protocols, 25µg of HDM protein was dissolved in 35µL saline and administered by droplet into the nostril.

3.4 Drug Formulations

3.4.1 Simvastatin for subcutaneous (s.c.) injection

Simvastatin was prepared by the method described by McKay et al.(173). Simvastatin was prepared as a 4mg/ml stock 'A' by dissolving 4 mg of simvastatin in 100µL of 100% ethanol. NaOH (150µl; 0.1N) was added to 100µL stock 'A' and the mixture was incubated at 50°C for 2h. The pH was adjusted to 7 using 0.1N hydrochloric acid, and the volume was made up to 1ml with phosphate buffered saline (PBS). The systemic dose of simvastatin used in mice was 40mg/kg/day s.c.

3.4.2 Simvastatin for intranasal (i.n.) administration

A working stock 'B' of 1 mg/mL was prepared in 100% ethanol by dilution of stock 'A'. PBS (990µL) of was added to 10µL of stock 'B' to obtain a stock 'C' with 10µg/ ml concentration. Since simvastatin in stock 'C' is unstable, it was prepared fresh every day before dosing. To obtain a dose of 6µg/kg /day ($\approx$ 125ng/mouse of 20g), 12.5µL of stock 'C' was used, and the final dosing volume was made up to 35µL for i.n. administration. Other doses of simvastatin (3, 1.5, 0.6 and 0.06 µg/kg) were formulated in a similar manner.

3.4.3 Fluticasone for i.n. administration

A 400 μ g/mL stock 'F1' of fluticasone suspension was prepared by the method described by Ulrich et al. and Nials et al. (215, 216). To make a stable suspension, 2 mg fluticasone propionate was mixed with 1.25mL 2% Tween 80 using a magnetic stirrer and glass beads at 12,000 rpm. To this, 4.75 mL PBS was added slowly, and the stirring was continued for 24 h in a fume hood (215, 216). Stock 'F1' was further diluted to obtain a working stock 'F2' at 160 μ g/mL and 'F3' at 80 μ g/mL; 12.5 μ L of stock 'F2' and 'F3' gave doses of 100 and 10 μ g/kg fluticasone propionate, respectively.

3.5 HDM Challenge and Treatment Protocol

All mice were anaesthetized in a plexi-glass chamber with isoflurane (Forane, Baxter, USA) mixed 1:5 with oxygen, prior to the HDM challenge or treatment protocols unless otherwise stated.

3.5.1 Acute HDM Challenge and Treatment Protocols

Mice were administered 25 μ g HDM i.n. daily, 5 days/wk for 2 weeks. Drug treatments were divided into prevention and reversal studies. For specific details please refer chapter 4-6.

3.5.2 Chronic HDM Challenge and Treatment Protocols

For chronic HDM challenge protocol, animals received 5-wk of HDM challenge at the same dose as used in acute HDM challenge protocol to induce airway inflammation, remodelling and hyperresponsiveness. Simvastatin was evaluated for its reversal potential in the presence or absence of allergen. For specific details please refer chapter 7.

3.6 Lung Mechanics (Lung Function Test)

Mice were anaesthetized with sodium pentobarbital (90 mg/kg, i.p) and the trachea was exposed ventrally. Tracheostomy was performed in anaesthetized mice, and a 20-gauge polyethylene catheter was sutured in the trachea, which was then connected to the flexiVent small animal ventilator (Scireq Inc., Montreal, PQ, Canada). Mice were ventilated with a tidal volume of 10mL/kg body weight, 150 times per min. A positive end expiratory pressure (PEEP) of 3cmH₂O was used for all studies.

Mice were subjected to an increased dose of nebulized methacholine (MCh) challenge protocol to assess dose-response characteristics of the mouse respiratory system. Saline (30μL) containing 0–50 mg/mL MCh was delivered over 10 s using an inline nebulizer at the beginning of each dose point. Low frequency forced oscillation technique was used to assess the effects of the MCh challenge on respiratory mechanics. During the low frequency forced oscillation, mechanical ventilation was interrupted, and then a volume perturbation signal was applied using a preset flexiVent Prime-8 protocol to obtain respiratory mechanical impedance (Zrs). By fitting Zrs to the constant phase model (217), the flexiVent software calculated central conducting Newtonian resistance (R_N), peripheral tissue damping (G), and tissue elastance or stiffness (H). Each parameter was normalized according to body weight. Values for each parameter were calculated as the mean of all 20 perturbation cycles performed after each MCh challenge.

Fall in FEV1 by 20% in response to MCh challenge (PC₂₀) is equivalent to 43.5% rise in respiratory resistance (218). Thus, in our model, the MCh PC₂₀ was calculated assessing the MCh concentration required to increase R_N by 43.5% from the base.

3.7 BALF Collection

Following lung function testing in anaesthetized mice, lungs were lavaged through the catheter with 1 mL cold sterile saline, twice, to obtain a total 2 mL BALF. The collected BALF was centrifuged at 1100 RPM (100g) for 10 min, and the supernatant was isolated and stored at -80°C. The cell pellet was resuspended in 1 mL of cold saline.

3.8 Inflammation Analysis

Re-suspended cell pellets were assessed for infiltration of immune cells as inflammatory markers such as total leukocyte cell count and differential cell count to estimate specific immune cells such as eosinophils, neutrophils, macrophages, and lymphocytes.

3.8.1 Total Cell Count

Total leukocytes in the lavage were counted using a hemocytometer. Ten microliters (10 µL) of the cell-pellet suspension was loaded in the hemocytometer, and the number of cells was counted in 4x1 mm² corner squares and total leukocytes were estimated using the following formula

$$\text{Total Leukocyte/mL BALF} = (\text{Total No of cells in 4 squares}) * \frac{10^4}{4}$$

3.8.2 Differential Cell Analysis

For differential cell counts, the cell suspension was further diluted as required to obtain a final concentration of 1-2x10⁵ cells/mL (generally a 1:10 dilution), and 100µL of this final suspension was cytospun on the glass slide using Cytospin® (Shandon Inc.) at 1000 rpm (112g) for 5 min. Glass slides were air-dried and then stained with modified Wright-Giemsa

stain (Hema 3® Stat Pack, Fisher Scientific), and cell distributions were analyzed by manually identifying and counting eosinophils, lymphocytes, macrophages, and neutrophils in six randomly chosen fields of view examined with a light microscope at 200X magnification. Cell counts were averaged across the six fields of view to calculate the absolute number and normalized to BALF volume and represented as cells/ml of BALF

3.8.3 Cytokine Analysis

Proinflammatory cytokines (IFN- γ , IL-1 β , IL-10, IL-12 p70, IL-2, IL-4, IL-5, IL-6, KC/GRO, TNF- α) were profiled in BALF supernatant either by using Proinflammatory Panel1 (mouse) Kit (1 Plate) V-PLEX™ kit from Meso Scale Discovery (Maryland; USA)(as per manufacturer's instruction), or by using the Discovery Assay service provided by Eve Technologies, Calgary for mouse 31-Plex cytokine/chemokine Array and 3-Plex Array for TGF- β .

3.9 Lung Tissue Histology

For lung histology, lungs were collected from mice that were subjected to the lung function test only (no lavage). Thoracic cavities of these mice were opened to expose lungs. Lungs were carefully removed from the thoracic cavity and using the catheter planted post tracheostomy, lungs were inflated with 1ml of 10% formalin, under constant pressure of 25cm of H₂O, then incubated in 10% formalin in a 50mL conical centrifuge tube for at least for 24 h for formalin fixation. Post fixation, the left lobe of the lung was dissected out and processed in a series of dehydration and hydration steps with alcohol and xylene before embedding in paraffin wax. Serial sections (6 μ m thick) were cut from the paraffin embedded left lung using a microtome and stained with hematoxylin and eosin (H&E, to detect inflammation at the tissue level), Periodic acid Schiff (PAS) to detect goblet cell hyperplasia and picrosirius red (to detect

collagen deposition as an index of lung tissue remodelling). Specimens were viewed and photographed using an upright light microscope equipped with a SPOT CCD camera (Olympus, Canada).

3.10 Morphometric Assessment of Lung Histology

3.10.1 Goblet Cell Hyperplasia

Goblet cells were quantified using Image-Pro-Plus software where the length of the basal membrane was calculated by assigning the region of interest using the free hand tool. The basal membrane length obtained in pixels was divided by a factor of 30 to convert it to μm . Based on the color differential between the PAS stained goblet cells (purple) and other cells of the basal membrane, the software counted the number of goblet cells within the assigned region of interest. Finally, the total number of goblet cells was divided by the total basal membrane length in μm to obtain the number of goblet cells/ μm of basal membrane.

3.10.2 Collagen Estimation

For collagen estimation, the picrosirius red stained slides were viewed in 'Ryan Brightfield' settings and then keeping the exposure parameters same, settings of the microscope were changed to 'Kim Red' and using a polarizing filter the polarized image of collagen fibres were taken. Both picrosirius red and polarized images from the same airway were imported to the Image-Pro-Plus software. The picrosirius red image was used to estimate the basal membrane length, and the polarized image was used to estimate the total area occupied by the collagen; the estimates were obtained in the number of pixels. The basal membrane length in μm was

calculated as described previously. The total number of pixel count for collagen/airway was divided by the basal membrane length in μm for that particular airway to obtain collagen units/ μm of the basal membrane.

3.11 Estimation of simvastatin in BALF and serum

Simvastatin and simvastatin acid were estimated in BALF and serum as described by Bews et al. 2014 (219). Both serum and BALF samples were subjected to the solid phase extraction process, using Oasis hydrophilic-lipophilic balance (HLB) cartridges (3 cm^3 size, 60 mg sorbent; Waters Cooperation, Taunton MA). The internal standard (20 ng, simvastatin and simvastatin hydroxy acid) was added to 50 μL of the extracted liquid, and the volume was made up to 1 mL using Milli-Q water. The cartridges were preconditioned with 2mL of methanol, followed by 2mL of Milli-Q water. The samples were passed through the SPE cartridges at 0.5 mL/min, and the compounds were then eluted out of the cartridges with 3mL of methanol under constant flow rate (1 mL/minute). The methanol was evaporated under vacuum (Savant Speed Vac SC110) at 40°C to dryness. The eluted compounds were reconstituted immediately prior to analysis in 0.4 mL of a 20:80 (v/v) solution of acetonitrile: 20mM sodium phosphate monobasic (pH 7.4). Reconstituted samples were vortexed and filtered through 13 mm, 0.22 μm polytetrafluoroethylene syringe filters (Chromatographic Specialties, Brockville, ON) before instrumental analysis(219).

For instrumental analysis, 30 μL samples were run through the Agilent (Mississauga, ON) 1200 ultra high performance liquid chromatograph coupled to a 6410B triple quadrupole tandem mass spectrometer (UHPLC/MS/MS) with electrospray ionization in positive mode, under nebulized nitrogen gas 300°C, with a flow rate of 10 L/min. The nebulizer pressure was maintained at 55 psi and the capillary voltage at 4000 V. The sodiated adducts of both

simvastatin and simvastatin hydroxy acid were analyzed and quantified using 5-point calibration curves for simvastatin and simvastatin hydroxy acid (219).

3.12 Estimation and quantification of OxPCs

Phospholipid extraction from BALF

Phospholipids were extracted from the BALF and lung tissue as described by Folch et al. 1957 (220). The detailed procedure is as follows: phospholipids were extracted from the BALF using the Folch extraction method (220), and analysis was performed as described by Hasanally et al. 2017 (221). BALF supernatant samples stored at -80°C were thawed. Sample aliquots of 100µL were transferred to 15mL glass centrifuge tubes, and 100µL ice-cold phosphate buffered saline (PBS) as well as 850µL chloroform:methanol (2:1) containing 0.01% butylated hydroxytoluene (BHT) was added. Before phospholipid extraction, each sample was spiked with 100µL of internal global standard and 50µL of 20:0 PC (10 µg/mL). The internal global standard consisted of 1,2-dinonanoyl-sn-glycero-3-phosphocholine (09:0 PtdCho, 0.1 ng/µL), 1-heptadecanoyl-2-hydroxy-sn-glycero-3-phosphocholine (17:0 lyso PtdCho, 0.1 ng/µL), 1,2-diarachidoyl-snglycero-3-phosphocholine (20:0 PtdCho, 1.0 ng/µL) and 1-heptadecanonyl-2-(9Z-tetradecenonyl)-sn-glycero-3-phospho-(1'myo-inositol)(ammonium salt) (31:1 PtdIns, 1.0 ng/µL). The mixture was vortexed briefly and then centrifuged at 3500 rpm for 5 min. The lower organic layer containing the phospholipids was collected, and to the remaining aqueous phase, 600 µL of chloroform was added. The mixture was again vortexed and centrifuged at 3500 rpm for 5 min. The organic phase was collected and pooled with the previously collected organic phase in a 5 mL glass centrifuge tube. The organic solvent was evaporated under nitrogen gas stream, and the remaining phospholipids in the tube were reconstituted in 500µL chloroform:

methanol (2:1). The final solution was divided into 2 aliquots of 400 and 100 μ L. For OxPC estimation, 400 μ L aliquot was evaporated under nitrogen stream and reconstituted in 80 μ L 65:30:5 (v/v/v) acetonitrile:isopropanol:water (reverse phase solvent). The entire procedure was carried out at 4°C.

HPLC-MS/MS analysis of OxPCs

For this, we used the HPLC (Shimadzu Corporation, Canby, Oregon, USA) system coupled to a 4000 QTRAP® triple quadrupole mass spectrometer system with a Turbo V electrospray ion source from AB Sciex (Framingham, Massachusetts, USA). For the analysis of OxPCs, thirty microliters of sample were injected onto an Ascentis Express C18 RP-HPLC column (15 cm $\times$ 2.1 mm, 2.7 μ m; Supelco Analytical, Bellefonte, Pennsylvania, USA). Separation of analytes was accomplished using a binary solvent system with solvent A consisting of 60:40 (v/v) acetonitrile:water and solvent B, 90:10 (v/v) isopropanol:acetonitrile. Both solvent systems contained 10mM ammonium formate and 0.1% formic acid. The mobile phase composition used for compound separation commenced with 32% solvent B, slowly ramped from 1.5 min until 4 min up to 45% B, followed by 52% B at 5 min, 58% B at 8 min, 66% B at 11 min, 70% B at 14 min, 75% B at 18 min, 97% B at 21 min and then 32% B at 25.10 min until the end of the 30.10 min run time. A flow rate of 260 μ L/min was used for chromatographic separation, with the column and sample trays maintained at temperatures of 45 and 4°C, respectively.

The mass range for precursor ion scans of mouse lung lavage and tissue extract oxidation products was set between 400 and 1300 Da in negative ion mode and was able to detect OxPCs species in a 30 min global scan for all masses. The precursor ion scan settings were as follows: declustering potential, -80 V; entrance potential, -10 V; collision energy, -60

V; collision cell exit potential, -20 V and a dwell time of 0.5 s. For PtdIns compounds, detection was carried out in negative ion mode by multiple reaction monitoring (MRM) of a total of 71 OxPtdIns, lyso PtdIns and non-oxidized PtdIns mass transitions using a PtdIns-specific product ion (241.1 m/z, Da) with an additional precursor ion/product ion transition monitored, characteristic of fragmentation occurring at the sn2 position. The ESI voltage was set to -4500 V and temperature of the ion source was set to 500 °C. High purity nitrogen was used as curtain gas at 26 psi, and high purity air was used as nebulizer and heater gas with pressures set at 40 and 30 psi, respectively. The MRM settings were as follows: declustering potential, -80 V; entrance potential, -10 V; collision energy, -60 V; collision cell exit potential, -20 V and a dwell time of 100 ms.

3.14 Statistical Analysis

Statistical analysis was done using SPSS20.0 software unless otherwise stated. The lung function, inflammation, and lung tissue remodelling data were analyzed using univariate or multivariate analysis of variance (MANOVA) with Bonferroni/Tukey HSD posthoc test (for normally distributed data) or Games-Howell test (for data that failed Levene's test of homogeneity). Results were represented as mean $\pm$ SEM. The cut-off for statistical significance was kept at $p=0.05$. The OxPC and 32plex cytokine array data were analyzed using Principal Component Analysis (PCA, unsupervised) and Partial Least Square Analysis (PLSDA, supervised) and Agglomerative Hierarchical Cluster (AHC) analysis using R statistical software, to analyze the interaction among different OxPC subspecies and their interaction with different treatment groups. Following this exploratory analysis, the multivariate analysis with Bonferroni posthoc test was performed as previously explained.

Chapter 4: Prophylactic benefits of systemically delivered simvastatin treatment in a house dust mite challenged murine model of allergic asthma

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Jules Carlson: Estimation of simvastatin and simvastatin hydroxy acid in biological samples

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4.1 Background

Statins are competitive inhibitors of hydroxyl-methyl-glutaryl Co-A reductase, the proximal rate-limiting enzyme of the mevalonate cascade. Beyond their capacity to block *de novo* cholesterol synthesis, statins inhibit formation of isoprenoid lipid chains that are needed for membrane anchorage and activation of signalling proteins that regulate cell proliferation, migration, contraction and secretory function (193, 200, 222, 223). Thus, orally administered statins have pleiotropic systemic effects on endothelial function, platelet aggregation, and inflammation that enhance their therapeutic benefits on cardiovascular health (224, 225). Retrospective epidemiological investigation shows that long-term statin use can also be associated with health benefits for asthmatics, evidenced by reduced emergency room visits and corticosteroid use (207, 226). Some clinical evidence suggests that short-term statin use can enhance the anti-inflammatory effects of inhaled corticosteroids (212, 214). However, systematic review of clinical studies, which to date have not included prospective assessment of the prophylactic effects of long-term statin use, reveals a paucity of evidence for short-term oral statin therapy having a significant impact on lung function in asthmatics (210, 211, 213, 227-229). This stands in contrast with evidence from preclinical studies showing that acute systemic statin therapy can prevent lung inflammation, and may reduce airway hyperreactivity in ovalbumin-sensitized rodents (173, 209, 222, 230, 231).

Systemic delivery of simvastatin at the onset of ovalbumin administration in mice prevents airway inflammation by inhibiting lung leukocyte influx, promoting leukocyte apoptosis, and blunting biogenesis of pro-inflammatory cytokines and mediators (173, 209, 222, 232). Simvastatin treatment can also prevent ovalbumin exposure-induced airway goblet cell hyperplasia (233). *In vitro* studies indicate that statins can have direct effects on lung structural

cells: for example, simvastatin promotes apoptosis and inhibits proliferation of airway smooth muscle cells, blocks TGF β ₁-induced extracellular matrix protein production by airway fibroblasts and airway smooth muscle cells, and blocks cytokine release by airway epithelial cells (71, 193, 200, 234, 235). In spite of this evidence for the effects of statins on lung inflammation and remodelling, there has been limited investigation of airway hyperresponsiveness, a hallmark feature of asthma. Lovastatin appears to reduce contractility of bronchi isolated from ovalbumin challenged rodents (231, 236). Systemic simvastatin treatment reportedly prevents increased lung resistance in ovalbumin exposed mice (222, 230). Recent pre-clinical studies in mice suggest that inhaled statins may affect lung function (237), but targeted delivery of statins to the lungs is not approved for humans. Real world interpretation and translation of results from pre-clinical studies of systemically delivered statins is limited: none have investigated the effects of long-term, prophylactic treatment or monitored the concentration of statins that accumulate in the lung. Moreover, the use of ovalbumin as an aeroallergen is clinically irrelevant (160, 238, 239).

The current study compares the effects of prophylactic and acute systemic simvastatin therapy on lung inflammation, airway goblet cell remodelling, and airway function in a murine model of allergic asthma using repeated lung challenge with house dust mite (HDM). We monitor pharmacokinetics and accumulation of simvastatin in serum, lung tissue and lung lavage. We reveal that repeated systemic delivery of simvastatin results in its accumulation in serum, but not in the lungs. Prophylactic systemic delivery of simvastatin increases anti-inflammatory effects; however, consistent with clinical studies, systemic simvastatin has no effect on airway hyperreactivity.

4.2 Methods

Simvastatin Single Dose Kinetics:

Balb/c mice were administered a single dose of simvastatin (40mg/kg; prepared as described previously in General Methods section) s.c., and blood was collected post euthanasia from 3 mice at 0, 8, 16, 32 and 48 h. Serum was extracted from blood samples as described after centrifugation at 5000 rpm for 10 min, and stored at -80°C until measurement. Lung samples were homogenized in cell lysis buffer in the presence of protease and phosphatase inhibitors, centrifuged at 5000 rpm for 15 min at 4°C, and the supernatant was collected. The simvastatin was analyzed in both serum and lung tissue as per the already described protocol.

HDM Challenge and Drug Treatment

The Balb/c mice were randomized to different groups. One group received only HDM acute challenge for 2 weeks. Mice to be treated with simvastatin were further divided into 3 groups: (i) the acute treatment group received simvastatin concomitantly with HDM for 2 weeks (5day/week), (ii) the prophylactic treatment group received 3 s.c. injections of simvastatin on alternate days 1-wk prior to receiving acute treatment protocol, and (iii) an age-matched naïve control group received simvastatin in the absence of HDM challenge (Figure 4.1). At 8 and 48 hrs post last HDM/ PBS challenge, various physiological end points were assessed as follows:

- i) Assessment of simvastatin and simvastatin acid in BALF, lung tissue and serum
- ii) Lung function test
- iii) Assessment of inflammation in BALF – total, differential leukocyte count and cytokine array using MSD platform, lung tissue histology using H&E staining
- iv) Lung tissue morphometry for goblet cell hyperplasia

All procedures were carried out as already described in General Methodology section.

Statistical Analysis

Data are presented as mean values $\pm$ standard error mean (SEM). Means were compared by univariate or one-way ANOVA and Tukey multiple comparisons test, using SPSS_20 software (IBM Inc) or Graph Pad Prism software 5.0. A p value <0.05 was interpreted as statistically significant among the mean values.

Figure 4.1

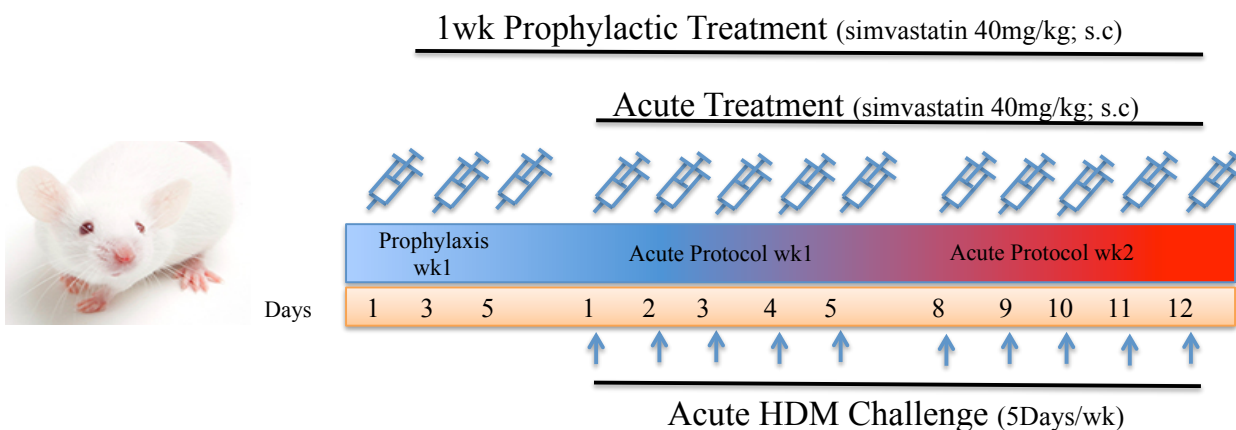


Figure 4.1: Schematic of acute HDM challenge with or without simvastatin treatment.

Arrows represent the days when mice received i.n. HDM, and injections represent days in which mice received s.c. simvastatin (40 mg/kg). Animals received HDM 25 $\mu\text{g}/\text{day}$ i.n.. Groups of mice that underwent acute treatment received simvastatin 40mg/kg s.c. concomitantly to HDM challenge. The prophylaxis group received s.c. injections of simvastatin 40mg/kg on 3 (1wk Prophylaxis) alternate days 1 week before the beginning of Acute Treatment. Note: A 2-day break occurred between each week of challenge/ treatment. The study consisted of treated and age-matched naïve control groups.

4.3 Results

Simvastatin Pharmacokinetics

The peak serum concentration of pharmacologically active simvastatin hydroxy acid (151 ± 87.5 $\mu\text{g/L}$) was reached 8 h after a single s.c. administration of simvastatin (40mg/kg); this declined sharply 16 and 32 h post-administration (3.1 ± 3.4 and 4.8 ± 4.8 $\mu\text{g/L}$, respectively), and was not detectable after 48 h (Figure 4.2A). Levels of simvastatin hydroxy acid were below the level of detection in lung tissue and BALF at all time points. Having established the kinetics of single dose simvastatin, we next profiled pharmacologically active simvastatin that developed at experimental endpoints for both the acute and prophylactic treatment regimens. Eight hours after the final administration of simvastatin, serum concentration was 46.7 ± 3.7 $\mu\text{g/L}$ in animals receiving acute treatment, and this was more than doubled in animals that received prophylactic treatment (116 ± 24.5 $\mu\text{g/L}$, $p < 0.05$) (Figure 4.2B). Both treatment regimens established a similar steady state concentration of simvastatin in the systemic circulation as measured 48 h after final administration (80.3 ± 23.2 $\mu\text{g/L}$ and 57.7 ± 0.7 $\mu\text{g/L}$ for acute and prophylactic protocols, respectively; n.s, $p > 0.05$, $n = 3$). This suggests that serum simvastatin hydroxy acid concentration follows a dynamic pattern after each s.c. administration, and with repeated treatment establishes stable levels in systemic circulation. In striking contrast, neither the acute nor the prophylactic treatment protocol was sufficient to establish levels of simvastatin hydroxy acid within the level of quantification (LOQ) for UHPLC/MS/MS of lung tissue or BALF.

Figure 4.2A

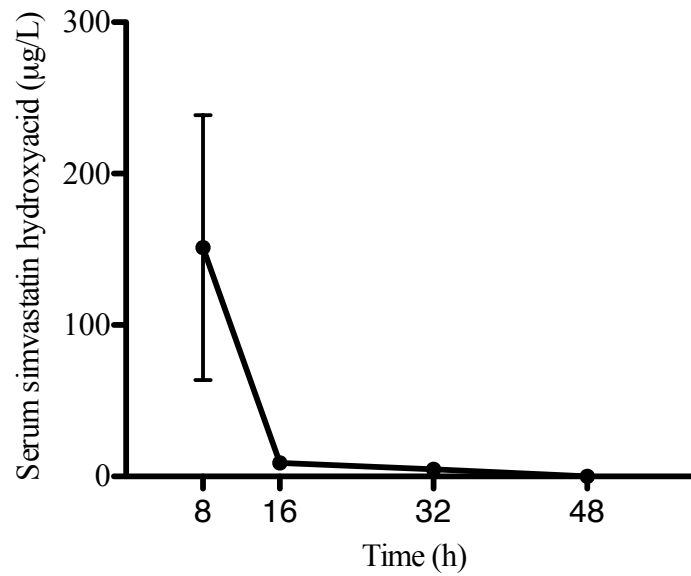


Figure 4.2B

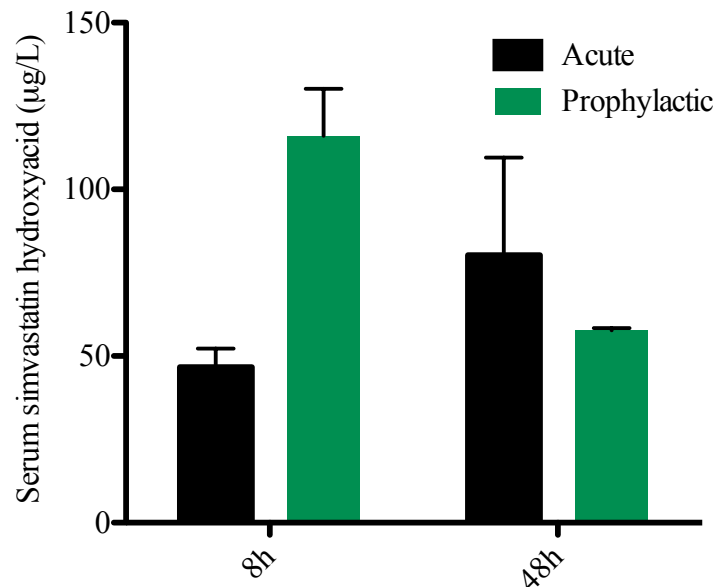


Figure 4.2: Serum kinetics of simvastatin injection.

A) Simvastatin hydroxy acid concentration in serum 8 h after a single administration of simvastatin (40mg/kg; s.c.) as measured by UHPLC-MS/MS. B) Serum concentration of simvastatin hydroxy acid 8 h and 48 h after acute and prophylactic treatment protocols. All data represent the mean from 3 animals at each time point and treatment. Data are shown as mean $\pm$ SEM. As determined by Student's t-test, * $p < 0.05$, ** $p < 0.01$.

Inflammatory cell count

Eight (8) Hours Post-HDM Challenge

Influx of inflammatory cells in the lung was induced markedly by HDM challenge, reaching $100.6 \pm 12.7 \times 10^4$ cells/mL BALF, as compared to the allergen naïve mice ($2.0 \pm 0.2 \times 10^4$ cells/mL). Acute treatment with simvastatin blunted HDM challenge-induced inflammatory cell number in BALF by approximately 25% ($75.3 \pm 10.8 \times 10^4$ cells/mL; $p < 0.05$). Prophylactic simvastatin treatment inhibited HDM challenge-induced total inflammatory cell influx by 70% ($30.4 \pm 5.7 \times 10^4$ cells/mL; $p < 0.05$), an impact that was nearly twice that achieved with the acute treatment protocol (Figure 4.3A). HDM challenge induced pronounced lung neutrophilia ($45.2 \pm 5.9 \times 10^4$, $p < 0.05$ vs. naïve), but acute treatment with simvastatin did not significantly inhibit neutrophil accumulation. However, prophylactic treatment inhibited neutrophil accumulation in BALF by 77%. HDM challenge-induced eosinophilia ($25.9 \pm 3.0 \times 10^4$ cells/mL BALF) was not significantly reduced by acute treatment, but was inhibited by 78% with prophylactic treatment. Neither acute nor prophylactic treatment was sufficient to change the number of macrophages/monocytes after HDM challenge, whereas prophylactic (but not acute) simvastatin treatment inhibited HDM-induced lung lymphocyte influx by over 75% ($p < 0.01$).

Forty-eight (48) Hours Post-HDM Challenge

HDM challenge-induced lung inflammatory cell accumulation in BALF was sustained for 48 h beyond final allergen exposure ($104.6 \pm 42.4 \times 10^4$ cells/mL; $p < 0.001$ vs. naïve; $n=6$). Both acute simvastatin treatment and prophylactic treatment significantly prevented HDM-induced total lung leukocyte infiltration ($50.6 \pm 0.9 \times 10^4$; $p < 0.01$, and $23.4 \pm 5.5 \times 10^4$, $p < 0.001$, respectively) (Figure 4.3B). Eosinophils predominated in BALF from HDM challenged mice ($43.5 \pm 10.2 \times 10^4$ cells/mL). Of note, eosinophil accumulation was reduced by 55% with acute

simvastatin treatment ($13.7 \pm 4.3 \times 10^4$ cells/mL; $p < 0.05$ vs. HDM) and even more so by prophylactic treatment that reduced eosinophil number by nearly 85% ($6.9 \pm 1.7 \times 10^4$ cells/mL; $p < 0.001$ vs. HDM). Lung neutrophilia persisted 48 h after HDM challenge ($22.4 \pm 7.6 \times 10^4$ cells/mL BALF); this was blunted significantly by prophylactic treatment ($3.1 \pm 1.12 \times 10^4$ cells/mL; $p < 0.01$ vs. HDM) but not by the acute treatment regime ($P > 0.05$ vs HDM). As we observed 8 h post-allergen challenge, neither acute nor prophylactic treatment was sufficient to limit the number of macrophages/monocytes 48 h after HDM challenge, whereas prophylactic (but not acute) simvastatin treatment inhibited HDM-induced lung lymphocyte influx by 61% ($p < 0.01$).

Figure 4.3A

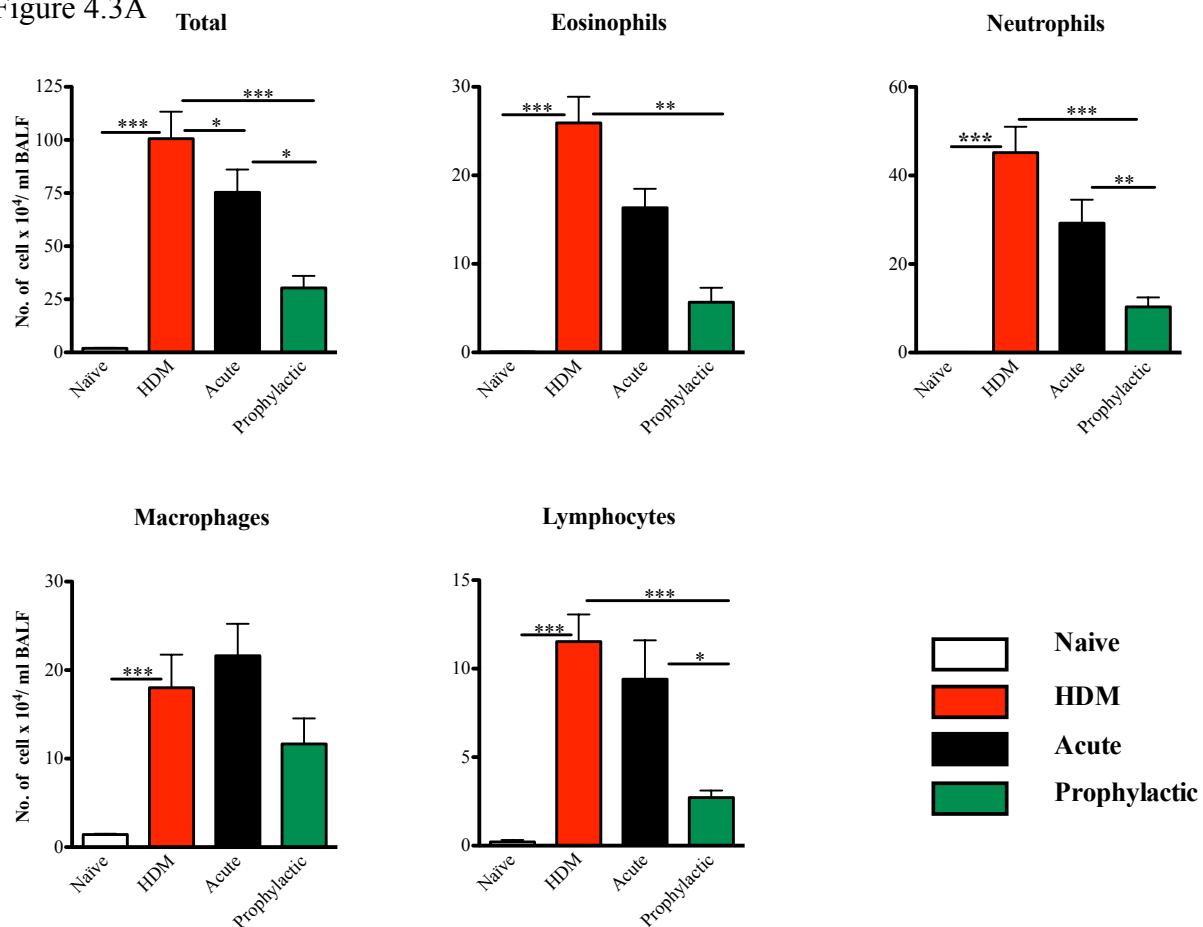


Figure 4.3B

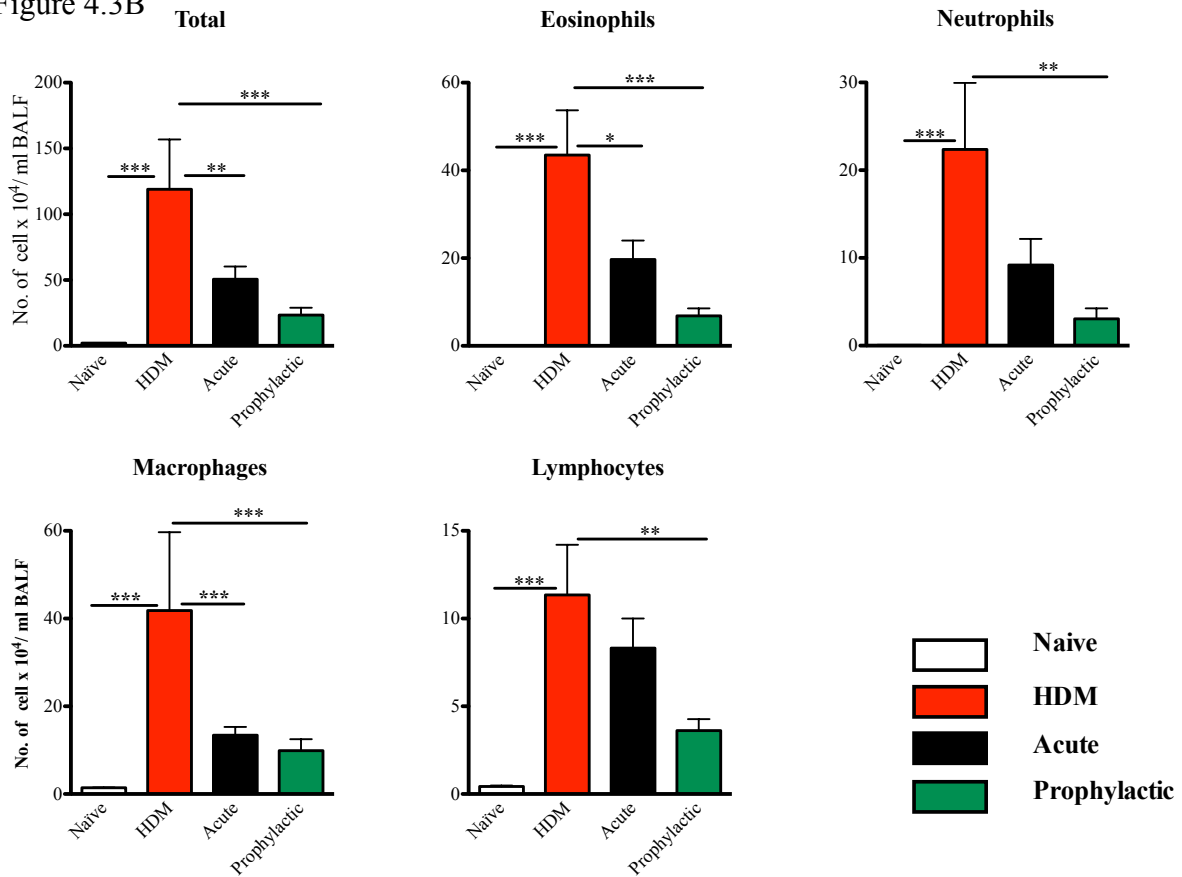


Figure 4.3: Effects of HDM challenge and acute and prophylactic simvastatin treatment on leukocyte number and differential in BALF.

Cell counts determined A) 8 h and B) 48h after completing HDM challenge, with and without simvastatin treatment. For all data, cell number is normalized to initial BALF volume and presented as mean $\pm$ SEM from 6 animals per group. Analysis was done using one-way ANOVA with Tukey multiple comparison test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Cytokine Analysis

As the profile of leukocytes in BALF was different 8 and 48 h after HDM challenge, being predominantly neutrophilic and eosinophilic, respectively, we profiled a panel of 10 pro-inflammatory cytokines and chemokines in BALF at both experimental end points.

Eight (8) Hours Post-HDM Challenge

Compared to allergen naïve mice, HDM challenge induced significant accumulation of a number of mediators, including: TNF α (100-fold, $p < 0.01$), KC/GRO (45-fold, $p < 0.001$), IL-6 (300-fold, $p < 0.05$), IL-4 (190-fold, $p < 0.01$), IL-5 (240-fold, $p < 0.05$), IFN- γ (800-fold, $p < 0.001$), and IL-1 β (80-fold, $p < 0.01$) (Figure 4.4A). Neither IL-2 nor IL-12 was detected in naïve mice; however, post HDM challenge levels in BALF of both cytokines rose to 5.2 ± 1.1 pg/mL and 13.9 ± 4.9 pg/mL, respectively. Both acute and prophylactic simvastatin treatment protocols inhibited HDM challenge-induced accumulation of a number of pro-inflammatory cytokines by 60-95%, including: TNF α , KC/GRO, IL-6, IL-4, IL-1 β , and IFN- γ . The level of IL-12 in BALF was also reduced to below detection limits by both treatment protocols. In contrast, neither acute nor prophylactic treatment affected HDM challenge-induced IL-2 (not shown). Notably, though acute treatment had no significant impact on IL-5 in BALF from HDM challenged mice, prophylactic treatment was sufficient to inhibit IL-5 level by 95% ($p < 0.05$). Interestingly, for the anti-inflammatory cytokine IL-10, acute and prophylactic simvastatin treatment suppressed HDM challenge-induced accumulation in BALF by $84.6 \pm 6.9\%$ and $94.5 \pm 1.4\%$, respectively ($p < 0.05$).

Forty-eight (48) Hours Post-HDM Challenge

As shown in Figure 4.4B, 8h compared to naïve mice, 48h after allergen exposure we measured significant elevation in four pro-inflammatory cytokines in BALF, including IL-1 β

(23-fold), IL-4 (7-fold), IL-5 (38-fold), and KC/Gro (6-fold). HDM challenge also induced a 7-fold increase in the anti-inflammatory cytokine IL-10 (Figure 4.4B). Conversely, though a trend for elevated levels was apparent, we did not observe a statistically significant change in the abundance of IFN- γ , IL-12, IL-2, IL-6 or TNF α compared to allergen naïve animals (data not shown). Acute and prophylactic treatment with simvastatin prevented HDM challenge-induced accumulation of IL-1 β by 70% and 85%, respectively. Notably, prophylactic but not acute simvastatin treatment markedly blunted HDM-induced accumulation of IL-4 and IL-5 by over 75%, and prophylactic treatment was significantly more effective than acute treatment in damping allergen challenge-associated accumulation of the Th2-cytokine IL-4, as well as the pro-neutrophilic chemokine KC/Gro ($p < 0.01$). In contrast to the effects we measured 8 h after allergen challenge, neither acute nor prophylactic simvastatin treatment abrogated the accumulation of anti-inflammatory IL-10 in BALF 48h after allergen exposure.

Figure 4.4A

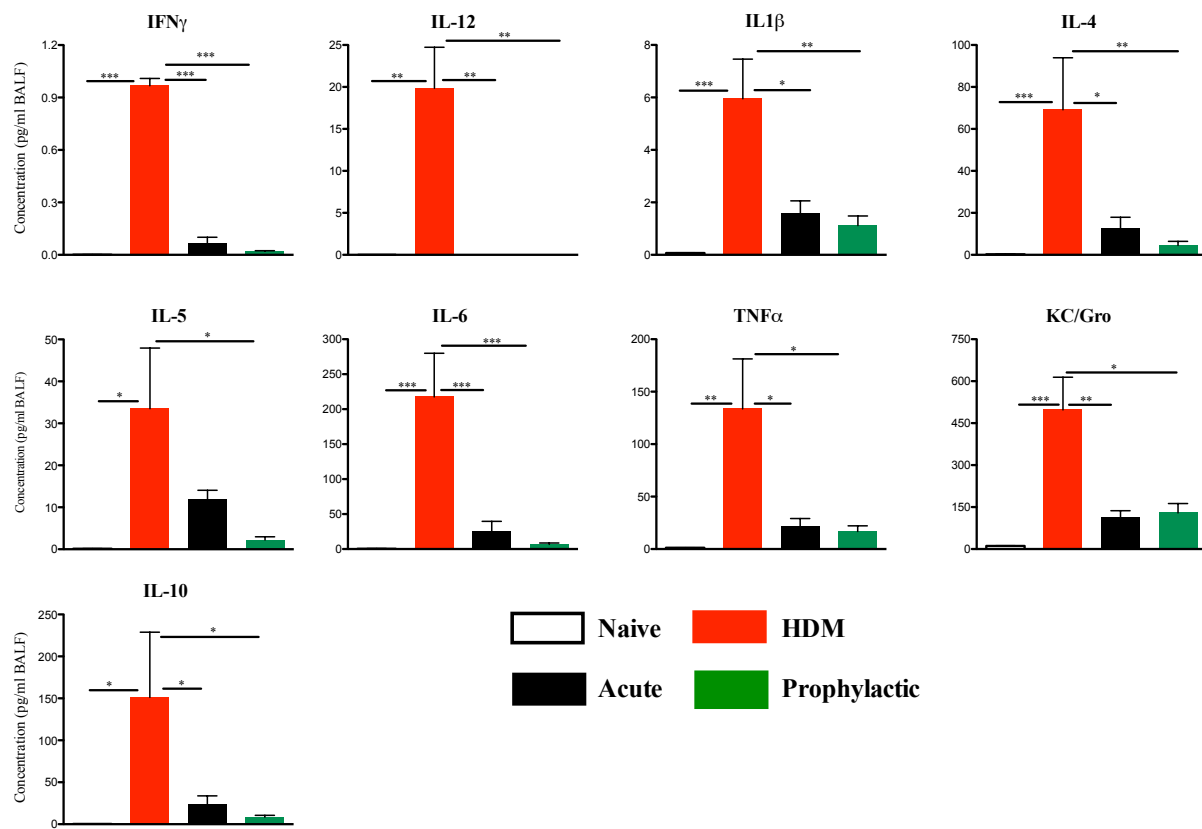


Figure 4.4B

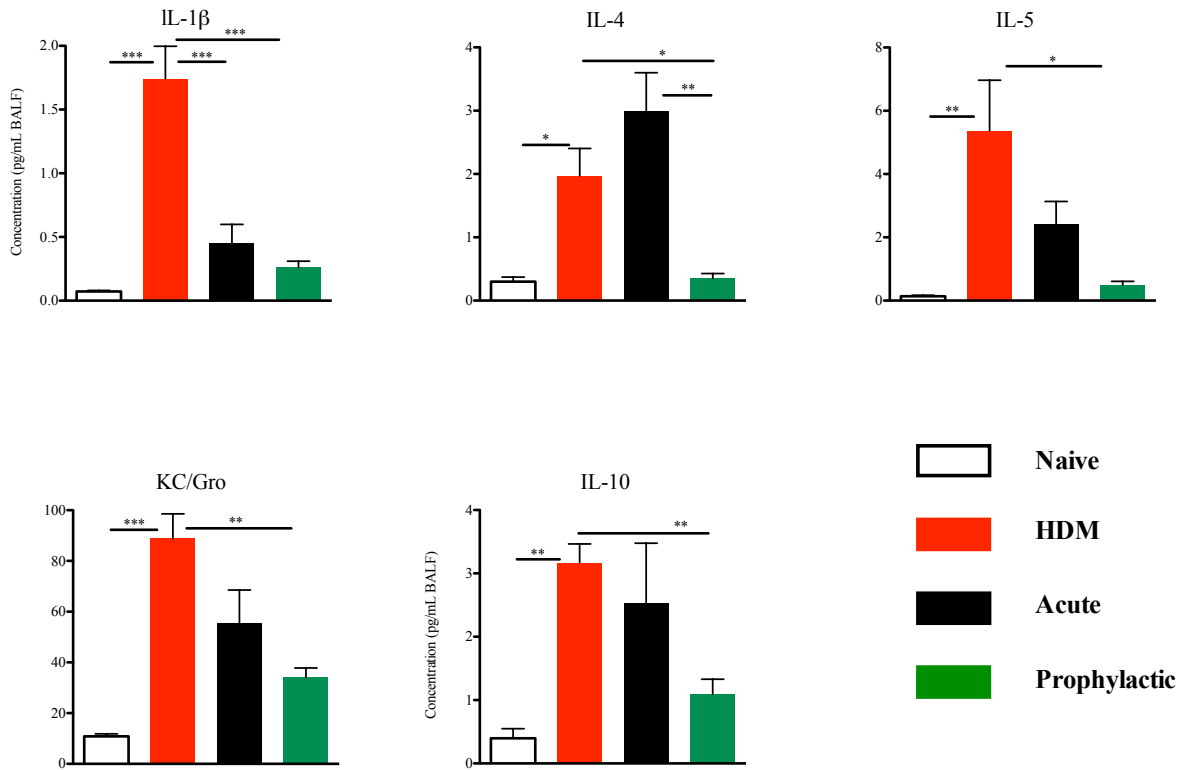


Figure 4.4: Effects of HDM challenge and acute and prophylactic simvastatin treatment on cytokine accumulation in BALF.

Cytokine abundance determined by 10-plex MSD array A) 8h and B) 48h after completing HDM challenge with and without simvastatin treatment. For all data, cell number is normalized to initial BALF volume and presented as mean $\pm$ SEM from 6 animals per group. Analysis was done using one-way ANOVA with Tukey multiple comparison tests: *p<0.05, **p<0.01, ***p<0.001.

Lung Histology

Lung histology was performed to assess airway goblet cell hyperplasia using PAS staining and quantitative microscopy. Goblet cells were absent in peripheral airways of allergen naïve animals (mean airway perimeter $161.1 \pm 28.5 \mu\text{m}$, $n = 36$). In contrast, in similar sized airways, both 8 and 48 h after completing allergen challenge robust goblet cell staining per unit of basement membrane was evident (Figure 4.5A-D). Both acute and prophylactic simvastatin treatment were sufficient to virtually abrogate the development of goblet cell hyperplasia after allergen challenge.

Figure 4.5A

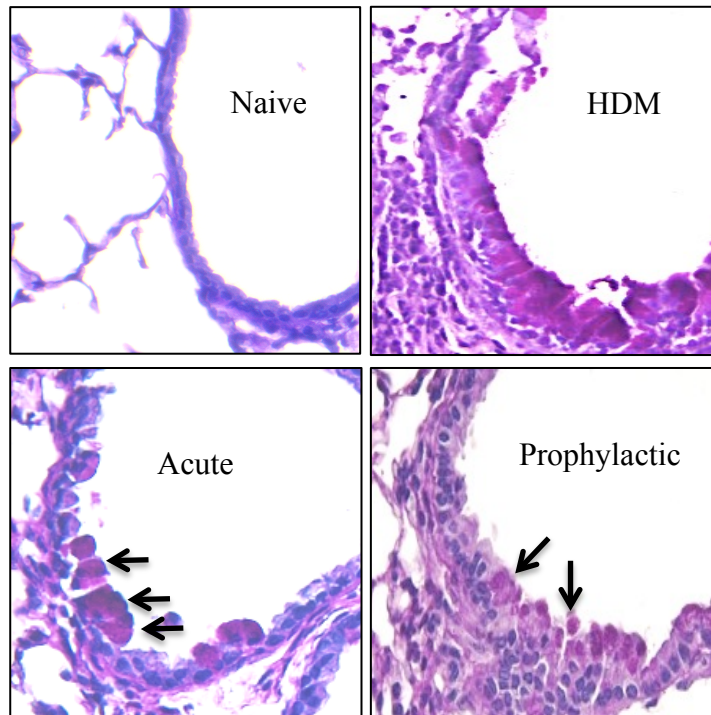


Figure 4.5B

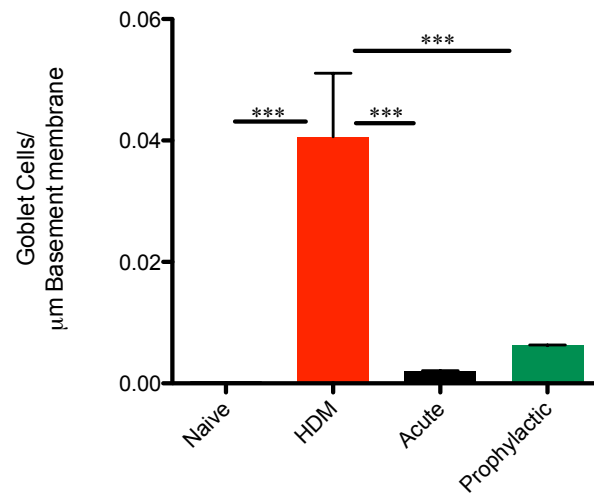


Figure 4.5C

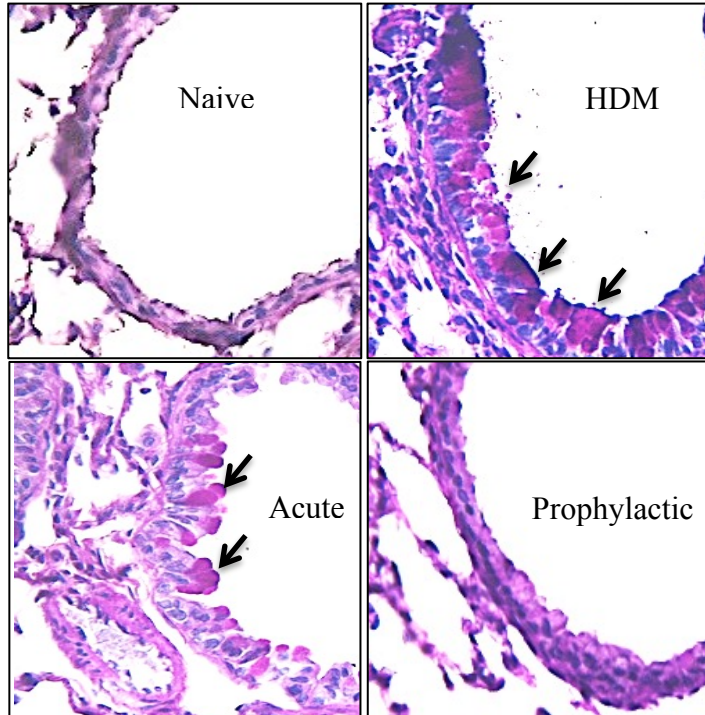


Figure 4.5D

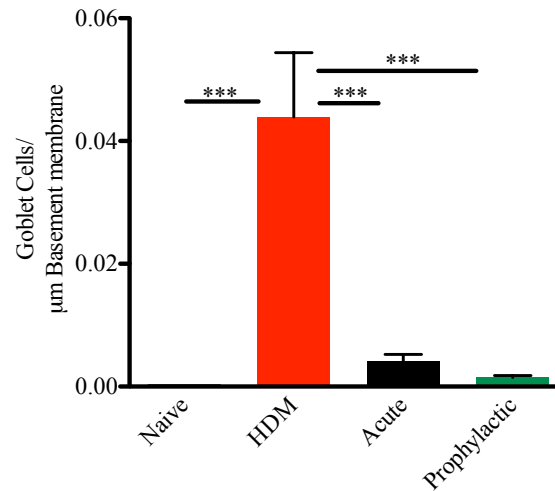


Figure 4.5: Effects of HDM challenge and acute and prophylactic simvastatin treatment on airway goblet cell number.

Images of typical PAS staining of goblet cells in bronchioles A) 8 h and C) 48 h after completing HDM challenge, with and without simvastatin treatment. Images were acquired using 200x magnification. Arrows indicate presence of purple mucous staining. Quantification of goblet cells using Image Pro software B) 8 h and D) 48 h after completing HDM challenge, with and without simvastatin treatment was used to determine goblet cell number in similar sized airways in each mouse (perimeter $161.1 \pm 28.5 \mu\text{m}$). Data represent mean $\pm$ SEM from 9 airways from 3 animals per group. Comparisons were done using one-way ANOVA with Tukey multiple comparison test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Lung function

We used a small animal ventilator to measure airway reactivity to inhaled MCh, 8 and 48 h after completing the HDM exposure protocol. Consistent with our prior studies (240, 241), airway resistance (R_N) was not statistically different from that in allergen naïve animals at the 8 h experimental end point, but 48 h after final HDM challenge we observed a significant increase in R_N in response to 25 and 50 mg/mL MCh ($p < 0.001$ and 0.01 respectively) (Figure 4.6A). Therefore, we only assessed the impact of acute and prophylactic simvastatin treatment on lung function 48 h after completing HDM challenge. In contrast the inhibitory effects of simvastatin therapy on allergen-induced lung inflammation and goblet cell hyperplasia, neither acute nor prophylactic simvastatin treatment prevented airway hyperreactivity (evidenced by increased R_N) 48 h after HDM exposure (Figure 4.6A). Similarly, simvastatin therapy did not affect lung elastance (H) caused by allergen challenge (Figure 4.6B). Tissue damping (G), an indicator of the combined contribution of small airways resistance and lung elastic recoil, was increased after HDM challenge, but this effect was blunted significantly by both acute and prophylactic simvastatin treatment when mice were exposed to high concentrations of MCh (Figure 4.6C). Consistent with hyperreactivity, HDM challenge induced hypersensitivity to MCh (PC_{20} for HDM challenged mice was 3.2 ± 2.2 mg/mL compared 25.2 ± 4.5 mg/mL for naïve mice; $p < 0.001$) remained untouched by both acute and prophylactic treatment with simvastatin.

Figure 4.6

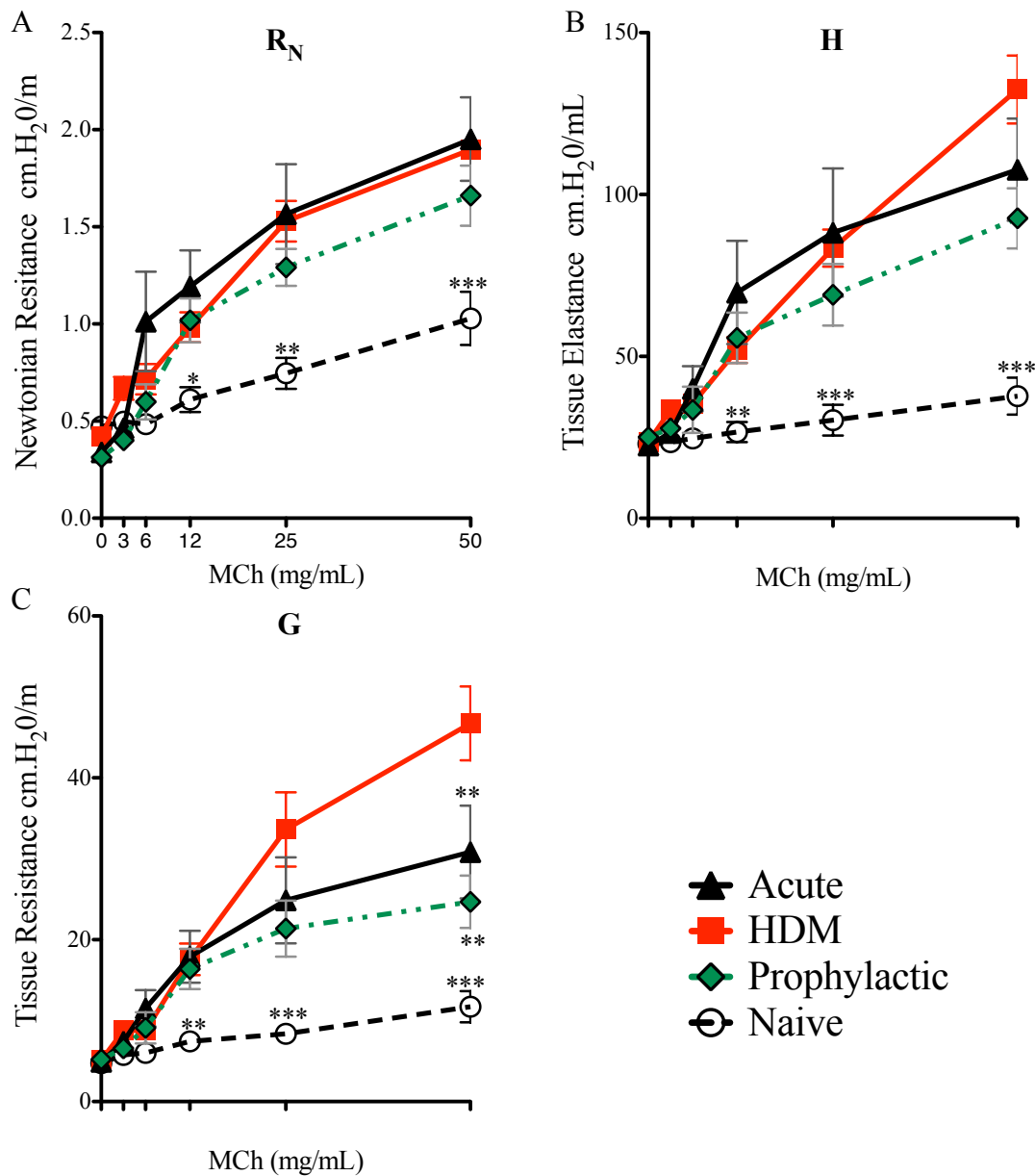


Figure 4.5: Effects of HDM challenge and acute and prophylactic simvastatin treatment on respiratory mechanics.

Lung function was assessed using a flexiVent small animal ventilator 48 h after completing HDM challenge with and without simvastatin treatment. A) Newtonian Resistance (R_N), B) Tissue Elastance (H) and C) Tissue Resistance (G) were measured in response to sequential challenge with nebulized saline (0) or MCh (3-50mg/mL). Data are presented as mean $\pm$ SEM from 6 animals per group. Comparisons of naïve and each treatment were performed against HDM challenge using Univariate analysis followed by Tukey multiple comparison test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4.4 Discussion

This study addresses the disparity in results from pre-clinical small animal model studies and human clinical trials pertaining to the effects of systemically delivered statins on asthma pathophysiology. Using a murine model of allergic asthma induced by repeated delivery of a clinically relevant aero-allergen, (HDM) to the lungs (160, 242), we show that acute systemic delivery of simvastatin in parallel with allergen challenge blunts allergic inflammation by more than 50%, including eosinophil and neutrophil influx and pro-inflammatory cytokine release, as well as abrogating airway goblet cell hyperplasia. Moreover, prophylactic simvastatin treatment, which establishes sustained levels of pharmacologically active simvastatin in the circulation, nullifies all indices of allergic lung inflammation even more effectively than acute treatment, and abrogates airway goblet cell hyperplasia. Despite these marked inhibitory effects, no effect was observed on airway hyperreactivity, a hallmark of asthma. These findings indicate that with systemic delivery, pharmacologically active simvastatin does not accumulate in the lungs, indicating that beneficial effects to prevent lung inflammation are likely associated with a mechanism of action in the systemic circulation and the behaviour of leukocytes therein.

Though the pharmacodynamics of oral statin therapy has been described in humans (243), for lung diseases it has not been a primary point of assessment for systemically delivered statins in pre-clinical studies. We show that serum simvastatin concentration increases sharply after s.c. delivery, but this declines rapidly for a single administration and is not detectable 48 hours later. This is consistent with human pharmacokinetic trials that show peak plasma concentrations within 4 hours of oral simvastatin therapy, and that this is reduced to only 10% of the peak concentration 8 hours later (243). A caveat of our study is that we did not measure peak simvastatin hydroxyl acid until 8 hours after treatment. Thus, we may not have determined the

maximum peak that could have been attained. Nonetheless, we did use our previously established UHPLC/MS/MS protocol, designed to specifically monitor pharmacologically active simvastatin hydroxy acid (219) to show that prophylactic treatment (repeated delivery of simvastatin every second day for one week) is sufficient to establish a steady-state serum concentration of $\sim 50\mu\text{g/L}$ simvastatin hydroxy acid. Similar circulating levels of pharmacologically active simvastatin were likely also achieved within one week of initiating acute treatment in parallel with HDM challenge. Notably, we show that levels of active simvastatin 8 hours after drug delivery were more than doubled to $>120\mu\text{g/L}$ with continued simvastatin administration during the HDM challenge arm of the prophylactic treatment protocol. The disparate level of circulating, pharmacologically active simvastatin that is achieved by prophylactic therapy, combined with the dynamic nature of peak and steady state circulating simvastatin hydroxy acid that we describe, is important to consider in deciphering the possible mechanisms for the effects of simvastatin on the evolution of lung inflammation in HDM-challenged mice.

Our findings are consistent with a number of studies that show the systemic delivery of statins is associated with a blunted influx of inflammatory cells and goblet cell hyperplasia upon allergen challenge (173, 209, 222, 244). These prior studies used mice subjected to ovalbumin sensitization and challenge to induce eosinophilic lung inflammation. Thus, the primary benefit of statin therapy is reported to be the prevention of lung eosinophilia and eosinophil-associated cytokines such as IL-5 and eotaxin-1 (173, 222, 235). A distinction of the HDM-challenge model is that it employs a complex multi-allergen, proteolytic aero-exposure that includes lipopolysaccharide, and as such induces a complex inflammatory response involving interplay between multiple immune and resident lung cells (239). Our data

demonstrate that repeated HDM challenge induces overlapping neutrophil, eosinophil, macrophage/monocyte and lymphocyte accumulation in the lung. This includes an early peak in neutrophil accumulation (8 hours post-allergen) and a later peak in eosinophil accumulation (48 hours post-allergen). Therefore, our experiments can distinguish the effects of simvastatin therapy on different immune cell types. Prophylactic simvastatin treatment was significantly more effective than acute treatment in suppressing neutrophil, eosinophil and lymphocyte recruitment to the lungs at all experimental endpoints. Indeed, acute simvastatin therapy was sufficient neither to significantly reduce peak influx of neutrophils 8 hours after HDM challenge nor to limit sustained neutrophilia 48 hours after HDM challenge. The differential effects of prophylactic treatment could be attributed to the higher serum concentration of simvastatin hydroxy acid that was achieved.

Our data are consistent with previous observations by McKay, Leung (173) who reported only high doses of intraperitoneal simvastatin support a histological reduction in inflammatory infiltrates in the lungs, an effect that may be linked to processes that involve vascular cell adhesion and interstitial cell adhesion molecules (173, 244). In line with these studies, Zeki, Bratt (245) demonstrated that intra-tracheal instillation of pravastatin, which is retained in the lung and does not accumulate significantly in plasma, is not sufficient to reduce BALF total leukocyte or eosinophil cell count in ovalbumin exposed mice. Collectively, our observations support a general concentration-dependent role for simvastatin hydroxy acid in the circulation to disrupt the influx and accumulation of inflammatory cells in the lung in response to allergen challenge.

Unpublished data from our group suggests that simvastatin also negatively impacts the abundance of prenyl transferase enzymes, responsible for prenylation of small G-proteins and

subsequent proinflammatory responses. Thus, it is possible that the prophylactic use of simvastatin pre-sets the system at a higher threshold for prenylation of small G-proteins, required to mount the inflammatory responses to the allergen insult, a clear benefit over the acute treatment. However, to unravel the exact mechanism we are currently pursuing a ‘multi-omics’ approach followed by horizontal data integration.

In line with previous reports using ovalbumin-challenged mice, we show that acute and prophylactic simvastatin treatment is sufficient to prevent goblet cell hyperplasia (173, 222, 246). This effect is likely attributable to our findings that simvastatin therapy inhibits eosinophil infiltration and associated release of mediators that promote mucous production (247).

To complement our studies comparing the magnitude of the effect of acute and prophylactic simvastatin therapy on lung inflammatory cell influx and goblet cell hyperplasia, we also used 10-plex technology to profile and quantify pro- and anti-inflammatory cytokines and chemokines in BALF. HDM challenge induced a complex inflammatory response, increasing pro-inflammatory cytokines that support eosinophilic and neutrophil inflammation, Th1- and Th2-like responses, as well as the anti-inflammatory cytokine, IL-10. This is attributable to the complex allergenic constituents of HDM and is consistent with HDM-associated human asthma (160, 242). As reported in prior studies using ovalbumin exposed mice (173, 209, 222, 235), we also observed a broad inhibitory effect of systemic simvastatin therapy on cytokine accumulation in the lungs. This was most evident 8 hours after HDM challenge, when a broader spectrum of cytokines and chemokines was observed, and was inhibited by both acute and prophylactic therapy: IFN γ , IL-12, IL-1 β , IL-6, and TNF α . We observed a statistically unique effect of prophylactic simvastatin therapy to prevent lung neutrophilia; this effect is mirrored by the inhibition of KC/Gro accumulation in BALF 8 and 48 hours after HDM

challenge. Similarly, pro-eosinophilic IL-5 and IL-4 in BALF, as well as lung eosinophil number, are inhibited by prophylactic simvastatin therapy 8 and 48 hours after allergen challenge, a pattern that appeared to be mirrored by acute treatment, though the trend for the latter was not validated by statistical analyses. Interestingly, accumulation 8 and 48 hours after HDM challenge of anti-inflammatory IL-10, as well as the Th2 cytokine IL-4, was prevented by prophylactic treatment, but acute simvastatin therapy only delayed their accumulation, as peak allergen-induced levels were still reached 48 hours after HDM challenge. This is inconsistent with some reports that simvastatin can up-regulate IL-10 in human subjects with chronic obstructive lung disease (248). A limitation of our experiments is that though we observed a suppression of lymphocyte influx with simvastatin therapy, our cytokine array did not include IL-17, which is down-regulated by statin treatment in ovalbumin challenged mice (209, 237). Measuring IL-17 abundance could shed light on the impact of therapy on Th17/Treg cell balance as a mechanism for Treg-associated suppression of Th2 cell-mediated inflammation. Overall our data indicate that in parallel with inhibitory effects on inflammatory cell influx, compared to acute simvastatin treatment, prophylactic therapy more effectively suppresses the breadth and magnitude of the cytokine and chemokine milieu that develops in the lung with repeated HDM challenge.

A principal goal of any asthma therapy is to improve lung function by mitigating the debilitating tendency for paroxysmal bronchial spasm associated with airway hyperresponsiveness. *In vivo*, preclinical studies by Zeki, Franzi (222) show that systemically delivered simvastatin tempers the increased respiratory resistance induced by ovalbumin exposure at baseline and in response to low concentration inhaled MCh. Using a chronic ovalbumin challenge model of airway remodelling, but with low levels of lung inflammatory cell

infiltrate, Ahmad, Mabalirajan (230) show that simvastatin treatment improves indices of lung and airway resistance. There is a general lack of concordance of such studies with clinical trials in asthmatics, which have not revealed any substantive improvement in airway function resulting from oral statin therapy. Indeed one study suggests statin treatment may lead to a decline in FEV1 (forced expiratory volume in 1 second) (249), whilst others show no effects of statin treatment on lung function (211, 227, 229, 250). One randomized controlled trial did report that in a subgroup of asthmatics who can tolerate withdrawal of inhaled corticosteroid therapy, oral simvastatin (40mg/day) showed a 150mL improvement in pre-bronchodilator FEV1 (210). For the current study, we used a repeat HDM challenge protocol to mimic the complex inflammatory and tissue remodelling microenvironment associated with human allergic asthma. Using this approach, we show that though acute and prophylactic systematic simvastatin treatment significantly diminish lung inflammation, they did not improve the indices of lung function such as R_N or H, but interestingly G was significantly improved by both acute and prophylactic simvastatin treatment. R_N is a measure of the constriction of central airways and accounts for 80% of total airway resistance; H represents alveolar tissue stiffness, whereas G is a measure of alveolar tissue constriction. Our data suggest that systemic simvastatin (both acute and prophylactic) relieves alveolar tissue constriction. However, the lungs remain stiff with the restricted central airways, which will lead to enhanced gas trapping and compromised lung function. Thus, systemic simvastatin treatment is not sufficient to prevent HDM challenge-induced decline in lung function. This is consistent with oral statin clinical trials in human asthmatics.

Our pharmacokinetic data suggest that the refractory nature of airway hyperreactivity to systemically administered simvastatin may be associated with the failure of its

pharmacologically active form to accumulate in the lungs, thereby preventing direct effects on the resident epithelium, smooth muscle and other structural cells. The absence of simvastatin hydroxy acid in the lungs may be due, in part, to its lipophilicity that likely favours deposition in lipid-rich organs, compromising already-low bioavailability <5% (243). Another mechanism for limiting the accumulation of simvastatin is the presence of the simvastatin metabolizing enzyme CYP3A11 in mouse lung (CYP3A4 in humans) (251-254). Recent evidence indicates that direct delivery of statins to the lung may be needed to reverse airway hyperreactivity and hypersensitivity (237, 245). Thus, our evidence indicates that systemic administration of simvastatin is insufficient to mitigate airway dysfunction, in spite of its capacity to prevent immune cell influx and release of pro-inflammatory cytokines.

In summary, using a murine model of allergic asthma that employs repeated challenge with the clinically relevant aeroallergen HDM, we demonstrate that systemic delivery of simvastatin is sufficient to inhibit both lung infiltration by inflammatory cells and release of Th1 and Th2 cytokines. Furthermore, a prophylactic treatment that establishes a steady state circulating concentration of simvastatin hydroxy acid prior to allergen challenge is more effective than commencing treatment at the onset of allergic insult. In a manner that parallels human clinical trials, we confirm that systemic delivery of simvastatin, acutely or prophylactically, is without impact on allergen challenge-induced airway hyperreactivity. Our findings support the need for developing approaches to deliver statins directly to the lung, perhaps in concert with systemic delivery, to assess the real-world potential of this therapeutic approach for asthma control.

Chapter 5: Intranasal delivery of simvastatin blunts HDM-induced airway inflammation and hyperresponsiveness in a murine model of allergic asthma

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Andrew Halayko: Guided, Supervised and facilitated the entire study

5.1 Background

Interest in the use of statins for the treatment of asthma was heightened in 2004 when McKay et al. first showed the anti-inflammatory properties of simvastatin in mice challenged with ovalbumin (173). Since then many preclinical studies have shown the potential of statin therapy to reduce various features of asthma including airway inflammation, smooth muscle hyperresponsiveness, airway hyperreactivity, and mucus overproduction in animals challenged with allergens (209, 222, 231, 233, 236, 244, 255, 256). However, most of these studies have used ovalbumin, a less physiologically relevant allergen compared to house dust mite (HDM) (257). Also, all preclinical studies have used very high, clinically incompatible doses of statins that would be well beyond the highest recommended daily dose in humans. For example, 40mg/kg simvastatin in a mouse is equivalent to 240mg for a man of 75kg weight, which is 2-fold higher than the maximum recommended dose of simvastatin (243, 258). Such a high dose of simvastatin is not compatible for clinical use, as the risk of rhabdomyolysis and liver enzyme dysfunction increases with increasing dose of simvastatin (243).

Perhaps unsurprisingly, clinical trials to date that use clinically relevant dosages of statins (10-40 mg/day) have not replicated the benefits observed in animal studies (210, 211, 213, 229). In line with these findings, we showed that in an acute model of HDM challenge in mice, systemic (subcutaneous) simvastatin had a modest impact on lung inflammation but did not rescue the decline in lung function. Notably, a stable level of pharmacologically active simvastatin was detected in serum but not in lung tissue or lavage fluid (219). This led to efforts in direct targeting of lungs with simvastatin via intranasal (i.n.) instillation, as a means to boost the presence of simvastatin locally and thus determine any effects on lung function. Previous attempts to deliver simvastatin through an inhaled route have had several flaws, including high

doses comparable to that used for systemic delivery in prior pre-clinical studies. Furthermore, the vehicle used for inhaled delivery included as high as 20% ethanol (v/v). Lastly, efficacy was not compared against standard asthma treatments such as inhaled corticosteroids (256, 259). These factors need to be addressed before considering clinical use of simvastatin for asthma therapy.

Here, we test the effectiveness of i.n. simvastatin at a dose of 6µg/kg, based on serum concentration of pharmacologically active simvastatin, as described in Chapter 4. This concentration of simvastatin is well within its aqueous solubility range (0.0122mg/mL) (260) and thus there is a residual ethanol content of only 0.35% in the final formulation. We compared the effects of i.n. simvastatin on airway inflammation, and hyperresponsiveness against relatively low (10µg/kg) and high (100µg/kg) doses of i.n. fluticasone that mimic therapies for mild-moderate and severe asthmatics, respectively. We hypothesized that directly targeting lungs with inhaled simvastatin will prevent allergen-induced airway inflammation and hyperresponsiveness in mice, to an extent comparable to inhaled fluticasone.

5.2 Methods

Female Balb/c mice were procured as explained previously in General Methodology section. The working stocks of simvastatin (1mg/mL in 100% ethanol) and fluticasone propionate (160µg/mL) were used to prepare drug formulations. The working stock of simvastatin was diluted using PBS, to obtain doses of 0.06, 0.6 and 6.0 µg/kg in 35µL saline. Fluticasone working stock was diluted with PBS to obtain doses of 10 and 100 µg/kg in 35µL saline.

HDM Challenge and Drug Treatment

Each Balb/c mouse was assigned an identity number (to be accessed by the study coordinator only) and was randomized based on body weight to different treatment groups to minimize dosing variability and to carry out all experiments in a blinded and bias-free manner. As a control, one group of mice received only HDM-acute-challenge for 2 weeks to induce airway inflammation and hyperreactivity. The remaining animals were divided into 5 groups that received HDM challenge concomitantly with different dosages of simvastatin or fluticasone. An age-matched allergen naïve control group (Naive) was also included in the study (Figure 5.1). At 48 h following the last HDM/PBS challenge, various physiological end points were assessed and include

- v) Assessment of simvastatin and simvastatin acid in BALF, lung tissue and serum
- vi) Lung function test
- vii) Assessment of inflammation in BALF: total and differential leukocyte counts, as well as cytokine array (MSD platform) and lung tissue histology using H&E staining
- viii) Lung tissue morphometry for goblet cell hyperplasia

All procedures were carried out as already described in Section General Methodology.

Statistical Analysis

Data are presented as mean values $\pm$ standard error of the mean (SEM). Means were compared by multivariate and Tukey multiple comparisons test, using SPSS_20 software (IBM Inc) and the representative graphs were created using Graph Pad Prism software 5.0. A p value <0.05 was interpreted to be statistically significant.

Figure 5.1

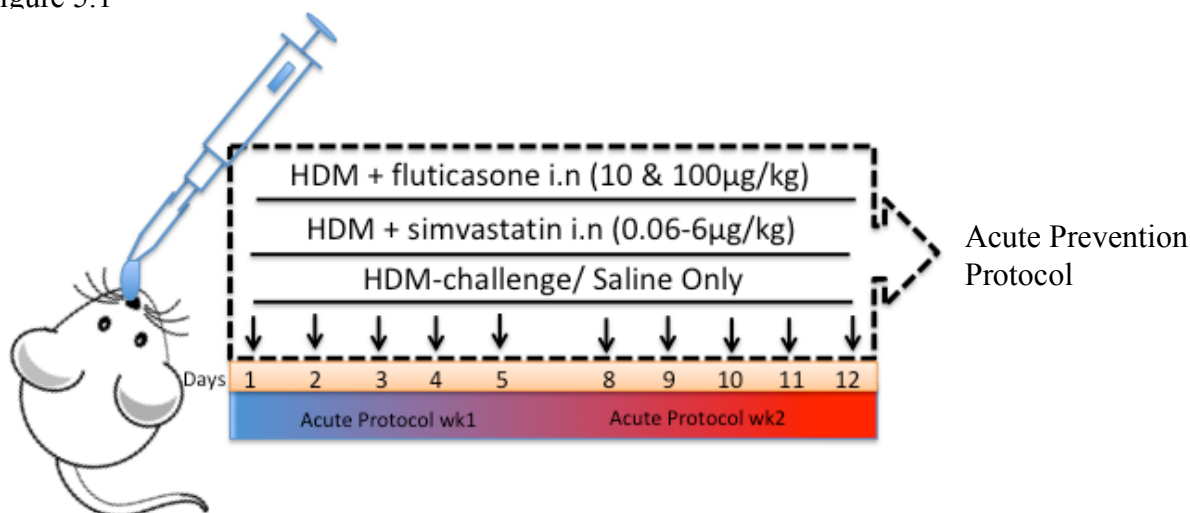


Figure 5.1: Schematic of acute HDM challenge with or without i.n. simvastatin or fluticasone treatment.

Acute murine model of allergic asthma with prevention and reversal treatment arms. For the prevention study, all animals received i.n. HDM challenge (25µg/day) for 2 weeks (5days/wk) either alone or concomitantly with i.n. simvastatin (0.06-6µg/kg) or fluticasone (10 & 100µg/kg). Age matched naïve (no HDM) control animals also received similar treatment.

5.3 Results

Quantification of simvastatin:

Forty-eight (48) hours following the last dose of simvastatin, we detected up to 40% of the initially administered amount of simvastatin (in the active form of simvastatin hydroxy acid) in the BALF obtained from the mice (Figure 5.2). However, neither simvastatin nor its active form were detectable in tissue homogenate or serum.

Figure 5.2

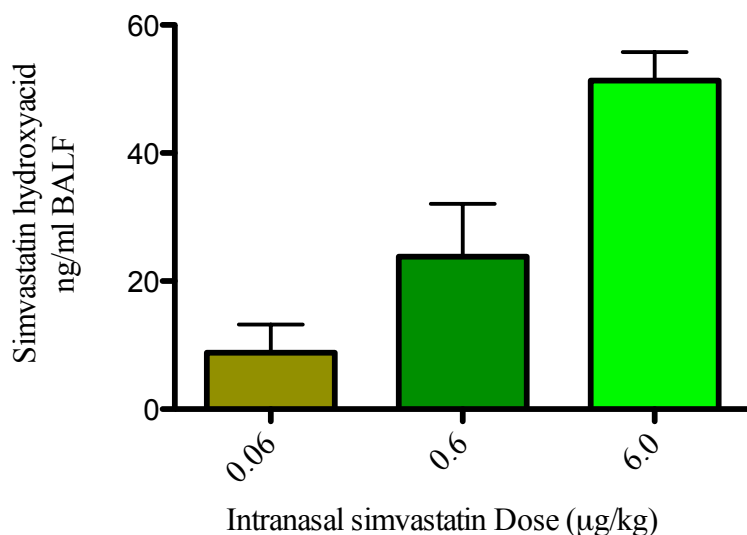


Figure 5.2: BALF accumulation of Simvastatin hydroxy acid.

Concentration of the active hydroxy acid form of simvastatin in BALF shows dose-dependent accumulation of an active form of simvastatin in BALF (n=3/group), measured by LC-MS/MS.

Intranasal simvastatin decreases lung inflammation

Consistent with our previous study (Chapter 4), we found that HDM challenge increased the influx of total cells in BALF by almost 140 fold (Figure 5.3A, $p < 0.001$), an effect

that included an increase in eosinophils and neutrophils over 300-fold ($p<0.001$). Macrophages and lymphocytes were elevated by 50- and 70-fold, respectively ($p<0.001$). As expected, fluticasone at both low and high doses blunted the allergen-induced influx of total inflammatory cells in the lungs by 53.1% ($p<0.01$) and 75.3% ($p<0.001$), including a significant reduction in eosinophils (Flut10: 46.9%, $p<0.05$; Flut100: 93.0%, $p<0.001$), neutrophils (Flut10: 41.1%, $p=0.054$; Flut100: 91.8%, $p<0.001$), macrophages (Flut10: 62.8%, $p<0.001$; Flut100: 62.0%, $p<0.01$) and lymphocytes (Flut10: 52.6%, $p<0.05$; Flut100: 67.7%, $p<0.001$; Figure 5.3B).

Intranasal simvastatin also blunted inflammation, measured by a decrease in total and differential cell counts in BALF from HDM challenged mice. The total influx of inflammatory cells was blunted by 45.5% ($p=0.4$), 43.4% ($p=0.3$) and 86.7% ($p<0.001$) by low (0.06 $\mu\text{g/kg}$), medium (0.6 $\mu\text{g/kg}$) and high (6.0 $\mu\text{g/kg}$) doses of simvastatin respectively (Figure 5.3A). A similar pattern was observed with differential cell counts, where simvastatin dose-dependently blunted the influx of eosinophils, neutrophils, macrophages and lymphocytes; however, as was the case for total cell count, only the 6.0 $\mu\text{g/kg}$ dose had a statistically significant impact on the influx of all inflammatory cells in the lungs, with decreases observed for eosinophils (80.8%, $p<0.01$), neutrophils (80.4%, $p<0.01$), macrophages (88.5%, $p<0.001$), and lymphocytes (91.3%, $p<0.001$) in BALF, compared to allergen challenge without simvastatin. At 0.6 $\mu\text{g/kg}$, simvastatin blunted the influx of lymphocytes by 68.1% ($p=0.035$), compared to allergen challenge alone, but the impact on the influx of other immune cells was statistically not significant at this dose. The lowest dose (0.06 $\mu\text{g/kg}$) did not have a significant impact on the influx of immune cells in the BALF compared to allergen challenge alone. High-dose i.n. simvastatin was significantly better than both low and medium dosages in blunting the influx of macrophage ($p\leq 0.05$) and lymphocytes ($p\leq 0.01$) (Figure 5.3A). The impact of i.n. simvastatin

(6 μ g/kg) on the allergen-induced influx of inflammatory cells in the lungs was comparable to the high dose fluticasone (100 μ g/kg), and it was better than the low dose fluticasone (10 μ g/kg). In fact, 6 μ g/kg simvastatin was significantly better than 10 μ g/kg fluticasone in blunting the influx of eosinophils and lymphocytes in the lungs with $p < 0.05$ and 0.01 , respectively (Figure 5.3B).

Figure 5.3.A

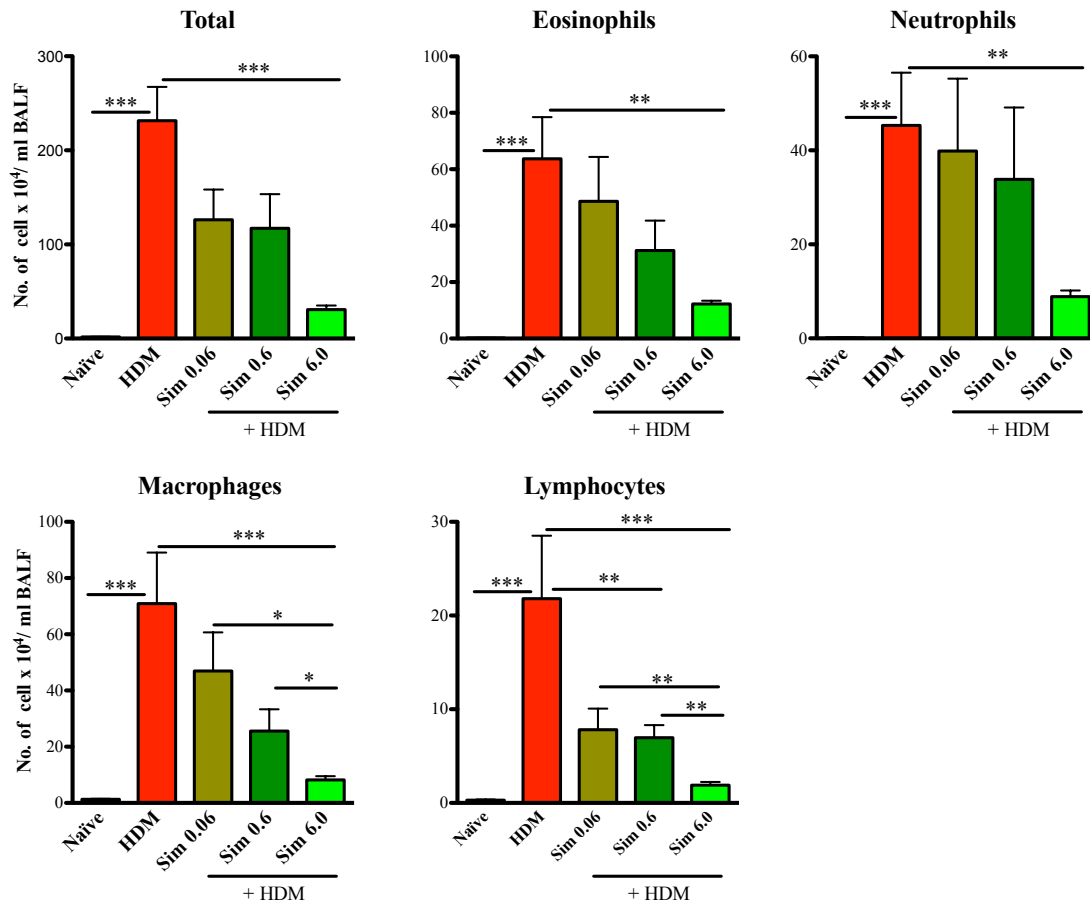


Figure 5.3.A: Impact of i.n. simvastatin on HDM induced influx of total leukocyte number and differential in BALF.

Measurements were taken 48 h after the final HDM challenge, with and without simvastatin treatment at 0.06, 0.6 and 6 μ g/kg. For all data, cell number is normalized to initial BALF volume and presented as mean $\pm$ SEM from 6 animals per group. Analysis was done using One Way ANOVA with Tukey multiple comparison test: *: $p \leq 0.05$, ** ≤ 0.01 and *** ≤ 0.001 (univariate analysis using SPSS20.0)

Figure 5.3.B

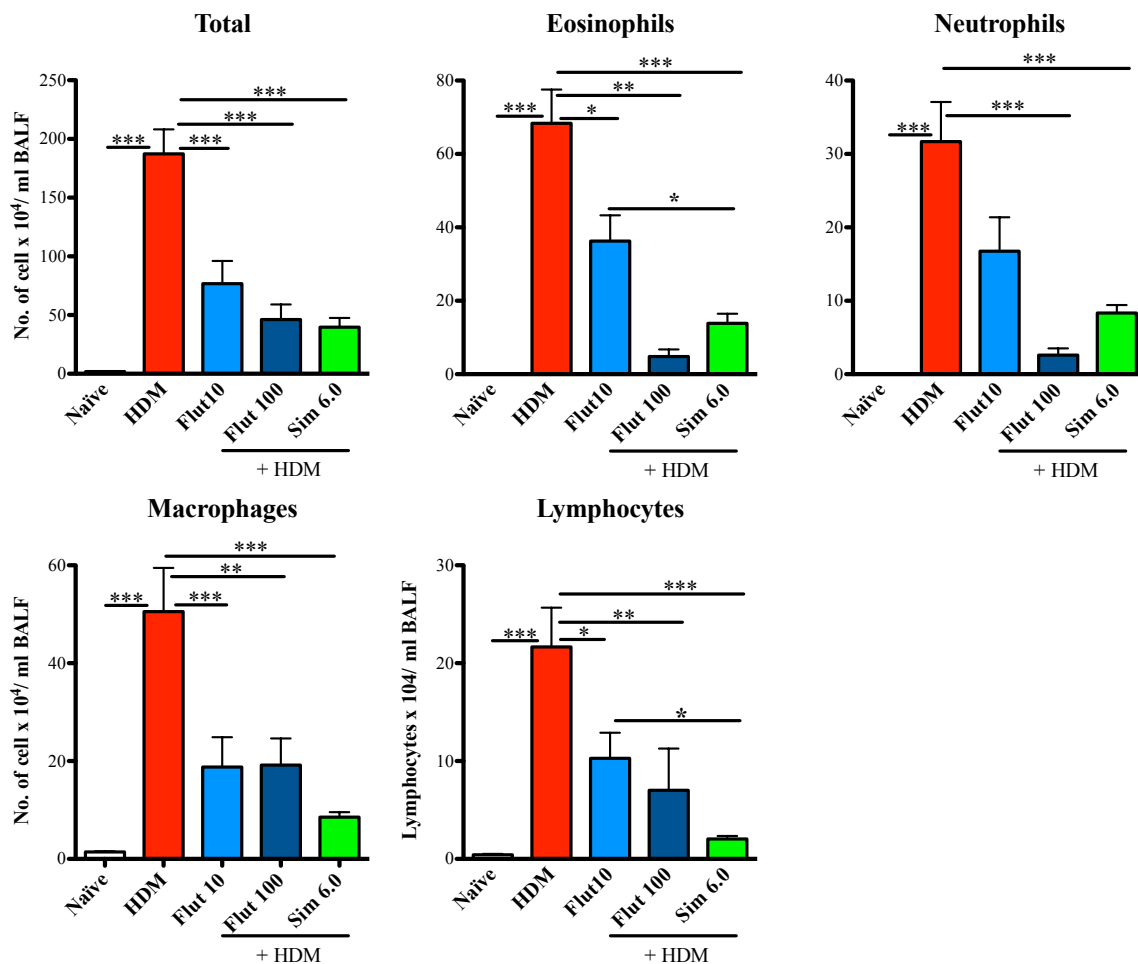


Figure 5.3.B: Impact of i.n. simvastatin in comparison to fluticasone on HDM induced influx of total leukocyte number and differential in BALF.

Measurements were taken 48 h after the final HDM challenge, with and without simvastatin or fluticasone treatment. For all data, cell number is normalized to initial BALF volume and presented as mean $\pm$ SEM from 6 animals per group. Analysis was done using one-way ANOVA with Tukey multiple comparisons test: *: $p \leq 0.05$, **: $p \leq 0.01$ and ***: $p \leq 0.001$ (univariate analysis using SPSS20.0)

Looking at the efficacy of the highest intranasal dose of simvastatin on airway inflammation, we evaluated its effect on the related cytokines, using an MSD multiplex cytokine array. We observed that HDM challenge significantly elevated IL-12 ($p < 0.001$), IL-4 ($p < 0.001$), IL-5 ($p < 0.01$), KC/Gro ($p < 0.001$), and TNF α ($p < 0.001$) compared to naïve controls (Figure 5.4).

Interestingly, HDM challenge also increased levels of the anti-inflammatory cytokine IL-10 ($p<0.001$). When compared to HDM-challenge alone, low dose fluticasone significantly blunted the surge in IL-4 ($p<0.01$) and IL-6 ($p<0.001$); however, other cytokines, including IL-5 and KC/Gro, were unaffected. The high dose of fluticasone significantly blunted the HDM-induced surge in IL-12 ($p<0.05$), IL-4, IL-6, and TNF α ($p<0.001$). Interestingly, high dose fluticasone did not significantly alter IL-5 in this model. We found that high dose fluticasone had a significantly greater impact on IL-12, IL-4, and TNF α in comparison to the lower dose. Treatment with 6 μ g/kg i.n. simvastatin blunted the HDM-induced levels of IL-12 ($p<0.001$), IL-4 ($p<0.001$), IL-5 ($p<0.05$), IL-6 ($p<0.001$), KC/Gro ($p<0.001$) and TNF α ($p<0.001$). Interestingly, inhaled simvastatin at 6 μ g/kg was significantly better than 10 μ g/kg fluticasone in reducing the HDM induced surge in IL-12 ($p<0.001$), IL-4 ($p<0.05$), KC/Gro ($p<0.001$) and TNF α ($p<0.001$). At the same time, the effect of 6 μ g/kg simvastatin was comparable to 100 μ g/kg fluticasone, except for IL-12, where simvastatin was significantly better than fluticasone.

Figure 5.4

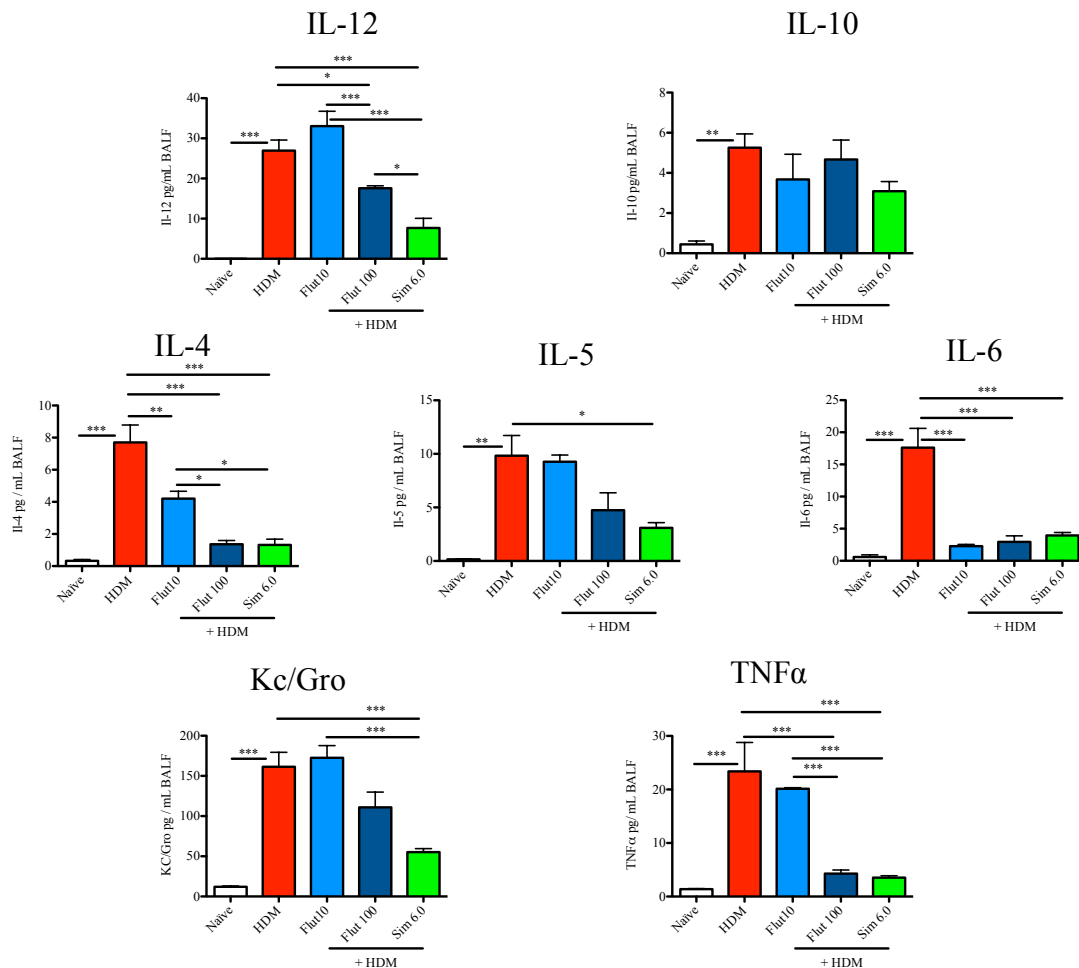


Figure 5.4: Impact of i.n. simvastatin in comparison to fluticasone on HDM induced accumulation of cytokines in BALF.

Cytokine abundance was determined using a 10-plex MSD array, 48 h after the final HDM challenge with and without simvastatin treatment. For all data, cytokine accumulation is normalized to initial BALF volume and presented as mean $\pm$ SEM from 6 animals per group. Analysis was done using one-way ANOVA with Tukey multiple comparisons test: *: $p \leq 0.05$, **: $p \leq 0.01$ and ***: $p \leq 0.001$ (multivariate analysis using SPSS20.0)

The H&E staining of the lung tissue sections shows qualitatively that HDM challenge significantly induced the influx of inflammatory cells in the tissue, as well as thickening of the epithelial cell layer. The two lower dosages of simvastatin had no

impact on HDM-induced lung tissue inflammation. The lower dose of fluticasone had moderate impact whereas 6 $\mu\text{g}/\text{kg}$ simvastatin and 100 $\mu\text{g}/\text{kg}$ fluticasone eradicated the tissue inflammation completely. Simvastatin dose dependently blunted the epithelial cell thickening; with 6 $\mu\text{g}/\text{kg}$ being the most effective dose, comparable to 100 $\mu\text{g}/\text{kg}$ fluticasone and better than 10 $\mu\text{g}/\text{kg}$ (Figure 5.5).

Figure 5.5

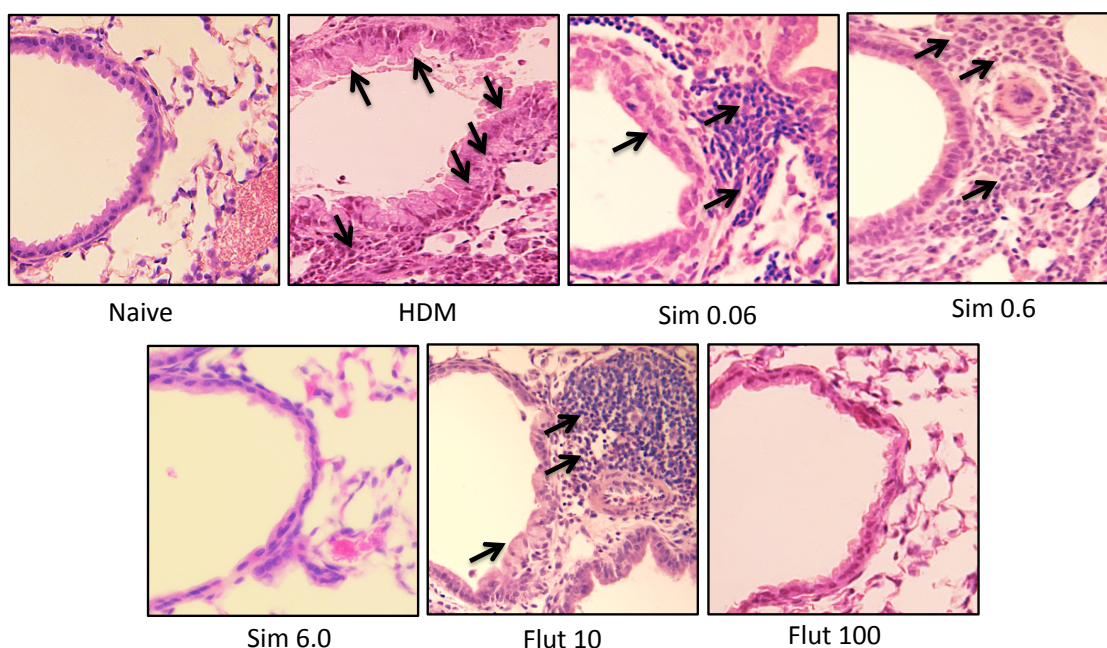


Figure 5.5: Impact of i.n. simvastatin on HDM induced tissue inflammation compared to fluticasone.

Images of typical H&E staining of tissue inflammation 48 h after the final HDM challenge, with and without simvastatin or fluticasone treatment (3 animals/group). Images were acquired using 200x magnification. Arrows indicate presence of inflammatory cells.

Intranasal simvastatin decreases goblet cell hyperplasia

HDM challenge induced goblet cell hyperplasia in mice (6.5 ± 1.2 cells/ μm of basement membrane) in comparison to the naïve ($p < 0.05$). Treatment with i.n. simvastatin inhibited this effect in a dose-dependent manner: goblet cells were reduced to 3.5 ± 0.3 ($p = 0.26$) and 0.7 ± 0.1 ($p < 0.05$) cells/ μm of basement membrane with 0.06 and 0.6 $\mu\text{g/kg}$ simvastatin, respectively. With 6.0 $\mu\text{g/kg}$ simvastatin, no goblet cells could be detected, similar to naïve controls (Figure 5.6.I&IIA). Fluticasone also improved the HDM-induced goblet cell hyperplasia in a dose-dependent manner: goblet cells were reduced to 1.97 ± 0.9 cells/ μm ($p < 0.05$) and 0.3 ± 0.1 cells/ μm ($p < 0.001$) of basement membrane with 10 $\mu\text{g/kg}$ and 100 $\mu\text{g/kg}$ fluticasone, respectively (Figure 5.6.IIB). The impact of 6.0 $\mu\text{g/kg}$ i.n. simvastatin was statistically similar to both doses of fluticasone.

Figure 5.6.I

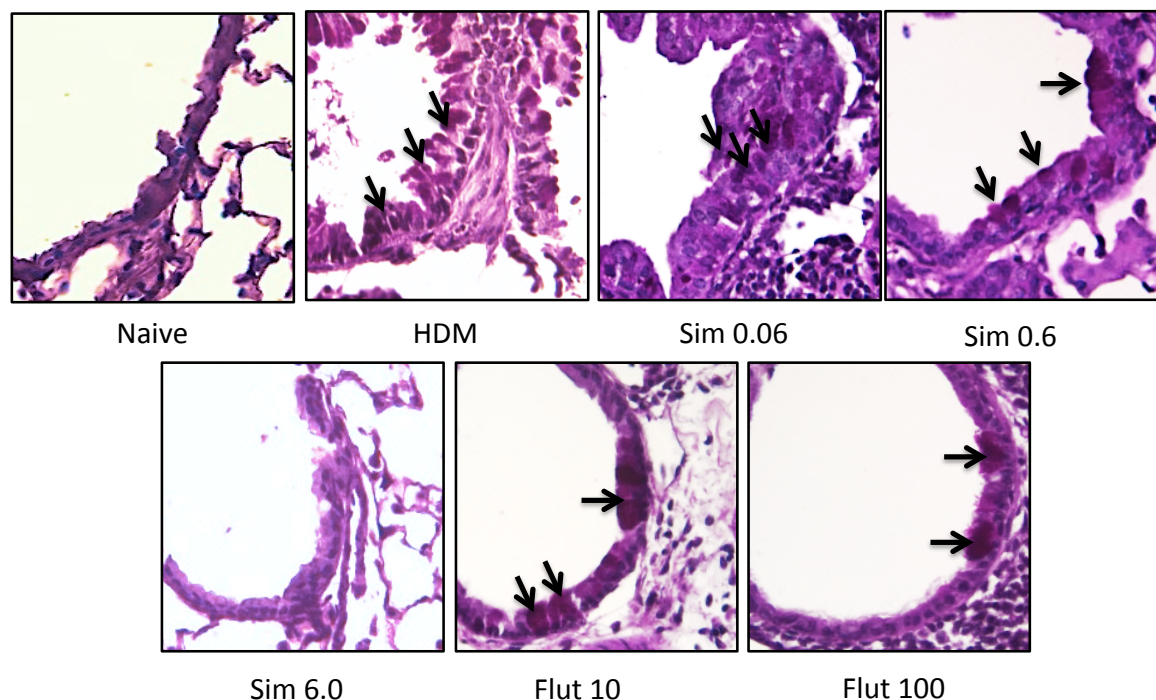


Figure 5.6.II

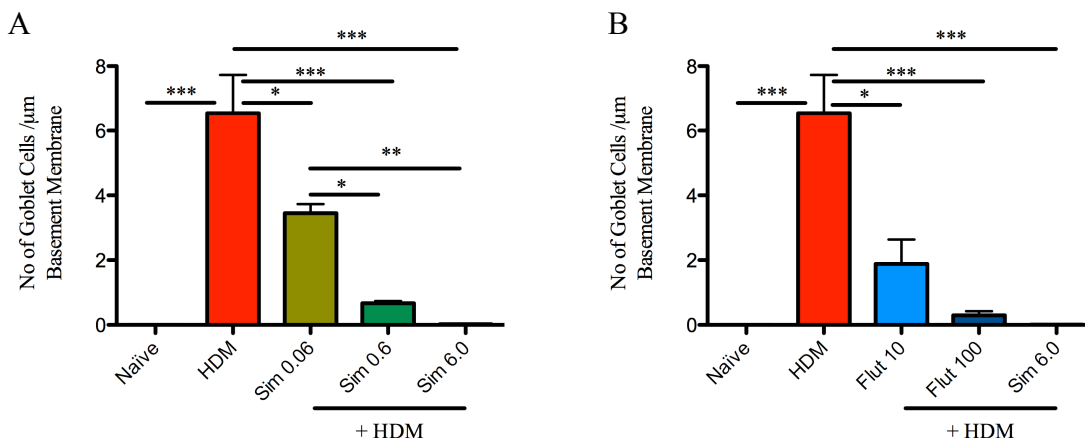


Figure 5.6 Impact of i.n. simvastatin on HDM induced goblet cell numbers compared to fluticasone.

I. Images of typical PAS staining of goblet cells in bronchioles 48h after completing HDM challenge, with and without simvastatin or fluticasone treatment. Images were acquired using 200x magnification. Arrows indicate presence of purple mucous staining.

II. Quantification of goblet cells using Image Pro software 48h after completing HDM challenge, with and without simvastatin or fluticasone treatment. Goblet cell number was determined in similar sized airways in each mouse (perimeter $161.1 \pm 28.5 \mu\text{m}$). Data represent mean $\pm$ SEM from 9 airways/animal (3 animals per group). Comparisons were done using one-way ANOVA with Tukey multiple comparison test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Intranasal simvastatin improves HDM-induced decline in lung function

HDM challenge significantly increased the constrictive response of airway to all doses of MCh. This included a two-fold increase in maximal R_N ($p < 0.001$) and an increase in MCh sensitivity, with a 6-fold decrease in the dose of MCh required to increase the R_N by 43.5% ($PC_{20} = 2.7 \pm 0.7 \text{ mg/ml}$; $p < 0.05$) compared to naïve controls ($PC_{20} = 17.4 \pm 4.6 \text{ mg/ml}$, Figure 5.7.I A & B). Interestingly, the two lower doses of simvastatin did not prevent the impact of HDM challenge on MCh PC_{20} or R_N ; however, 6 $\mu\text{g/kg}$ simvastatin significantly improved the HDM-induced increase in R_N ($p < 0.01$) and the sensitivity of the airway to MCh challenge, by lessening the decrease in PC_{20} to $11.2 \pm 2.0 \text{ mg/ml}$ ($p < 0.05$) compared to untreated, HDM-

challenged animals (PC_{20} : 2.7 ± 0.7 mg/ml, Figure 5.7.I B). A similar trend was observed with tissue resistance (G) and tissue elastance (H): HDM challenge resulted in a significant increase in G and H in response to MCh dose of 12, 25 and 50mg/ml ($p < 0.001$). The two lower dosages of simvastatin had no statistically significant benefit; however, 6 μ g/kg simvastatin dampened the HDM-induced increase in G and H at MCh dose of 25 and 50mg/ml ($p \leq 0.01$), and the overall effect at other dosages of MCh was significantly better than either 0.6 or 0.06 μ g/kg simvastatin ($p < 0.001$, Figure 5.7.I C & D).

Fluticasone at a low dose (10 μ g/kg) could not blunt the HDM-challenge induced increased R_N , G or H in response to graded dosages of MCh (Figure 5.6.II.A). Thus, unsurprisingly it did not improve MCh PC_{20} either (Figure 5.7.II.B). However, fluticasone at 100 μ g/kg significantly improved the HDM-induced rise in R_N at 25mg/mL ($p < 0.05$). At 50mg/mL MCh, statistical significance was lost although numerically R_N was improved. Fluticasone also suppressed the HDM-induced rise in G in response to MCh provocation at 25 ($p < 0.01$) and 50 mg/mL ($p < 0.001$, Figure 5.7.II.C). Similarly, it also improved the HDM induced surge in 'H' due to MCh challenge again at 25 ($p < 0.05$) and 50mg/mL dose ($p < 0.01$, Figure 5.7.II.D). In spite of this, PC_{20} was not improved in these mice. When the effects of 6.0 μ g/kg simvastatin, was compared to fluticasone, simvastatin outperformed 10 μ g/kg fluticasone significantly in all aspects of lung function assessed (R_N , G, H, and PC_{20}). The impact of simvastatin was comparable to 100 μ g/kg fluticasone.

Figure 5.7.I

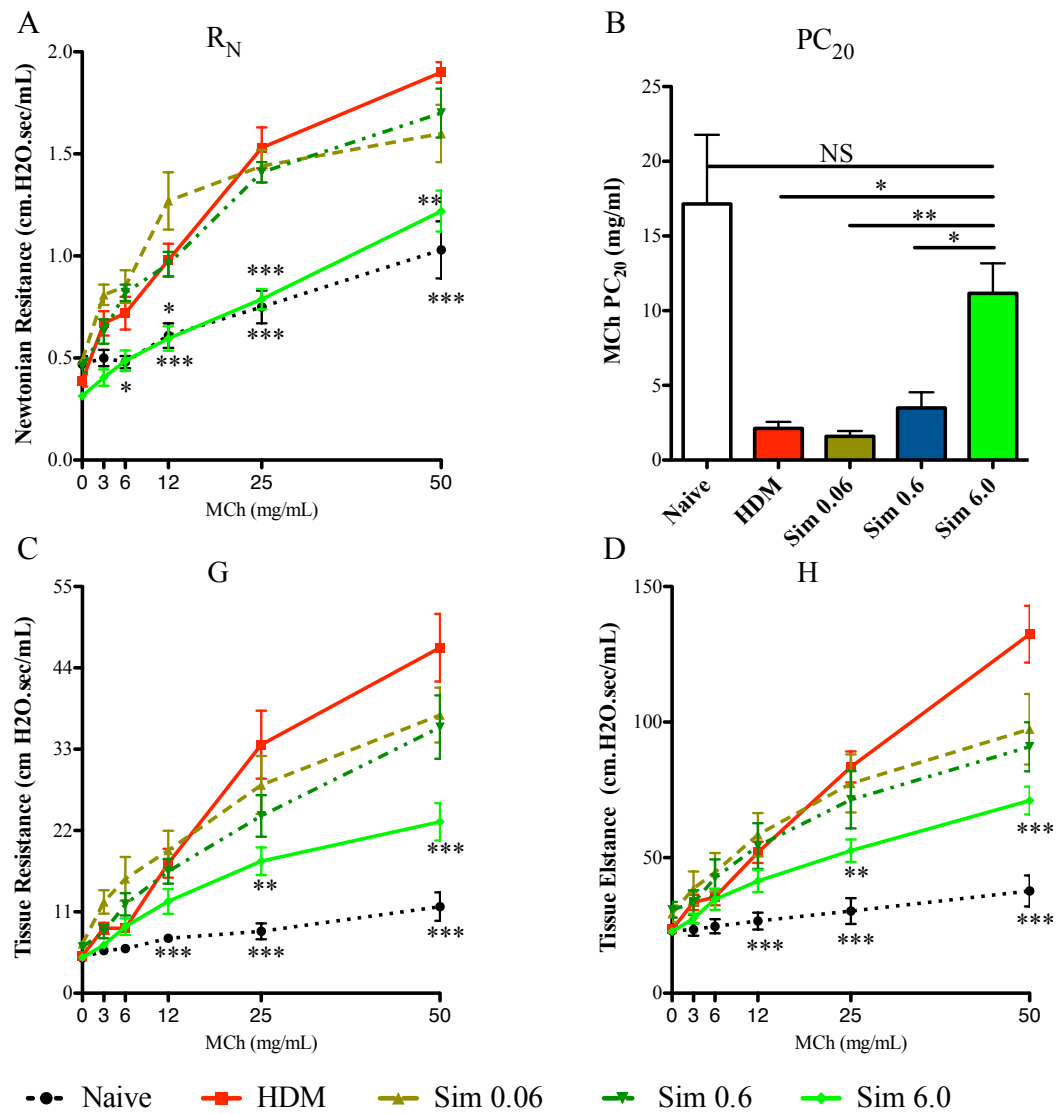


Figure 5.7.II

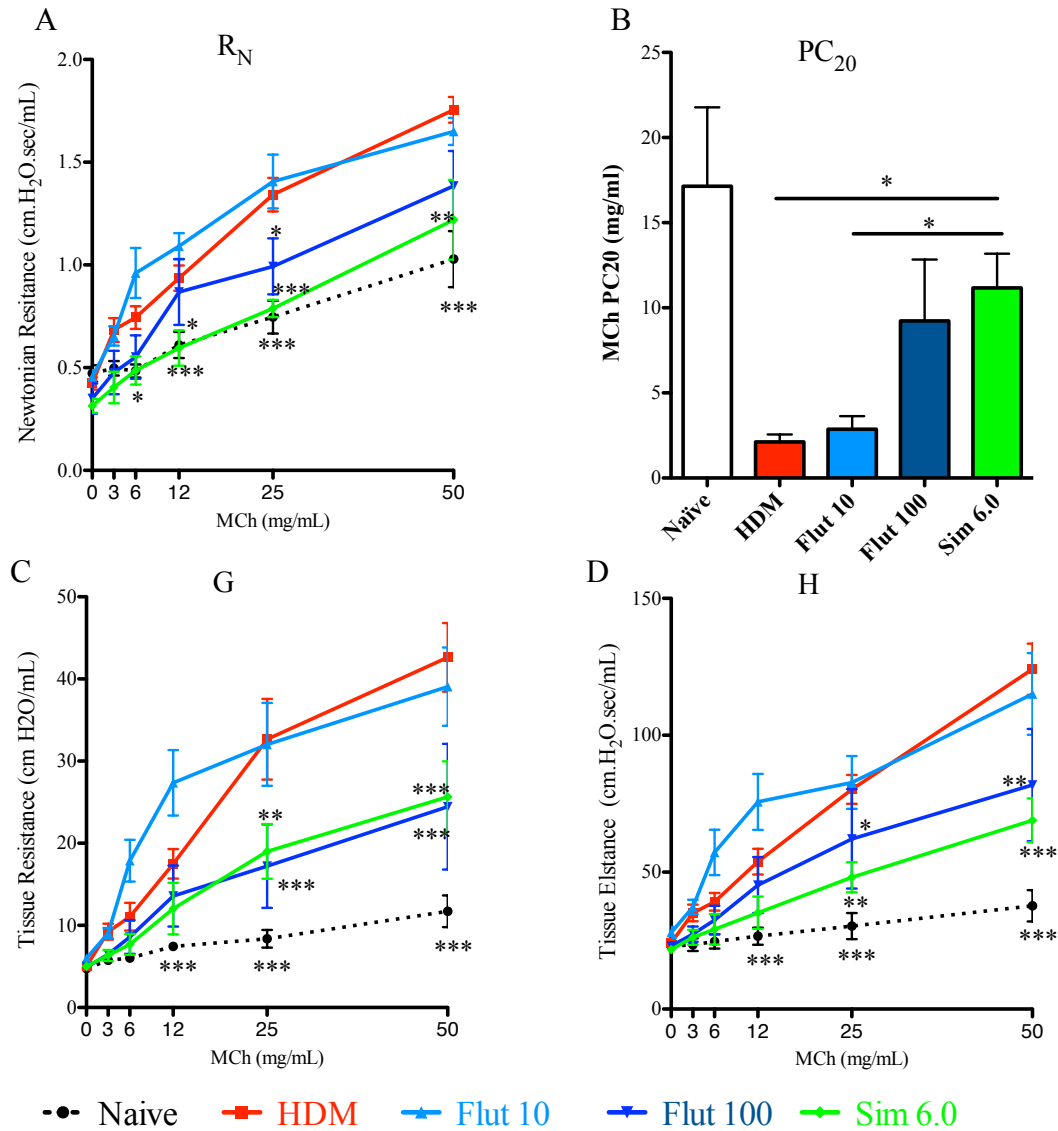


Figure 5.7: Impact of i.n simvastatin treatment on respiratory mechanics.

Lung function was assessed using a flexiVent small animal ventilator 48 h after the final HDM challenge, I) with and without increasing doses of simvastatin or II) with and without simvastatin or fluticasone treatment. A) Newtonian Resistance (R_N), B) Tissue Elastance (H) and C) Tissue Resistance (G) were measured in response to sequential challenge with nebulized saline (0) or MCh (3-50mg/mL). Data are presented as mean $\pm$ SEM from 6 animals per group. Comparisons of naïve and each treatment were performed against HDM challenge using Univariate analysis followed by Tukey multiple comparison test: * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ (multivariate analysis using SPSS20.0) compared against HDM challenge alone.

5.4 Discussion

The current study is an important step in bridging the existing knowledge gap between clinical and preclinical research regarding the use of simvastatin in asthma. In line with current knowledge from clinical trials, in the previous chapter we showed that systemically delivered simvastatin improved allergic airway inflammation but the decline in lung function remained unaffected. Surprisingly, no simvastatin could be detected in the lung after systemic delivery, which led to the change in route from systemic to intranasal, based on the serum concentration of simvastatin (125ng/ml) that previously showed optimum protection against allergen-induced lung inflammation. Thus, the selected dose was of the order of $1/7000^{\text{th}}$ of that used for systemic dosing in our previous study and that of other preclinical studies (173, 236, 255). In the current study, we were able to detect pharmacologically active simvastatin in BALF at a level that was up to 50% of the total amount of drug delivered intranasally. Even at such a low concentration, simvastatin blunted HDM-induced airway inflammation, goblet cell hyperplasia and most importantly, airway hyperresponsiveness to MCh challenge. We compared the impact of i.n. simvastatin against the gold-standard therapy for asthma, inhaled corticosteroid, and found that the beneficial effects of simvastatin on inflammation and lung function was equal to a high dose of fluticasone that would be required to treat severe asthma, and was superior to a lower dose of fluticasone used to treat mild asthma in humans (11).

Airway inflammation in asthma is marked by the presence of excessive total inflammatory cells, especially eosinophils (261, 262). However, in severe asthmatics, eosinophilic inflammation is also accompanied by the infiltration of neutrophils and complex sets of cytokines (262, 263). In our model, we showed that acute HDM challenge significantly increased the total influx of inflammatory cells in the BALF that includes an increase in eosinophils, neutrophils, macrophages as well as lymphocytes. In line with cellular infiltrates,

HDM also induced accumulation of proinflammatory cytokines of mixed Th1 and Th2 signatures, which is not surprising considering the complex constituents of HDM (160). For example, IL-12, the cytokine that switches naïve T-cells towards the Th1 phenotype (264), as well as IL-6 and TNF α , stayed significantly elevated even 48 h after the final HDM challenge. At the same time, Th2 defining cytokines (IL-4, IL-5, and CXCL1 (KC/Gro)) were also significantly elevated in the BALF of the HDM-challenged mice. IL-4 is a classic marker of the Th2 type immune phenotype. It promotes the differentiation, proliferation, and survival of Th2 lymphocytes and initiates the vicious cycle of Th2 mediated inflammation (265). IL-5 is another very important Th2 cytokine, which is chiefly responsible for production, recruitment, and survival of eosinophils (266, 267). Thus, HDM-induced BALF eosinophilia is strongly correlated to the elevated levels of IL-5 in BALF. CXCL1 is the murine equivalent of human IL-8, which is a pro-neutrophilic Th2 chemokine. Thus, elevated levels of BALF neutrophilia are consistent with HDM-challenge induced CXCL1 elevation. Interestingly, our model displays classic features of a severe or treatment-resistant asthma phenotype, with the presence of complex sets of Th1 and Th2 (especially IL-5 and 8) cytokines along with the presence of eosinophilia and neutrophilia (268-272).

We showed that although low dose fluticasone was able to decrease the influx of inflammatory cells to the BALF and lungs, it lacked any impact on cytokine accumulation except for IL-4 and IL-6. High dose fluticasone had a more comprehensive impact on the HDM-induced surge in inflammation with respect to both cellular infiltration and cytokine accumulation, further strengthening the notion that this acute HDM exposure represents a severe asthma phenotype in mice in the acute setting.

Simvastatin blunted the HDM-induced influx of inflammatory cells in the BALF as well as lungs. However, statistical significance was obtained with only the highest i.n. dose of 6

µg/kg. This dose of simvastatin was equal in effectiveness to 100µg/kg fluticasone. Thus, our results are consistent with other preclinical studies and clinical trials that supported the antiinflammatory benefits of simvastatin (173, 211, 212, 222, 244, 255), but this was achieved at a greatly reduced dose of simvastatin.

Since only the highest dose (6 µg/kg) of simvastatin had a significant impact on the parameters of lung inflammation, we chose to evaluate the effects of this dose only on HDM-induced cytokine accumulation. Interestingly, simvastatin completely obliterated the Th1-defining cytokine IL-12, at the same time significantly reducing the proinflammatory Th2-specific cytokines IL-4, 5 and CXCL1, as well as IL-6 and TNFα. The impact of 6 µg/kg simvastatin on cytokines was equivalent to 100µg/kg fluticasone, and significantly surpassed the impact of low dose fluticasone in all measures except for IL-4 and IL-6. The effect of simvastatin on cytokine accumulation is consistent with its ability to blunt the influx of inflammatory cells in the BALF.

We also showed that levels of the anti-inflammatory cytokine IL-10 were significantly elevated following HDM challenge. This is consistent with other preclinical studies using HDM challenge, but clinical data showed a decrease in Treg and IL-10 levels in human asthmatics (269, 273-275). Such a contrast could be explained by the chronic nature of the disease in humans, whereas short-term HDM challenge in animals elicits a very potent inflammatory response and elevation in the IL-10 could be a compensatory mechanism to control the outburst of acute inflammation (276). Thus, to mimic the IL-10 levels in human disease, a chronic model of HDM-challenge in mice would be more appropriate (277). To our surprise, neither treatment group affected the HDM-induced IL-10 levels. Apart from the impact of simvastatin on pro- and anti-inflammatory cytokines, its anti-inflammatory effect could also be attributed to its ability to inhibit chemotactic proteins such as vascular cell adhesion

molecules (VCAM) and interstitial cell adhesion molecules (ICAM) (173, 244). Simvastatin primarily affects lung inflammation by inhibiting the mevalonate cascade (222).

Histological evidence suggests that acute HDM challenge induced goblet cell hyperplasia an index of tissue remodelling. Treatment with simvastatin dose-dependently affects the goblet cell hyperplasia. However, even though the two lower dosages of simvastatin had little impact on the influx of the inflammatory cells in the lung tissue, they had significant impact on the goblet cell hyperplasia. Overall, simvastatin had a dose-dependent impact on goblet cell hyperplasia.

Fluticasone blunted the tissue inflammation and goblet cell hyperplasia dose-dependently. Interestingly, the impact of 6µg/kg simvastatin on these parameters was comparable to 100µg/kg fluticasone. These results indicate that intranasal simvastatin alone can prevent the worsening of asthma on par with fluticasone. Moreover, intranasal simvastatin may possess anti-remodelling properties, but this warrants further exploration. This is the first time that both anti-inflammatory and anti-remodelling benefits of simvastatin have been demonstrated at such a low concentration (200, 230, 233, 278).

A decline in lung function is one of the key clinical features of asthma, and any therapy aimed towards asthma must address this issue. Thus, we looked at the lung function in these mice using a Scireq small animal ventilator system that measures impedance to airflow, enabling calculation of Newtonian resistance (R_N), Tissue Damping (G) or Tissue Elastance (H) using a constant phase mathematical model (217). Since it is impossible to calculate the FEV₁ in animals, in order to generate more clinically relevant lung function in these mice we also calculated the dose of MCh required to increase baseline resistance by 43.5%, which is equivalent to a decrease in FEV₁ of 20% (PC₂₀) (218). We showed that HDM challenge significantly increased the maximal airway resistance to MCh and increased the sensitivity to

MCh by reducing the PC₂₀, thereby characterizing a hyperresponsive state. We also showed that HDM challenge increases G and H, a reflection of compromised lung elasticity, which could be due to ongoing inflammation, accumulation of fluid as well as remodelling (279, 280).

Interestingly, the most elusive effect of simvastatin in any clinical trial has been an improvement in lung function. Consistent with clinical trials, in the previous chapter we argued that in an acute model, systemically delivered simvastatin does not accumulate in its active form in the lungs, and as such has little impact on the HDM-induced decline in lung function. Conversely, i.n. simvastatin at 6µg/kg significantly dampened HDM-induced increases in R_N, G, and H in response to MCh challenge, associated with the prevention of reduction in MCh PC₂₀. Indeed, mice that received i.n. simvastatin during HDM challenge exhibited the same sensitivity to inhaled MCh as allergen-naïve controls. A limitation of this particular study is that it does not directly reveal a biological mechanism that underpins the effect of i.n. simvastatin.

Interestingly, i.n. simvastatin at 6µg/kg dose was superior to the high dose fluticasone (100µg/kg) in preventing the allergen induced decline in lung function. To our surprise, in our HDM-challenge model of allergic asthma, low dose fluticasone did not prevent the decline in any parameter of lung function induced by the HDM-challenge. The limited efficacy of low dose fluticasone may reflect that the HDM-challenge model we use mimics a phenotype for more severe asthma, although this warrants further investigation and is not the primary aim of the study.

In conclusion, this study is unique in its capacity to show that i.n. simvastatin at a clinically relevant low dose had equivalent or better impact in preventing allergen-induced lung inflammation, remodelling and hyperresponsiveness. Thus, we consider this study as a stepping-stone for further evaluation of i.n. simvastatin as a means to reverse the established features of asthma. This is important for clinical relevance, as patients are not diagnosed with asthma until

after the disease has been established. On a cautionary note, although mice provide an excellent model to study human disease, their physiology is very different from humans, a major reason for the failure of drugs in clinical trials (151). Although it is important not to extrapolate the success directly from an animal model in to a clinical setting, the results of the current study are promising enough to move to the next step: to evaluate if simvastatin can reverse the established features of the disease in a murine model of allergic asthma, and to explore a likely mechanism for its efficacy.

Chapter 6: Allergen challenge induces peroxidation of phosphatidylcholine in murine and human asthmatics lungs, an effect mitigated by intranasal simvastatin.

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6.1 Background

Phosphatidylcholines (PCs) are the major phospholipid components of the plasma membrane in mammals (40-50%), more so for lung surfactant (80%) (21, 22), and consist of a polar choline head and two nonpolar lipid tails. PCs in mammalian plasma membrane typically have a saturated lipid chain attached to the choline head at the S_N1 position, and a polyunsaturated fatty acid (PUFA) chain attached to the choline head at the S_N2 position (22). However, lung surfactant PCs are different from those in the plasma membrane in that they include a di-saturated molecule known as dipalmitoylphosphatidylcholine (DPPC), or 16:0/16:0-PC (where 16 indicates the number of carbon atoms in the fatty acid chain, and 0 indicates the number of double bonds). Other PCs present in lung surfactant may be unsaturated, such as 16:0/16:1-PC, 16:0/18:1-PC and 16:0/18:2-PC (21). The bulk of DPPC in the lungs is generated by the conversion of unsaturated fatty acid chains at the S_N2 position by various enzymes, involving hydrolysis and reacylation (281, 282). Importantly, PCs with unsaturated fatty acids are essential for maintaining both a healthy plasma membrane and healthy surfactant.

PCs are susceptible to oxidative stress, because the unsaturated bonds within their fatty acid chains undergo β -oxidation to form oxidized phosphatidylcholine (OxPCs). During oxidation, the unsaturated FA acid chain at the S_N2 position either changes in conformation, forming unfragmented OxPCs, or gets truncated to produce fragmented OxPCs. Both forms are highly bioactive and have a strong correlation with pathogenesis of diseases (221) including ischemia (283, 284), multiple sclerosis (285) and acute lung injury (286). Since oxidative stress is a key pathological feature of asthma (20), it would be of interest to know the role of these highly bioactive molecules in the pathophysiology of asthma. The first step would be to establish their distinct signature in animals and human asthmatics challenged with allergen.

Intriguingly, one of the pleiotropic benefits of statin therapy includes antioxidant effects, in part via induction of the heme-oxygenase (HO)-1 gene (202). In the previous chapter, we showed that the intranasal (i.n.) simvastatin could blunt the allergen-induced lung inflammation and a decline in lung function in mice, an effect that was more comprehensive than fluticasone treatment. Because current asthma therapies do not target oxidative stress specifically, it would be of interest to evaluate the potential of simvastatin to mitigate the OxPC biosignature.

To begin to decipher the mechanism of action of simvastatin delivered directly to the lungs, here we measure the signature of OxPCs in our animal model of allergen-induced asthma, as well as in human asthmatics. Next, we investigate the impact of simvastatin and fluticasone on HDM-induced accumulation in OxPCs in the lung, to gain insight into potential differences in the impact of these two drugs in our murine model of asthma. Finally, we compare OxPCs observed in our murine model of allergic asthma to those in human asthmatics to validate clinical relevance. We hypothesize that the allergen challenge both in our murine model as well as in atopic asthma in humans, induces the accumulation of OxPCs in the lungs, and that simvastatin and fluticasone have a differential impact on the allergen-induced accumulation of OxPCs.

6.2 Methods

Animal Experiments:

Female Balb/c mice were procured as explained previously in section General Methodology. Drug formulations were generated from working stocks of simvastatin (1 mg/mL in 100% ethanol) and fluticasone propionate (160 µg/mL). The working stock of simvastatin was diluted using PBS to a final dose of 6.0 µg/kg in 35µL saline. The fluticasone working stock was diluted with PBS to obtain a final dose of 10 µg/kg in 35µL saline.

HDM Challenge and Drug Treatment

Balb/c mice were assigned identity numbers (accessible only to the study coordinator) and randomized to a total of 4 groups by body weight (to minimize dosing variability and to ensure that all experiments could be carried out in a blinded and bias-free manner). As a control, one group of mice received only HDM, acute challenge for 2-wks, as described in Section General Methodology, to induce airway inflammation and hyperreactivity. Treatment groups received 6.0 µg/kg simvastatin or 10 µg/kg fluticasone, concurrent with HDM challenge. The fourth group was an age matched allergen-naïve groups (Figure 6.1). Forty-eight hours after the last HDM/ PBS challenge, animals were subjected to lung function test, and BALF was collected for assessment of inflammation as described in Section General Methodology. In this study, 0.1% (w/v) EDTA was added to the saline used for BALF extraction, to prevent further oxidation of OxPCs. OxPCs were measured in 100µL aliquots of BALF supernatant.

All other procedures were carried out as already described in Section General Methodology.

Figure 6.1

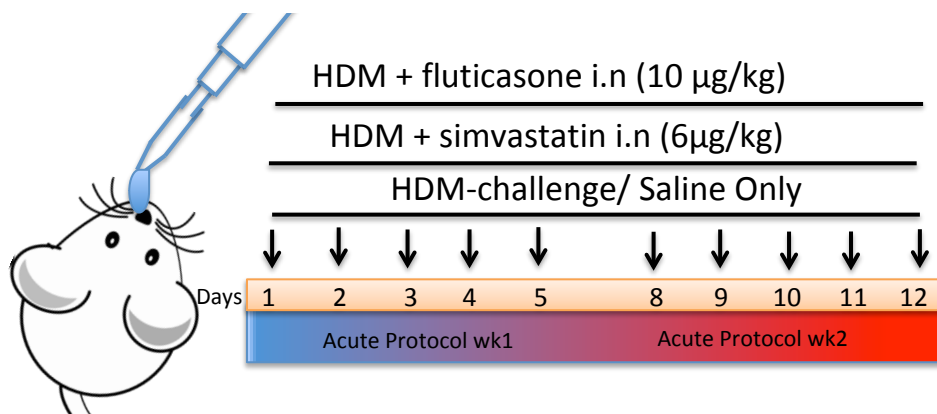


Figure 6.1: Schematic of acute HDM challenge with or without i.n. simvastatin or fluticasone treatment.

Acute murine model of allergic asthma with prevention and reversal treatment arms. For the prevention study, all animals received i.n. HDM challenge (25µg/day) for 2 weeks (5days/wk) either alone or concomitantly with i.n. simvastatin (0.06-6µg/kg) or fluticasone (10 & 100µg/kg). Age matched naïve (no HDM) control animals also received similar treatment.

OxPC estimation in Human Subjects:

Ten mild asthmatics with demographics shown in Table 6.1 received segmental challenge with either diluent or an allergen, to which they were sensitive (determined by the skin prick test), on separate days (at least one month apart). The asthmatics selected for the study were not taking corticosteroids or any other regular medication for asthma (or any other medical condition), apart from the occasional use of β_2 -agonist. After 24h, lung lavage was collected from the same segment of the lung where the challenge was performed and the lavage was subjected to OxPC analysis as described previously.

Table 6.1: Demographic details of Human Asthmatics

Parameter	N (Mean $\pm$ SEM)
N	10
Age	34 $\pm$ 5.1
Male	7
Female	3
FEV1 (%Predicted) pre-challenge	92.5 $\pm$ 2.5
Methacholine PC20 (mg/ml)	4.6 $\pm$ 1.2
EAR Diluent (Δ FEV1)	-2.5 $\pm$ 1.1
LAR Diluent (Δ FEV1)	-2.9 $\pm$ 1
EAR Allergen (Δ FEV1)	-38.5 $\pm$ 2.7
LAR Allergen (Δ FEV1)	-23.0 $\pm$ 3.3

Intranasal OxPAPC challenge

Commercial PAPC was oxidized in room air for 72 h at room temperature and then reconstituted in a 2:1 chloroform:methanol mixture at a concentration of 1 mg/mL. Quality control was performed to assess the extent of oxidation and presence of oxidized species, using LC-MS/MS analysis. Post quality control, Balb/c mice (female; 7-8 weeks old) received 400ng of OxPC (20 µg/kg) per day dissolved in saline over a period of 2 weeks (5 days/wk), similar to acute HDM challenge.

Statistical Analysis

Data are presented as mean values $\pm$ standard error mean (SEM). Means were compared by univariate analysis and the Tukey multiple comparisons test, using SPSS_20 software (IBM Inc), and the representative graphs were created using Graph Pad Prism software 5.0. A p value <0.05 was taken to be statistically significant among the mean values. We also used unsupervised (principal component analysis/PCA) and supervised (partial least square discriminant analysis/PLSDA) multivariate analysis to understand the signatures of OxPCs associated with different treatment groups; this was further illustrated by using the agglomerative hierarchical clustering on a heat-map for the expression of different OxPCs within a treatment group. Within PLSDA we also calculated the “Variable Importance in Projection” (VIP) scores for OxPCs to elucidate how well OxPCs define a treatment group. For this, we used the statistical packages R, XLSTAT, and Metaboanalyst 3.0. The cell count data from OxPC challenge was analyzed using the student T-test.

6.3 Results

Airway inflammation:

As expected, HDM challenge in mice induced a robust inflammation in the lungs, including a significant increase in the influx of total inflammatory cells $[(223.1 \pm 32.6) \times 10^4; p < 0.001]$ in comparison to naïve animals $[(1.9 \pm 0.2) \times 10^4]$. The surge in total inflammatory cells included a significant increase in eosinophils $[(63.6 \pm 4.5) \times 10^4; p < 0.001]$, neutrophils $[(43.4 \pm 15.2) \times 10^4; p < 0.001]$, macrophages $[(48.7 \pm 10.7) \times 10^4; p < 0.001]$ and lymphocytes $[(38.6 \pm 12.0) \times 10^4; p < 0.001]$ (Figure 6.2). Both fluticasone and simvastatin significantly blunted the HDM-induced influx of total inflammatory cells in the lungs by 73.9% ($p < 0.01$) and 85.4% ($p < 0.001$), respectively. Fluticasone also blunted influx in eosinophils (64.6%, $p < 0.05$), macrophages (86.1%, $P < 0.001$) and lymphocyte (83.7%; $P < 0.01$) (Figure 6.2). Fluticasone decreased the neutrophil influx by 62.2% but the impact was not statistically significant compared to the HDM-challenge only group ($p = 0.085$). In contrast, simvastatin significantly blunted HDM-induced influx in eosinophils (82.0%, $p < 0.001$), macrophages (85.6%, $p < 0.001$), lymphocytes (94.3%, $p < 0.001$), as well as neutrophils (77.4%, $p < 0.05$). Although mean values suggest simvastatin had a greater impact than low dose fluticasone, these differences were not statistically significant.

Figure 6.2

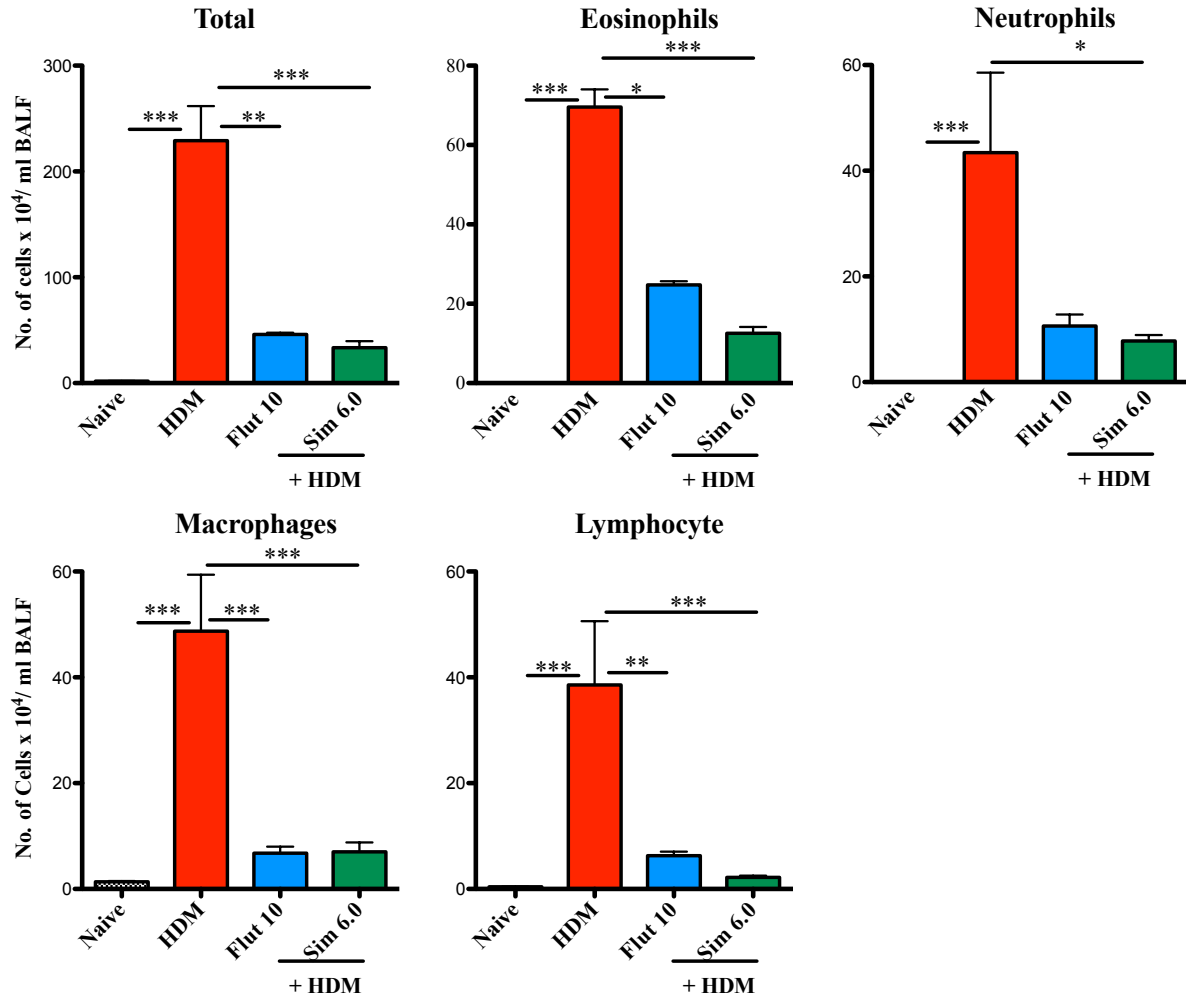


Figure 6.2: Impact of i.n. simvastatin compared to fluticasone on HDM- induced inflammatory cells in BALF.

Measurements were taken 48 h after the final HDM challenge. For all data, cell number was normalized to initial BALF volume and presented as mean $\pm$ SEM from 6 animals per group. Analysis was done using one-way ANOVA with Tukey multiple comparisons test: *: $p \leq 0.05$, ** ≤ 0.01 and *** ≤ 0.001 (univariate analysis using SPSS20.0)

Lung Function:

As expected, airways of HDM-challenged mice showed a significant increase in Newtonian resistance (R_N , Figure 6.3A), Tissue resistance (G, Figure 6.3B), and Tissue elastance (H, Figure 6.3C), in response to MCh challenge at doses of 12 ($p<0.001$), 25 ($p<0.001$) and 50 mg/mL ($p<0.001$). No impact of 10 μ g/kg fluticasone was observed on any parameters of lung function. However, 6.0 μ g/kg simvastatin significantly reduced the HDM-induced rise in R_N at MCh doses of 12 ($p<0.05$), 25 ($p<0.01$) and 50mg/mL ($p<0.001$). Simvastatin also blunted the HDM-induced rise in G and H at 25 ($p<0.01$) and 50mg/mL MCh ($p<0.001$). Overall, lung function in the simvastatin treatment group was similar to naïve controls, and significantly better than animals challenged with HDM with or without 10 μ g/kg fluticasone treatment ($p<0.001$).

Figure 6.3A

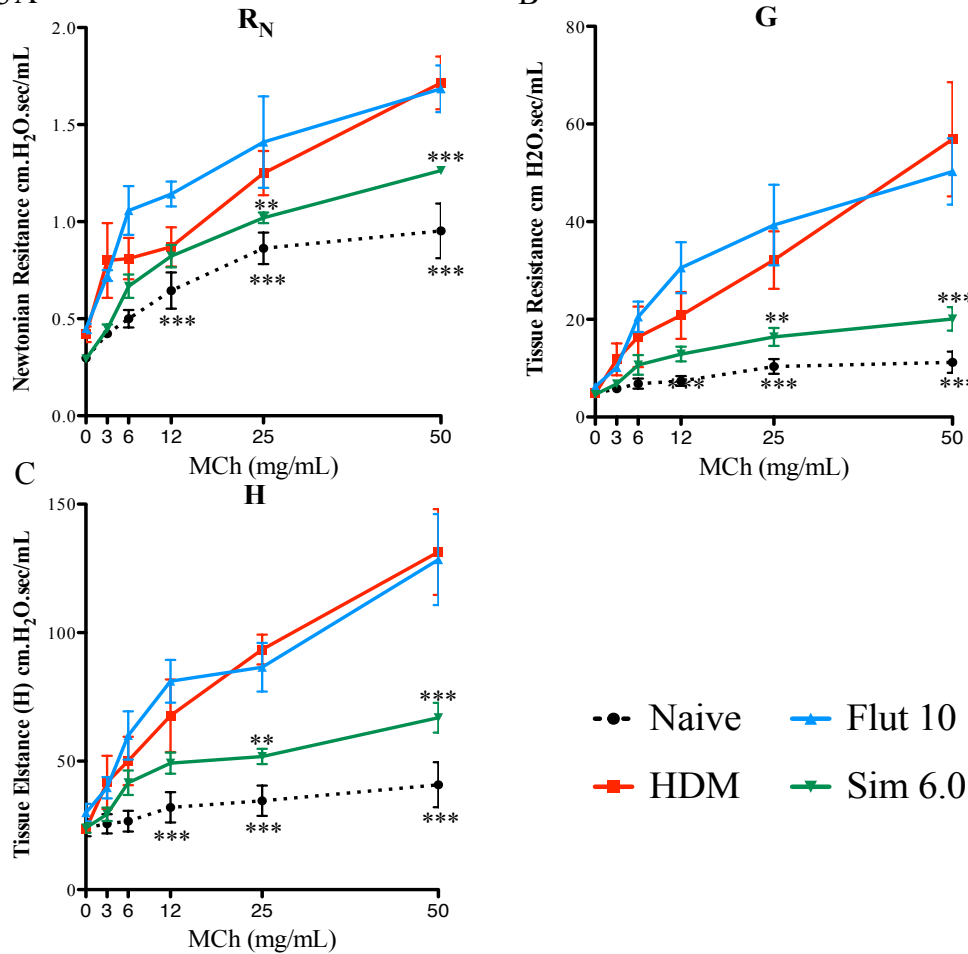


Figure 6.3: Impact of i.n. simvastatin treatment on respiratory mechanics.

Lung function was assessed using a flexiVent small animal ventilator 48 h after the final HDM challenge with and without simvastatin or fluticasone treatment. Naïve controls received PBS in lieu of HDM or drug treatment. A) Newtonian Resistance (R_N), B) Tissue Resistance (G) and C) Tissue Elastance (H) were measured in response to sequential challenge with nebulized saline (0) or MCh (3-50mg/mL). Data are presented as mean $\pm$ SEM from 6 animals per group. Comparisons of naïve and each treatment were performed against HDM challenge using Univariate analysis followed by the Tukey multiple comparisons test: * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ (multivariate analysis using SPSS20.0), compared to HDM challenge alone.

Estimation of OxPCs in mouse BALF:

The ‘multiple reactions monitoring’ (MRM) technique we employed had the capacity to quantify 85 oxidized products of PCs (OxPCs). Of these, we were able to detect 33 OxPCs in both HDM challenged and Naïve control groups. HDM challenge induced a 6-fold increase in the accumulation of total OxPCs in BALF. Both fluticasone and simvastatin treatment significantly inhibited the accumulation of OxPCs by $81.3 \pm 1.0\%$ ($p < 0.001$) and $94.2 \pm 0.4\%$ ($p < 0.001$), respectively. In fact, simvastatin was significantly more effective than fluticasone in blunting the accumulation of OxPCs in BALF ($p < 0.05$) (Figure 6.4).

Figure 6.4

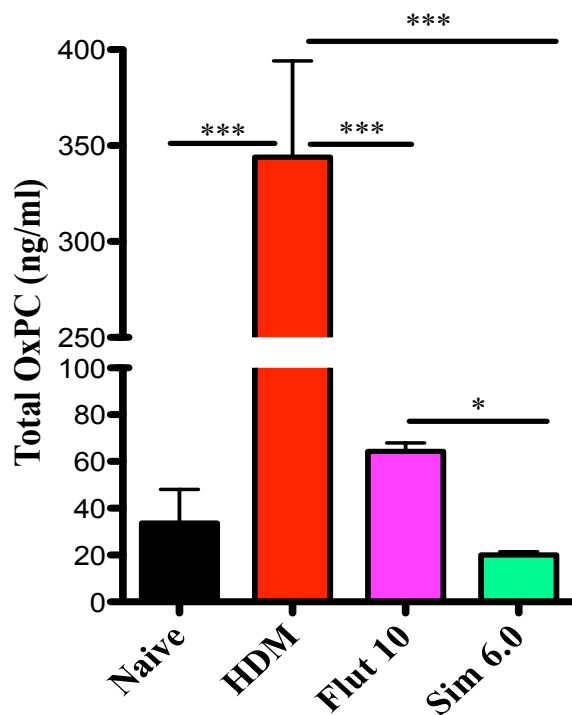


Figure 6.4: Impact of HDM challenge and fluticasone or simvastatin treatment (i.n.) on total OxPC accumulation in BALF.

OxPCs were measured by LC-MS/MS 48 h after the final HDM challenge, with or without simvastatin or fluticasone treatment. The total OxPC quantity was normalized to the initial BALF volume and is presented as mean $\pm$ SEM from 5 animals per group. * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ (multivariate analysis using SPSS20.0), compared against HDM-challenge only group. Legend: Naïve = Allergen naïve, HDM = HDM-only challenge, Flut 10 = HDM + Fluticasone (10 μ g/kg), Sim 6.0 = HDM + Simvastatin (6 μ g/kg).

The correlation of allergen challenge with accumulation of specific OxPC species, was evidenced by principal component analysis (PCA), where 29 of 33 OxPCs detected in BALF strongly correlated with HDM challenge (Figure 6.5). Naïve controls exhibited a strong negative correlation with accumulation of total OxPCs in the BALF. To assess the impact of different treatments on OxPC profile in BALF in comparison to the naïve or HDM-challenged only animals, we used supervised multivariate analysis- ‘partial least square discriminant analysis (PLS-DA) (Figure 6.6 and 6.7). Both fluticasone and simvastatin treatment groups clustered close to naïve controls. Although the probability ellipse for the naïve group overlapped with the probability ellipse for fluticasone treated mice, we surmised that this is most likely due to the presence of a possible outlier among the allergen-naïve animals. Simvastatin treated animals formed a very tight cluster close to most naïve animals.

This was confirmed in a heat map showing the differential impact of allergen challenge and treatment groups on individual OxPC species (Figure 6.8). In this regard, agglomerative hierarchical clustering analysis, shown above the top row of the heat map, reveals that simvastatin treatment is more closely associated with naïve controls, compared to fluticasone treatment. Importantly, naïve controls as well as both treatment groups formed clusters distinct from HDM-challenged controls.

To further investigate differences between simvastatin and fluticasone treatment groups, we calculated the Variance Importance in Projection (VIP) score within the PLS-DA algorithm using the Metaboanalyst 3.0 online tool (Figure 6.7). VIP scores reveal that accumulation of KOHA-PC, SLPC-OH, KOOA-PC, IsoPG(A2,J2)-SPC, KODA-PC, PONPC, 10-oxo-6,8-decedienoyl-PPC, SAPC-OOH, diketo and PLPC-OOH.OH strongly correlate to HDM challenge. All these OxPCs remained elevated with fluticasone treatment, which was not the case for naïve controls or simvastatin treatment. Indeed, the accumulation of HODA-PC, 10-

OH-5,8,11-tridecatrienoyl-PC (SAPC), 10-OH-5,8,11-tridecatrienoyl-PC(PAPC), KDiA-PC and SAPC-OH were most strongly associated with fluticasone treatment. Notably, fluticasone treatment was positively correlated to the HODA-PC and KDiA-PC expression, even more so than for HDM-challenge alone. The OxPCs most significantly associated with between-group differences were invariably lower in simvastatin treatment group. Interestingly, simvastatin treatment was also uniquely associated with increased abundance of PLPC-epoxy,keto, which was present in low abundance in naïve animals, and unaffected by HDM challenge, or fluticasone.

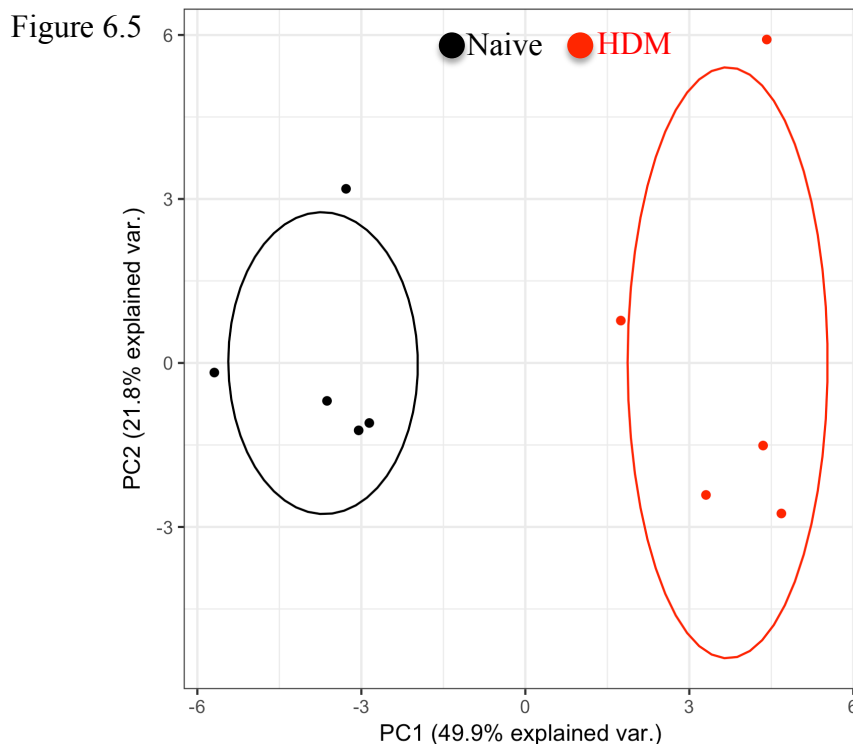


Figure 6.5: OxPC profile in BALF of mice with or without HDM challenge.

Principal Component Analysis (PCA; unsupervised multivariate analysis) of lipidomics data in BALF from naïve and HDM challenged mice showed distinct cluster of HDM-challenged animals (red: probability ellipse, dots represent individual animals) from the allergen-naïve animals (black: probability ellipse, dots represent individual animals) ($n = 5/\text{group}$). Probability ellipse defines 95% confidence interval of finding the mean within the ellipse. The accumulation of 27 out of 33 detected OxPCs were strongly associated with HDM challenge, whereas naïve controls were negatively correlated with increased accumulation of OxPCs. The graph is a scatter plot representing eigenvalues from two principal components (PC1 and 2) of the total OxPC expression values within individual treatment groups. The two principal components accounted for 71.7% variance within the data. Legend: HDM = Naïve = Allergen naïve, HDM-only challenge.

Figure 6.6

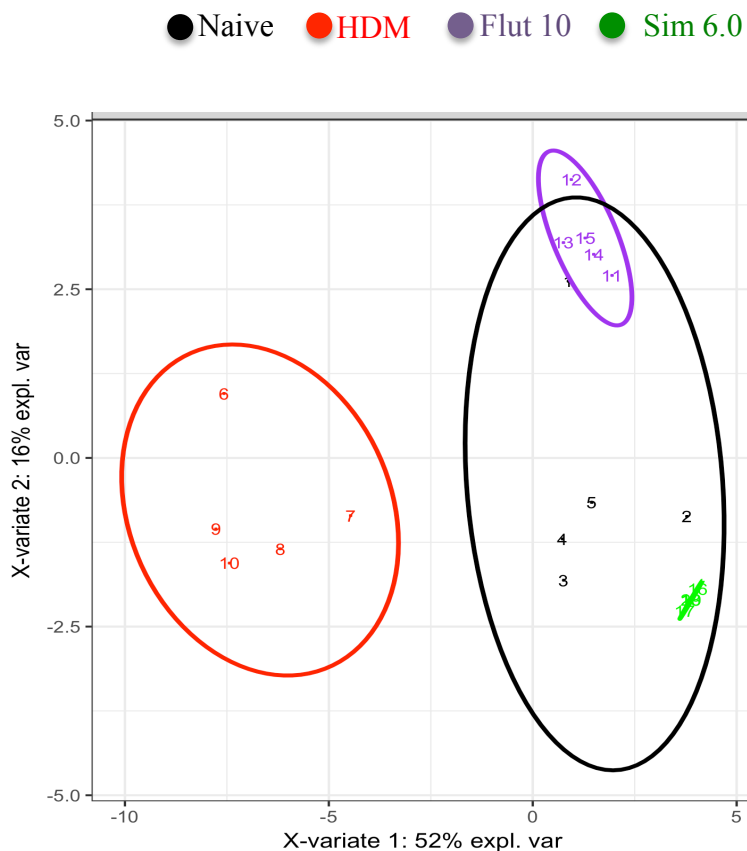


Figure 6.6: Impact of fluticasone and i.n. simvastatin treatment on OxPC profile in BALF of mice challenged with HDM.

Partial Least Square Discriminant Analysis (PLS-DA; supervised multivariate analysis) of lipidomics data in BALF from HDM challenged (red: probability ellipse with numbers representing individual animals) mice with or without fluticasone (purple: probability ellipse with numbers representing individual animals) or simvastatin (green: probability ellipse with numbers representing individual animals) treatment shows that both fluticasone and i.n. simvastatin treated animals formed clusters separate from HDM-challenged animals but closer to age-matched naïve control mice (black: probability ellipse with numbers representing individual animals); $n=5/\text{group}$. However compared to fluticasone treatment, i.n. simvastatin treated animals formed a separate cluster that was within the probability ellipse of the naïve animals. Probability ellipse defines 95% confidence interval of finding the mean within the ellipse. Suggesting OxPC profile in i.n. simvastatin treated animals were most similar to the naïve animals. The graph is a scatter plot representing eigenvalues from two principal components (PC1 and 2) of the total OxPC expression values within individual treatment groups. The two principal components accounted for 69.9% variance within the data. Legend: Naïve = Allergen naïve, HDM = HDM-only challenge, Flut 10 = HDM + Fluticasone (10 $\mu\text{g/kg}$), Sim 6.0 = HDM + Simvastatin (6 $\mu\text{g/kg}$).

Figure 6.7

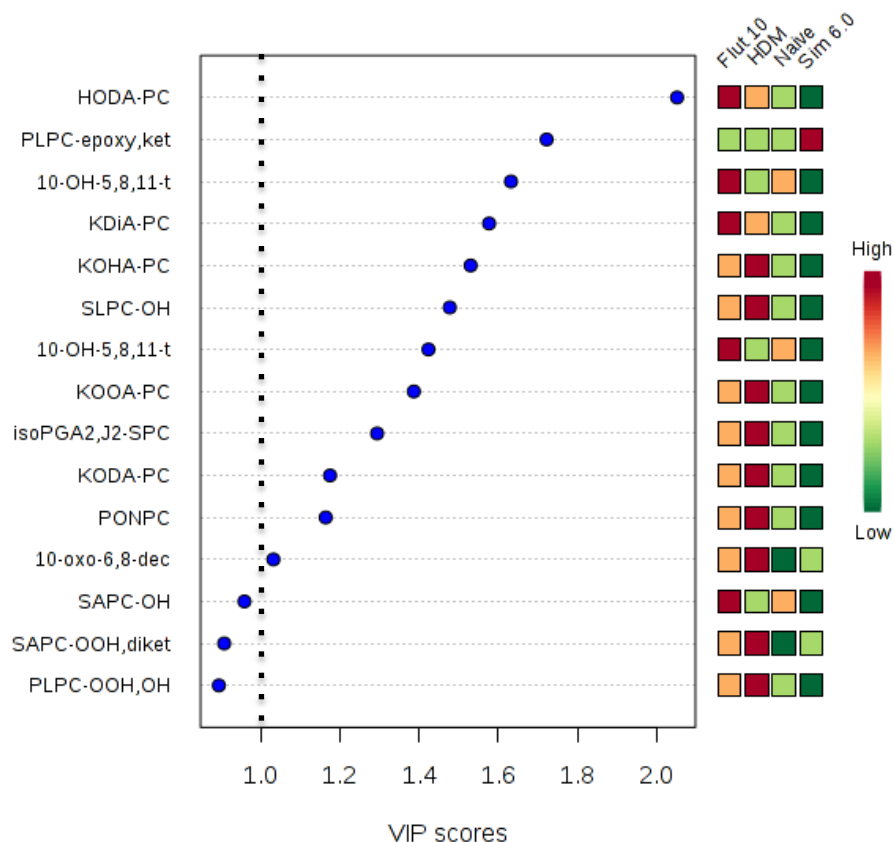


Figure 6.7: OxPCs most important in defining the treatment clusters based on Variance Importance in Projection (VIP) score.

A VIP score ≥ 1.0 is important discriminator for the cluster pattern observed with PLS-DA. The graph shows the scatter plot with the VIP scores (X-axis) for different OxPCs (Y-axis). The heatmap represents the relative concentrations of the OxPCs in each group for 15 OxPCs with highest VIP scores. Legend: Flut 10 = HDM + Fluticasone (10 μ g/kg), HDM = HDM-only challenge, Naïve = Allergen naïve, Sim 6.0 = HDM + Simvastatin (6 μ g/kg).

Figure 6.8

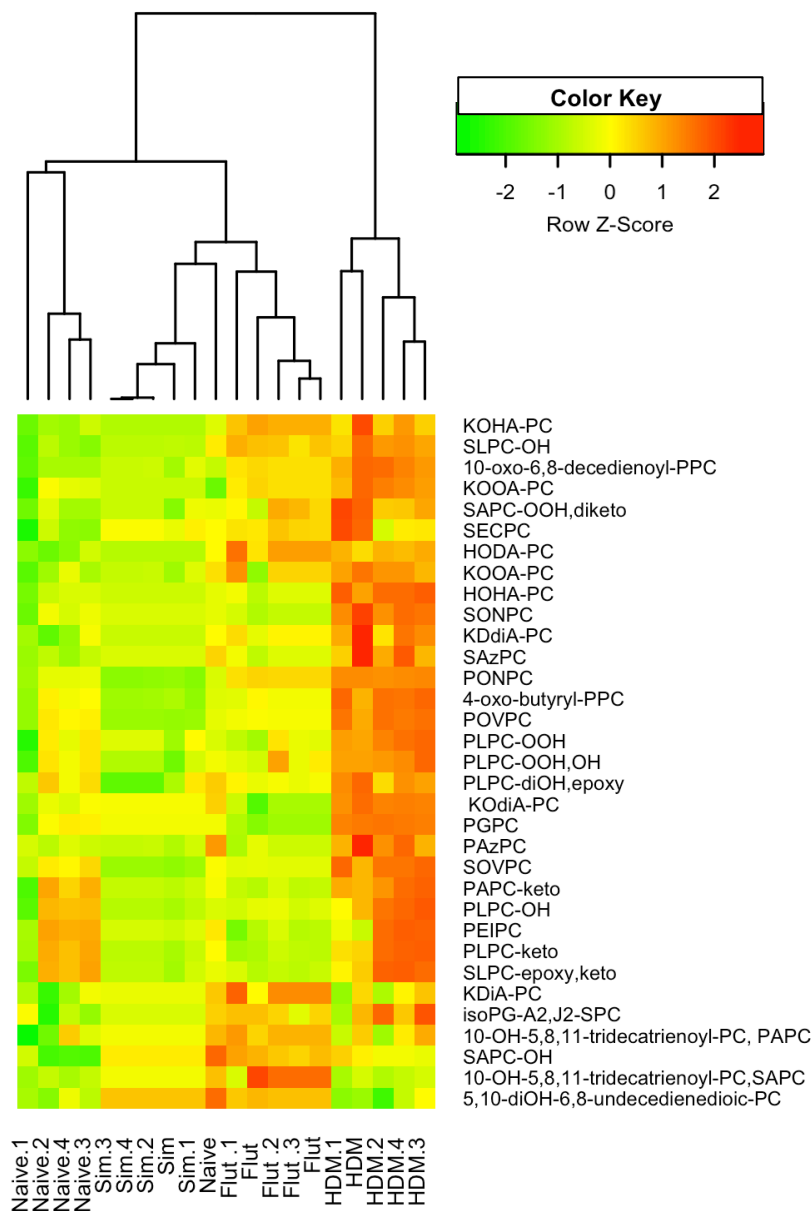


Figure 6.8: Heat-map showing OxPC profile in BALF of mice challenged with HDM with or without fluticasone or i.n. simvastatin treatment.

Y-axis contains all OxPCs detected in mouse BALF and X-axis represents the treatment groups (n= 5/group; indicated by numbers). The heatmap colour is based on the Row z score (color key). Cluster analysis of the treatment groups was performed using agglomerative hierarchical clustering (AHC), and dissimilarities were calculated based on the Euclidian distance represented by the dendrogram – the vertical length of the dendrogram is proportional to the dissimilarities between the groups and/ or individual animals. The horizontal connections of the dendrogram represent the individual animals and/ or clusters. The AHC clustering shows that the HDM-only challenged groups are different from both treatment, and the allergen naïve groups. Simvastatin treated animals are closest to the naïve animals. Legend: Flut 10 = HDM + Fluticasone (10µg/kg), HDM = HDM-only challenge, Naïve = Allergen naïve, Sim 6.0 = HDM + Simvastatin (6µg/kg).

OxPCs in Human BALF:

OxPC accumulation profiles were compared in segmental BALF samples from 10 well-characterized human asthmatics (Table 6.1), collected 24 h after saline and allergen challenge. In each of the 20 samples, 13 OxPCs were detected, and of these, 12 were common to those that we also detected in HDM-challenged mice (Figure 6.9).

Figure 6.9

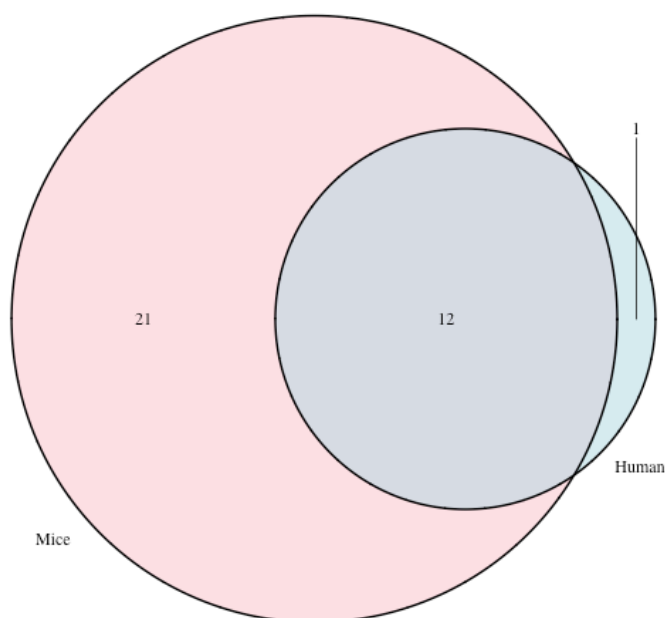


Figure 6.9: Venn diagram showing over 90% homology between the OxPCs identified in human asthmatics lavage and mouse lavage.

Total 13 OxPCs were detected in the BALF from human asthmatics, of these only 1 (blue) was unique to humans while 12 (grey) overlapped with the OxPCs observed in mice and 21 (pink) OxPCs were unique to mice.

Since the late asthma response (LAR) is an important clinical feature in defining control of and worsening symptoms of asthma, LAR severity after saline or allergen challenge was stratified into mild, moderate and severe (Table 6.2).

Table 6.2: Stratification based on severity of late asthma response (LAR)

Late Asthma Response (LAR)	Decline FEV1 % predicted
LAR1/ Mild	<10%
LAR2/ Moderate	10-20%
LAR3/ Severe	>20%

PLS-DA was applied to determine if the OxPC pattern correlated with LAR severity. We were able to distinguish LAR severity among asthmatics based on their OxPC profile, except for a few outliers (Figure 6.10A).

Figure 6.10A

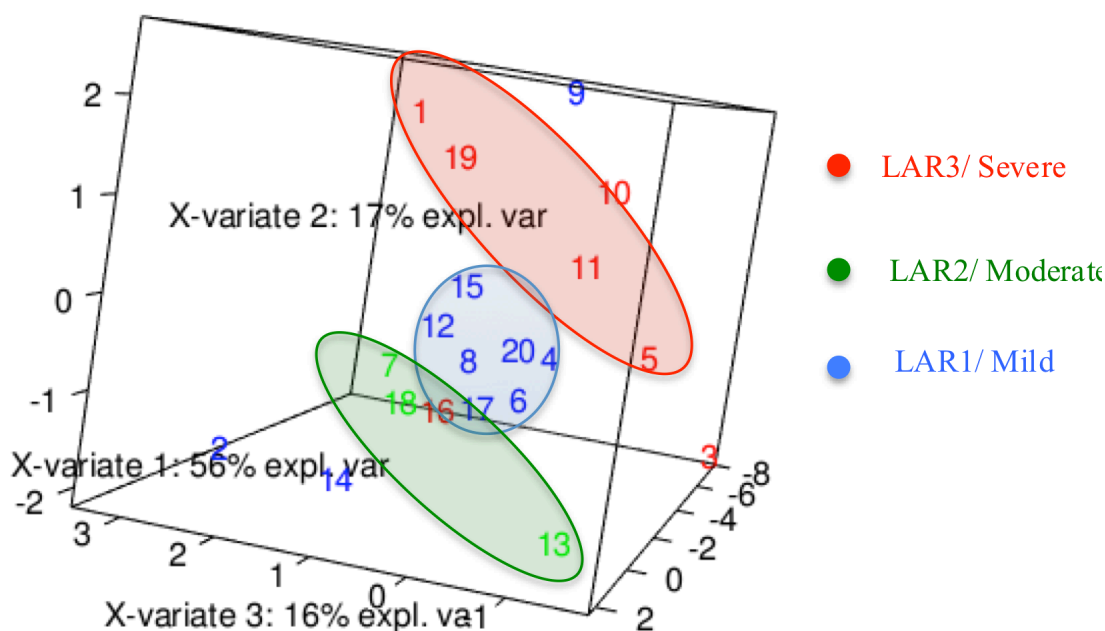


Figure 6.10A: OxPC profile in BALF of human asthmatics can define clusters of late asthma response (LAR) severity.

Partial Least Square Discriminant Analysis (PLS-DA; supervised multivariate analysis) of lipidomics data in BALF from human asthmatics challenged with allergen or diluent (same subjects were challenged with diluent or allergen 30 days apart; $n=10$). Based on OxPC expression in the BALF different categories LAR formed separate clusters. Mild, Moderate and Severe LAR are represented by blue, green and red probability ellipse containing individual subjects represented by a number, respectively. Probability ellipse defines 95% confidence interval of finding the mean within the ellipse. The graph is a scatter plot representing eigenvalues from three principal components (PC1, 2 and 3) of the total OxPC expression values within individual groups. The two principal components accounted for 86% variance within the data. Sample 3 and 16 within the LAR3 group, as well as and 2, 9 and 14 within the LAR1 group, are considered outliers. Legend: LAR1/ Mild = $<10\%$, LAR2/ Moderate = $10-20\%$ and LAR3/ Sever = $>20\%$ decline % predicted FEV1.

We next identified which specific OxPCs define patterns associated with response to a specific allergen or LAR severity, by evaluating the VIP scores obtained from PLS-DA modeling (Figure 6.10B). A VIP score of 1 or greater reflects a significant contribution of a particular OxPC in defining the separation of clusters observed in the PLS-DA plot (Figure 6.10A). Interestingly, all but one of the OxPCs that were important for defining LAR3 (severe)

were unfragmented, oxidized products of PLPC (PLPC-OH, PLPC-keto, PLPC-OOH,OH, PLPC-epoxy,keto, and PLPC-OOH) (Figure.6.10B).

Figure 6.10 B

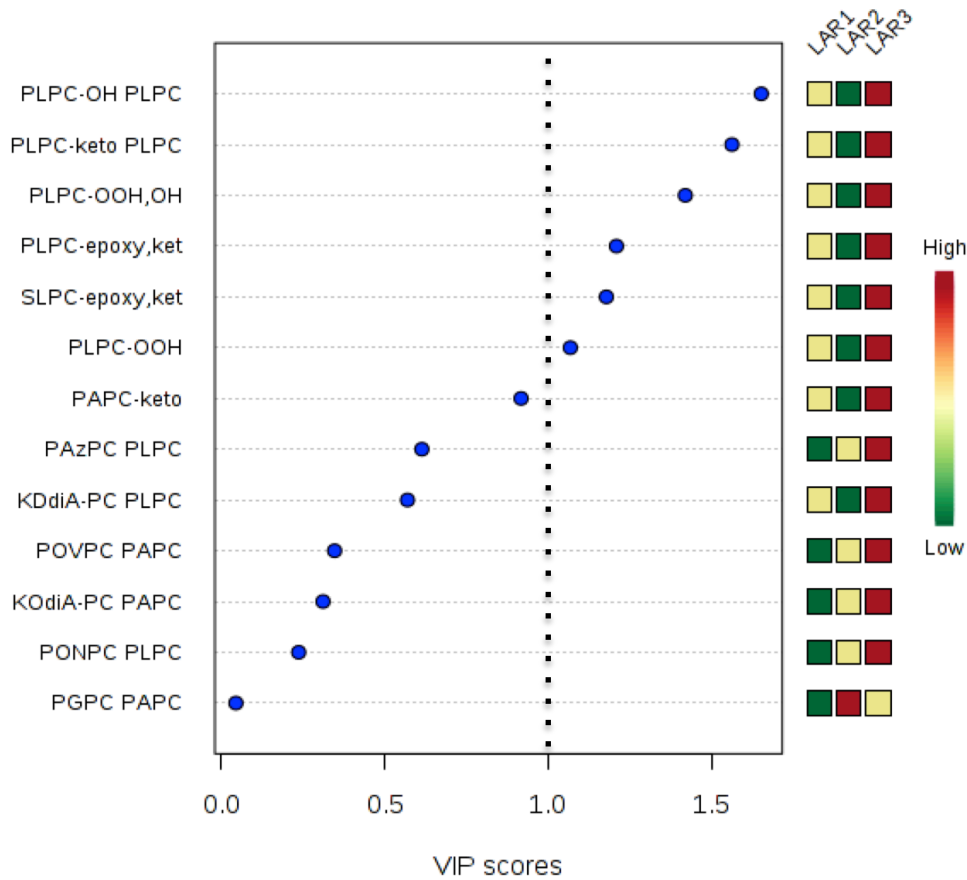


Figure 6.10B: OxPCs most important in defining the LAR severity in human asthmatics based on Variance Importance in Projection (VIP) score.

A VIP score ≥ 1.0 is important discriminator for the cluster pattern observed with PLS-DA. The graph shows the scatter plot with the VIP scores (X-axis) for different OxPCs (Y-axis). The heatmap represents the relative concentrations of the OxPCs in each group for all OxPCs detected in human lung lavage of these 6 had a VIP score of ≥ 1.0 . Legend: LAR1/ Mild = $<10\%$, LAR2/ Moderate = $10\text{-}20\%$ and LAR3/ Sever = $>20\%$ decline % predicted FEV1.

Further comparison of the two allergens that induced severe LAR (cat and HDM) we found that both allergens induced unique sets of OxPCs (Figure. 6.11& 12 A). The response to cat allergen was primarily defined (indicated by a VIP score ≥ 1) by the accumulation of

unfragmented OxPCs primarily of PLPC origin (PLPC-epoxy, keto, PLPC-OH, PLPC-keto), but also included one unfragmented OxPC derived from PAPC (Figure.6.11B).

Figure 6.11A

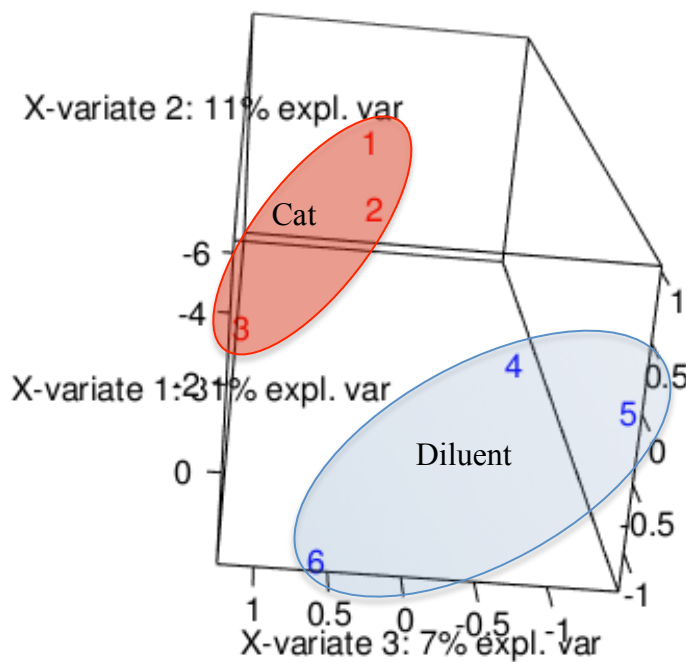


Figure 6.11A: Cat-allergen challenge induces distinct OxPC profile in BALF of human asthmatics.

Partial Least Square Discriminant Analysis (PLS-DA; supervised multivariate analysis) of lipidomics data in BALF from human asthmatics challenged with cat-allergen or diluent (to the same subject 30 days apart) showed that the OxPC expression profile could separate the cluster of cat-allergen challenge (red: probability ellipse containing individual subjects represented by a number) from their diluent control (blue: probability ellipse containing individual subjects represented by a number) (n=3). Probability ellipse defines 95% confidence interval of finding the mean within the ellipse. The graph is a scatter plot representing eigenvalues from three principal components (PC1, 2 and 3) of the total OxPC expression values within individual groups. The three principal components accounted for 49% variance within the data. Legend: Cat Allergen challenged, Dil: Diluent challenged.

Figure 6.11B

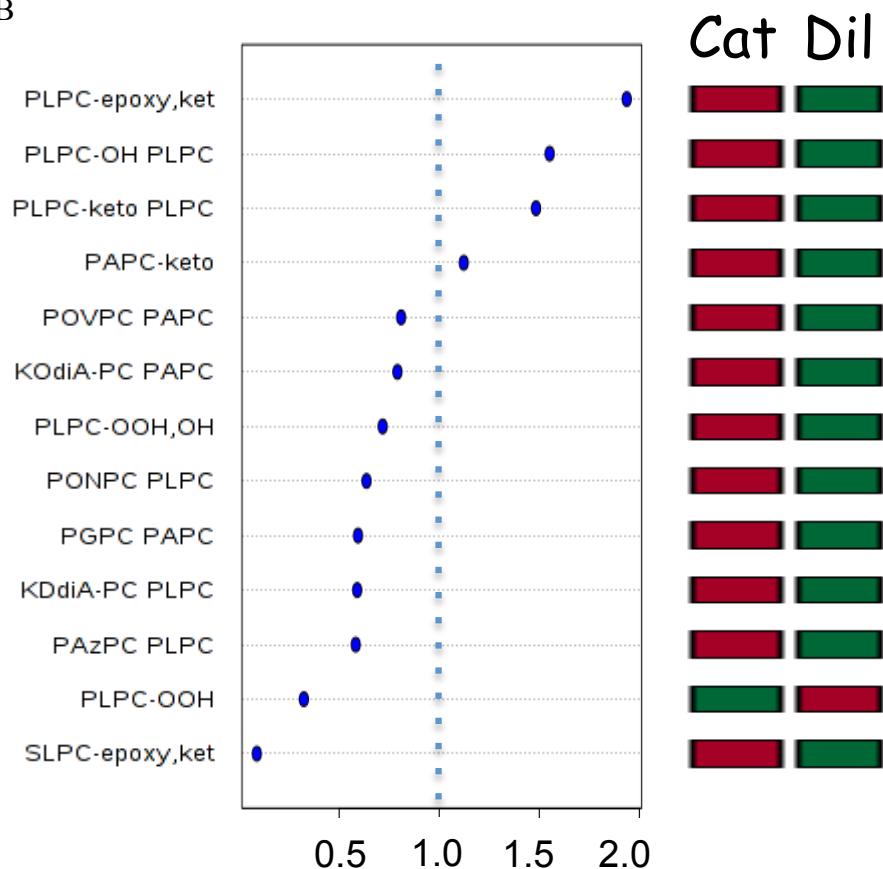


Figure 6.11B: OxPCs most important in defining cat-allergen challenge from the diluent challenge based on VIP score.

A VIP score ≥ 1.0 is important discriminator for the cluster pattern observed with PLS-DA. The graph shows the scatter plot with the VIP scores (X-axis) for different OxPCs (Y-axis). The heatmap represents the relative concentrations of the OxPCs in each group for all OxPCs detected in human lung lavage. Legend: Cat: Cat-allergen challenged, Dil: Diluent challenged.

For patients who were allergic to HDM, allergen challenge induced its own unique OxPC subset compared to diluent control (Figure.6.12B). The OxPCs that became elevated with HDM challenge and that were important for defining the clusters (VIP score ≥ 1) included PGPC, PONPC, KDdiAPC, and SLPC epoxy keto. Of these, 3 are fragmented OxPCs, PONPC is of PLPC origin, whereas PGPC and KDdiAPC are of PAPC origin. SLPC epoxy keto is an unfragmented OxPC species.

Figure 6.12A

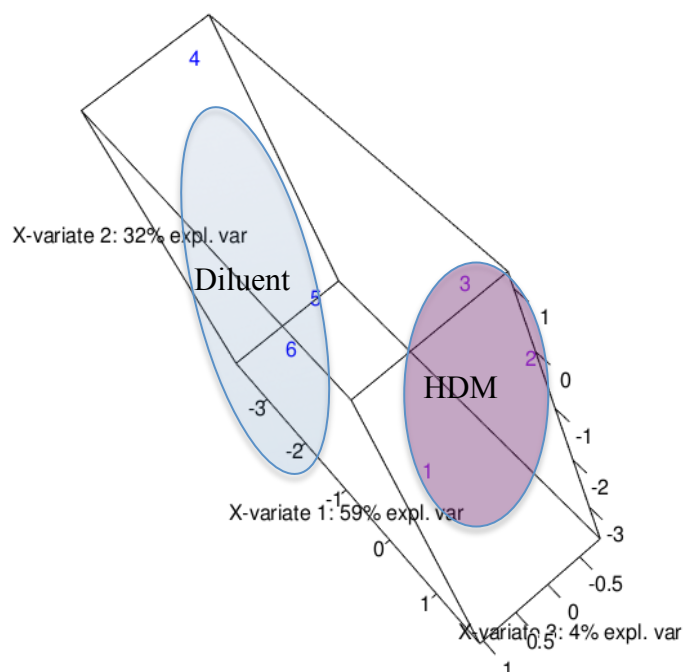


Figure 6.12A: HDM-allergen challenge induces distinct OxPC profile in BALF of human asthmatics.

Partial Least Square Discriminant Analysis (PLSDA; supervised multivariate analysis) of lipidomics data in BALF from human asthmatics challenged with HDM-allergen (purple: probability ellipse containing individual subjects represented by a number) or diluent (blue: probability ellipse containing individual subjects represented by a number) showed that the OxPC expression profile could separate the cluster of allergen challenge from their diluent control ($n=3$). Probability ellipse defines 95% confidence interval of finding the mean within the ellipse. The graph is a scatter plot representing eigenvalues from three principal components (PC1, 2 and 3) of the total OxPC expression values within individual groups. The three principal components accounted for 95% variance within the data. Legend: HDM: HDM-allergen challenged, Dil: Diluent challenged.

Figure 6.12B

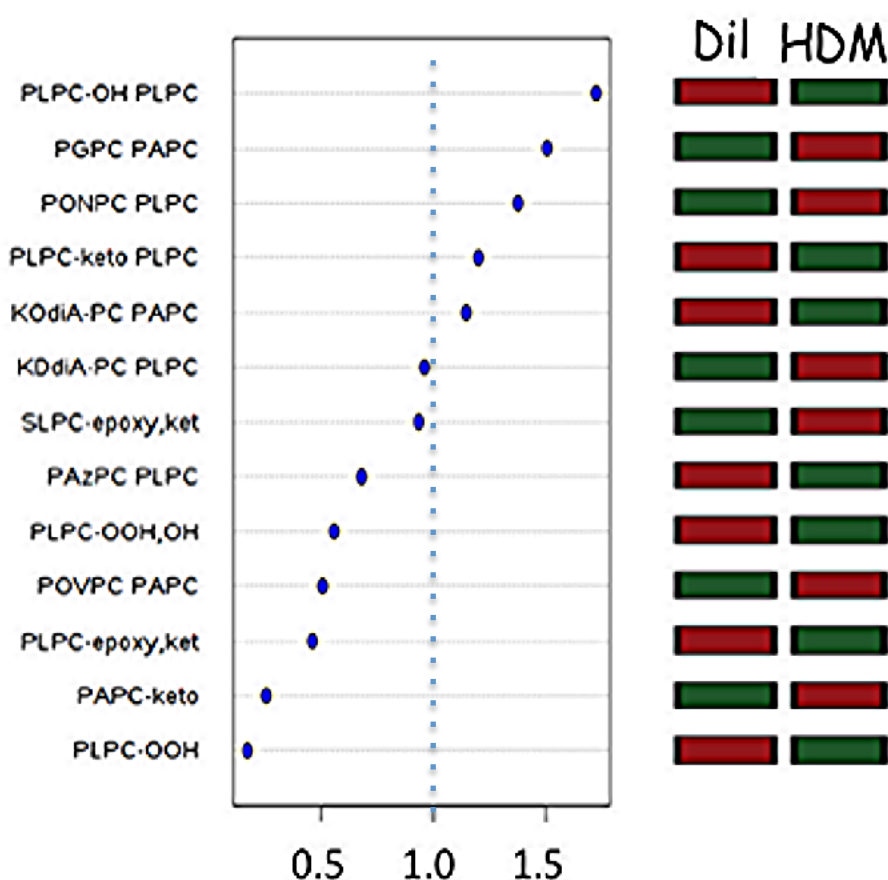


Figure 6.12B: OxPCs most important in defining HDM-allergen challenge from the diluent challenge based on VIP score.

A VIP score ≥ 1.0 is important discriminator for the cluster pattern observed with PLS-DA. The graph shows the scatter plot with the VIP scores (X-axis) for different OxPCs (Y-axis). The heatmap represents the relative concentrations of the OxPCs in each group for all OxPCs detected in human lung lavage. Legend: HDM: HDM-allergen challenged, Dil: Diluent challenged.

Impact of OxPC challenge on airway inflammation

Instillation of intranasal 20 µg/kg OxPC on its own did not significantly increase the total influx of inflammatory cells $[(4.1 \pm 0.6) \times 10^4]$ in BALF when compared to naïve controls $[(3.4 \pm 0.2) \times 10^4]$ (Figure. 6.13). However, data from cell differentials reveal that OxPC challenge significantly increased the influx of eosinophils (7 fold), neutrophils (4 fold), and lymphocytes (2 fold) ($p < 0.05$). Macrophages not change with OxPC challenge $[(3.0 \pm 0.3) \times 10^4]$ compared to naïve controls $[(2.9 \pm 0.6) \times 10^4]$.

Figure 6.13

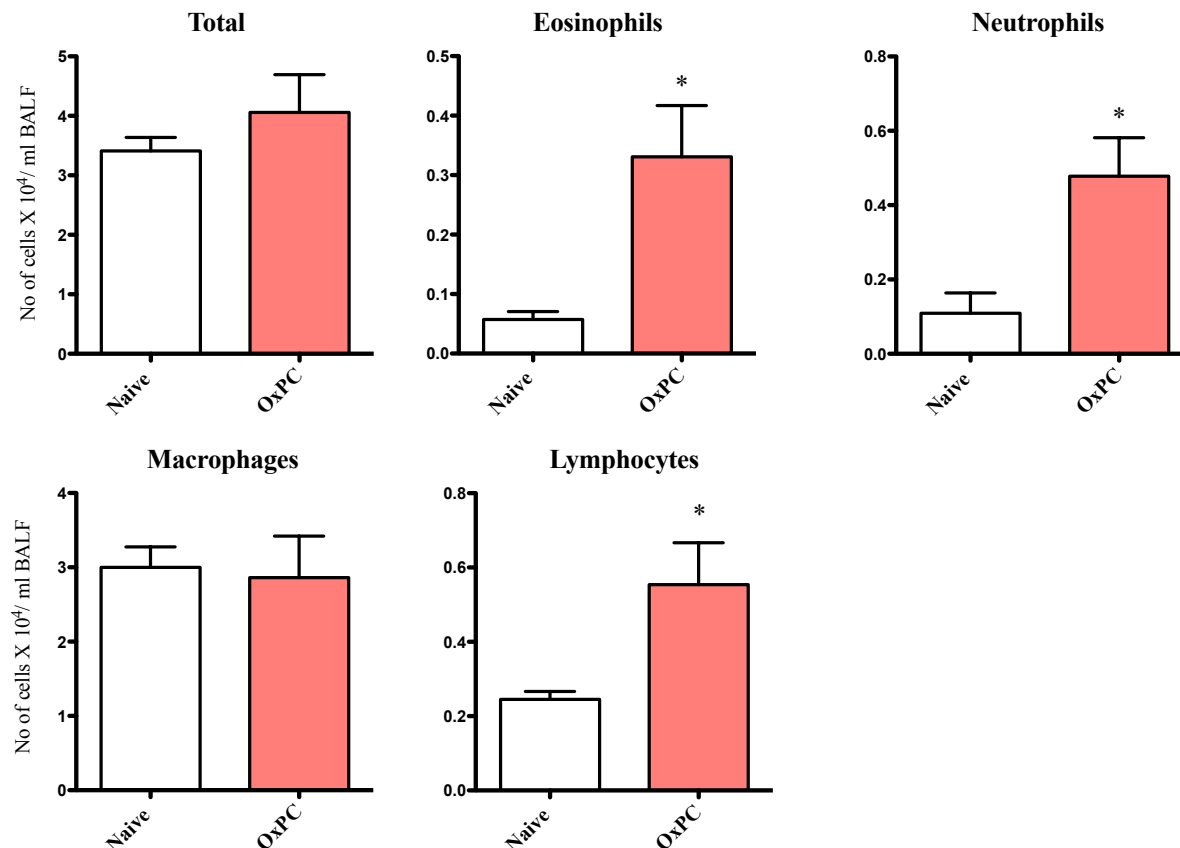


Figure 6.13: The impact of i.n. OxPCs challenge on the influx of total leukocyte number and differentials in BALF.

Measurements were taken 48 h after the final OxPC challenge. For all data, cell number was normalized to initial BALF volume and presented as mean $\pm$ SEM from 5 animals per group. Analysis by unpaired Student T-test. *: $p \leq 0.05$.

Impact of OxPC challenge on lung function

Intranasal OxPC challenge did not induce a decline in lung function compared to naïve controls; none of the lung function parameters measured (R_N , G, or H) were significantly upregulated post OxPC challenge (Figure. 6.14).

Figure 6.14

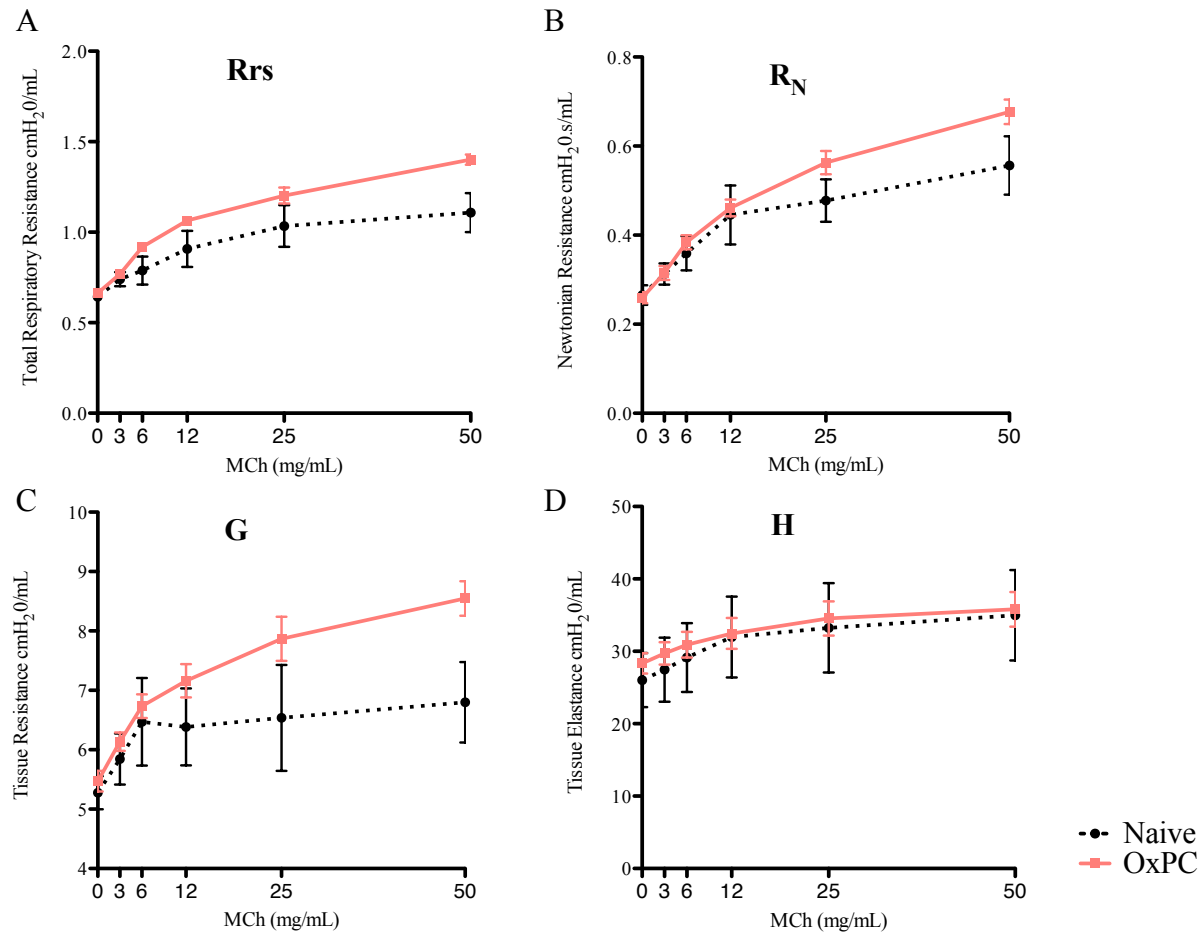


Figure 6.14: Impact of intranasal OxPC challenge on respiratory mechanics.

Lung function was assessed using a flexiVent small animal ventilator 48h after completing the OxPC challenge . A) Total Respiratory Resistance (R_{rs}) B) Newtonian Resistance (R_N), C) Tissue Resistance (G) and C) Tissue Elastance (H) were measured in response to sequential challenge with nebulized saline (0) or MCh (3-50 mg/mL). Data are presented as mean ± SEM from 5 animals per group. Comparisons of naïve and OxPC challenge was done using Univariate analysis followed by Bonferroni multiple comparison.

6.4 Discussion

PCs are the major constituents of the plasma membrane in lung cells as well as lung surfactant. The literature from cardiovascular research suggests that under oxidative stress PCs are oxidized, generating fragmented and unfragmented OxPCs that facilitate inflammation by disrupting the physical barrier to pathogens and also by inducing chemical cues for leukocyte migration to sites of stress (22, 24, 39, 287). However, the role of OxPCs in chronic lung diseases such as asthma, where oxidative stress plays a major role in pathogenesis, has been largely unstudied.

Our previous work, again confirmed in this study, has shown that HDM challenge induces robust allergic airway inflammation and AHR in mice. Both i.n. simvastatin and fluticasone prevented allergen-induced airway inflammation. However, only simvastatin prevented allergen-induced AHR. For the first time, we show that allergen challenge also induces the accumulation of OxPCs in the lungs of the mice as well as humans. In fact, the OxPCs found in the BALF of human asthmatics (regardless of allergen challenge status) had over 90% overlap with the OxPCs that accumulate in our murine model after repeated HDM challenge. In line with murine data, allergen challenge also induced a unique pattern of OxPC accumulation in BALF from human atopic asthmatics, compared to saline control in the same individuals. For example, human subjects challenged with inhaled cat allergen exhibited a unique accumulation of unfragmented OxPCs, whereas HDM challenge induced accumulation of predominantly fragmented OxPCs. Thus, specific allergen challenge induces a signatory panel of OxPCs. This work also identifies the OxPCs PGPC and PONPC as particularly important in defining the response to HDM challenge in both our murine model and human asthmatics.

To assess the relevance of OxPCs in asthma pathobiology, we looked at the LAR severity in human asthmatics in response to allergen challenge. LAR is observed 3 to 4 hours following allergen challenge, peaking at 6 to 12 hours. It is marked by eosinophilic inflammation and undefined airway bronchoconstriction and AHR. LAR and its severity predict risk for asthma exacerbations, and thus are an index of therapeutic control (288). Our cohort of human subjects had mild asthma with defined atopy, and all exhibited LAR in response to an allergen. Based on categories defined by decline in FEV₁, all asthmatics that received diluent challenge showed mild LAR (<10% decrease in FEV₁), while allergen challenge induced anywhere from moderate (define) to severe (define) LAR.

Based on their OxPC profiles, asthmatics with different LAR severities clustered separately from each other. As all these subjects had similar lung function with no record of ongoing lung inflammation, we used our animal model of allergic asthma to elucidate the relationship between the initiation of OxPC accumulation with airway inflammation and decline in lung function. Interestingly we observed that both simvastatin and fluticasone significantly inhibited HDM-induced OxPC accumulation in the lungs, consistent with their capacity to inhibit HDM-induced inflammation. Also, a significantly greater impact of simvastatin compared to fluticasone was observed, correlating with better control of eosinophilic and neutrophilic inflammation in mice with simvastatin treatment. This evidence is consistent with other studies showing the role OxPCs in the tethering and migration of inflammatory cells across the endothelial layer (30, 33, 39, 40). However, it is unknown how simvastatin is mitigating the accumulation of OxPCs in the lungs of mice exposed to the HDM. The unpublished ‘multiomics’ data from others within our group suggest that HDM challenge induces genes for pro-inflammatory proteins on HDL such as Serum Amyloid A (SAA) and Apolipoprotein-J, and this was significantly inhibited with simvastatin treatment. Simvastatin is

also known to enhance the ApoA1(289), an integral component of 'Functional HDL' (47). Thus, it is possible that simvastatin is blunting the HDM-induced decline in the 'Functional-HDL'. At the same time, it is known that simvastatin enhances HDL levels (48), and HDLS can take up and neutralize oxidized phospholipids. Thus, simvastatin is possibly enhancing the removal of OxPCs from the system.

Based on our data it is difficult to fully decipher whether OxPCs themselves drive inflammation or if they are by-products of oxidative stress induced by chronic inflammation (20, 22). Nonetheless, there exists a linear relationship between OxPC accumulation and influx of inflammatory cells in the lungs.

Interestingly, both i.n. fluticasone and simvastatin had a significant impact on the accumulation of OxPCs in the lungs, but fluticasone did not prevent HDM induced AHR in mice, whereas simvastatin abrogated AHR. The significantly stronger impact of simvastatin on OxPC accumulation compared to fluticasone could explain the differential effect on allergen-induced lung dysfunction. The failure of fluticasone to improve AHR could also be explained by the strong positive correlation that we observed for fluticasone treatment on a subset of OxPCs: HODA-PC, 10-OH-5,8,11-tridecatrienoyl-PC (SAPC), 10-OH-5,8,11-tridecatrienoyl-PC(PAPC), KDiA-PC and SAPC-OH. These OxPCs were upregulated by fluticasone treatment in HDM challenged mice. VIP scores also suggest that most of the OxPCs associated with the response to HDM challenge also remained elevated with fluticasone treatment, compared to naïve controls. This suggests that the impact of fluticasone on OxPCs is not sufficient to blunt HDM-induced AHR.

Our evidence suggests that beyond a threshold level, OxPCs in the lung can be pathogenic. A limitation of this study is that it does not enable us to determine a causal role. Thus, it is yet to be proven whether OxPCs are sufficient to drive the decline in lung function

observed with HDM challenge. In a preliminary attempt to explore this possibility, mice were challenged with i.n. OxPAPC, utilizing a protocol that mimics our acute HDM challenge model. In these initial experiments, OxPAPC challenge was not sufficient to induce an influx of total inflammatory cells in the lungs; however, it did significantly induce eosinophilia, neutrophilia and lymphocyte influx, although to a lesser extent than acute HDM challenge. Thus, unsurprisingly there was no observed impact on AHR. Although the results are preliminary, it provide some evidence that OxPCs can drive local inflammation by themselves, it is very promising. Future work to more fully test the requirement and/or sufficiency of OxPCs in driving asthma pathobiology are needed. Overall, body of work highlights the importance of OxPCs in asthma pathology and prompts further investigation that could lead to a marker for asthma control and/or endotype, as well as the unique mechanism through which simvastatin could impart additive benefits to inhaled glucocorticoid therapy.

Chapter 7: Effect of i.n simvastatin on airway inflammation, remodelling and hyperresponsiveness in a chronic murine model of allergic asthma

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Mohammad Soussi Gounni: Inflammatory cell count and collagen imaging

Sujata Basu: Lung function testing

Jacquie Schwartz: Histology

Andrew Halayko: Guided, Supervised and facilitated the entire study

7.1 Background

Asthma is a chronic disease of the lung characterized by lung inflammation and remodelling leading to airway hyperresponsiveness (AHR). Patients who present to a health care practitioner, hospital or emergency room for asthma-related symptoms typically already have established features of the disease. The current therapies of choice for the treatment of asthma include inhaled corticosteroids (ICS) and beta-adrenergic receptor agonists (β ARA); ICS are a controller medication that resolves airway inflammation and prevents future attacks of asthma, while β ARAs are relievers that relax bronchial smooth muscle to reduce chest tightness associated with asthma (11, 97, 98, 108). Proper use of current medication can prevent further deterioration of lung health in most cases (11); however, some asthmatics are refractory to current therapy and develop severe asthma, incurring a significant health and economic burden to society (106, 131, 132). Resistance to therapy could be attributed in part to the inability of medications to specifically address AHR and remodelling (290). In addition oxidative stress is another key feature of asthma that remains unaddressed by currently available therapies (44).

Previously, in Chapter 5 we showed that intranasal simvastatin could prevent the allergen-induced airway inflammation and hyperresponsiveness in an acute model of allergic asthma in mice. Up to this point, the efficacy of statins in a murine model of allergic asthma has been conducted only in an acute model, which was and is designed for prevention, where treatment with simvastatin is administered concurrently with allergen challenge (173, 209, 222, 231, 236). However, a more clinically relevant model would accommodate reversal, where treatment begins only after the essential features of the disease are established. In this chapter, we will begin with an acute reversal study, with allergen avoidance and exposure arms as a proof of concept, then proceed to explore the impact of simvastatin in a chronic reversal study.

Our hypothesis is that intranasal simvastatin will *reverse* allergen-induced lung inflammation, remodelling and hyperresponsiveness.

To our knowledge, this is the first study that has evaluated if intranasal simvastatin could reverse the established features of asthma in mice in both acute and chronic settings. Also, this is the first study that attempted to evaluate the impact of simvastatin in mice against chronic HDM challenge.

7.2 Methods

Female Balb/c mice were procured as explained previously in Section General Methodology. Drug formulations were made from the working stocks of simvastatin (1 mg/mL in 100% ethanol). The working stock of simvastatin was diluted using PBS to obtain 3.0 and 6.0µg/kg dose in 35µL saline.

HDM Challenge and Drug Treatment

Balb/c mice were assigned identity numbers (accessible only to the study coordinator) and randomized to different groups by body weight (to minimize dosing variability and to ensure that all experiments could be carried out in a blinded and bias-free manner).

Acute reversal Study:

All animals received i.n. HDM challenge once daily (25µg/35 µL) for 2 weeks (5d/wk) to establish airway inflammation and hyperresponsiveness. Post 2-wks of allergen challenge animals received i.n. simvastatin treatment with or without allergen avoidance (Figure 7.1A).

Chronic Reversal Study:

All mice received the i.n. HDM once daily (25µg/35 µL) for 5 days/wk for the first 2 weeks, after which they received i.n. HDM on alternate days (3 days/wk) for an additional 3

weeks. The 5 week protocol was designed to evaluate the disease reversal potential of i.n. simvastatin, in both allergen avoidance and exposure settings. In the allergen avoidance arm, mice were treated with i.n. simvastatin (3µg/kg) with allergen avoidance post 5 *weeks* of HDM challenge, (allergen avoidance; Figure 7.1B). In the allergen exposure arm, the animals received i.n. simvastatin treatment during the last 2 weeks of HDM challenge, concomitantly with the HDM-challenge (Figure 7.1B).

Outcomes measured for acute reversal studies include (i) lung inflammation (total and differential cell count in BALF) and (ii) lung function tests as described in Section General Methodology. Outcomes measured for chronic reversal studies include (i) lung inflammation (total and differential cell count in BALF, as well as cytokine array using Eve Technology 32-plex mouse cytokine array + Total TGFβ1, 2 and 3) and (ii) histology (H&E, PAS and Picrosirius Red staining of paraffin embedded lung sections). All procedures were carried out according to the detailed descriptions in Section General Methodology.

Statistical Analysis:

Data are reported as mean values ± standard error mean (SEM). Means were compared by the generalized linear multivariate and Bonferroni multiple comparisons test, using SPSS_20 software (IBM Inc), and the representative graphs were created using Graph Pad Prism software 5.0. A p value <0.05 was defined as statistically significance among mean values. Cytokine analysis for the chronic study was performed using a supervised multivariate analysis (PLSDA) using the statistical package R, followed by a generalized linear multivariate analysis and Bonferroni multiple comparisons test, using SPSS_20 software (IBM Inc). All representative figures for cytokine analysis were generated in R.

Figure. 7.1.A

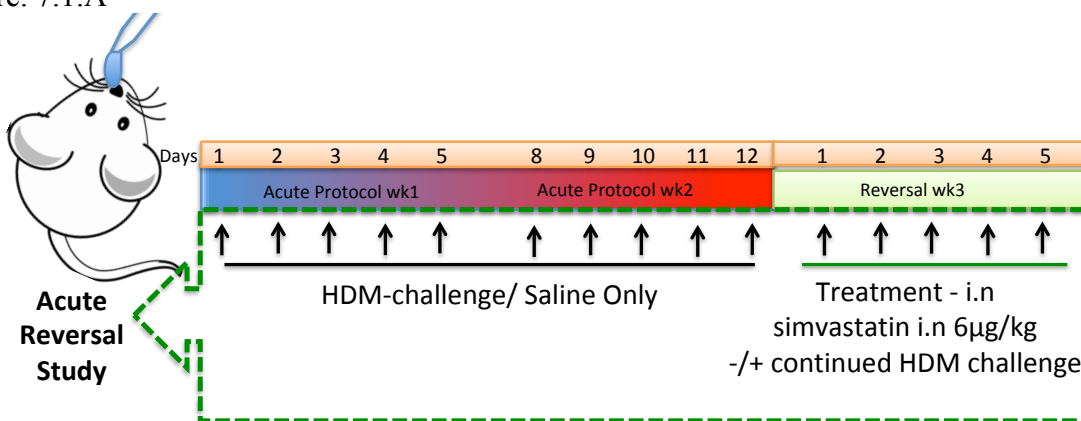


Figure 7.1A: Schematic of acute reversal study.

All animals received i.n. HDM for 2 weeks followed by treatment with i.n. simvastatin (6μg/kg) on third week with or without HDM challenge. The study also consisted of age matched naïve control group that received no HDM at all.

Figure 7.1B

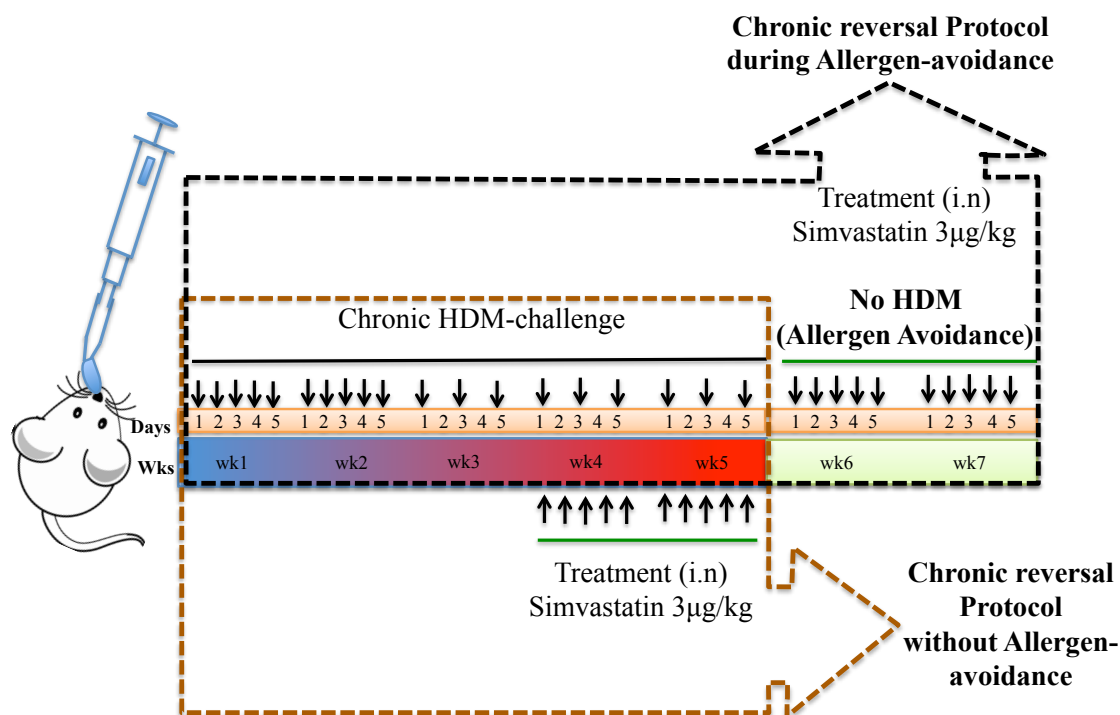


Figure 7.1B: Schematic of chronic HDM challenge with or without simvastatin treatment.

Mice were challenged with i.n. HDM 25μg/day for the first 2 weeks, followed by 3 weeks of challenge on 3 alternate days/wk, with a 2 day break between each week of dosing. The mice in the allergen avoidance arm of the study ((black rectangle) did not receive any allergen challenge for a final 2 weeks (weeks 6 & 7), but received treatment with 3μg/kg i.n. simvastatin 5days/wk. For allergen exposure arm, simvastatin treatment began in week 4 and continued for 2 weeks concomitantly with HDM challenge. Studies included age matched allergen naïve control group.

7.3 Results

7.3.1. Acute study:

Airway Inflammation:

One week of HDM avoidance significantly attenuated the inflammation induced by 2 weeks of HDM challenge (Figure 7.2). After one week of allergen avoidance, the allergen avoidance group had only $(33.2 \pm 3.8) \times 10^4$ cells in comparison to 2-wks of acute HDM-challenge $((187.3 \pm 20.8) \times 10^4$ cells; $p < 0.001$). Differential cell counts revealed an 11-, 8- and 6-fold decrease in eosinophils ($p < 0.01$), neutrophils ($p < 0.001$) and lymphocytes ($p < 0.05$), respectively, compared to HDM challenge alone. Simvastatin treatment made no difference to cell counts in BALF when used in combination with allergen avoidance, compared to allergen avoidance alone.

In the allergen exposure arm, allergen challenge continued for an additional week (3 times on alternate days). There was no statistical difference in cell counts among animals that received 2 or 3 weeks total allergen challenge (Figure 7.3). Treatment with simvastatin during the third week resulted in an almost 4-fold reduction in the total inflammatory cell count compared to HDM challenge alone ($p < 0.001$). This was associated with an almost 17-fold decrease in eosinophils ($p < 0.01$), a 2.5-fold decrease in neutrophils ($p < 0.05$), and a 2-fold decrease in lymphocytes ($p < 0.05$). However no statistically significant change in macrophage count was observed.

Figure 7.2

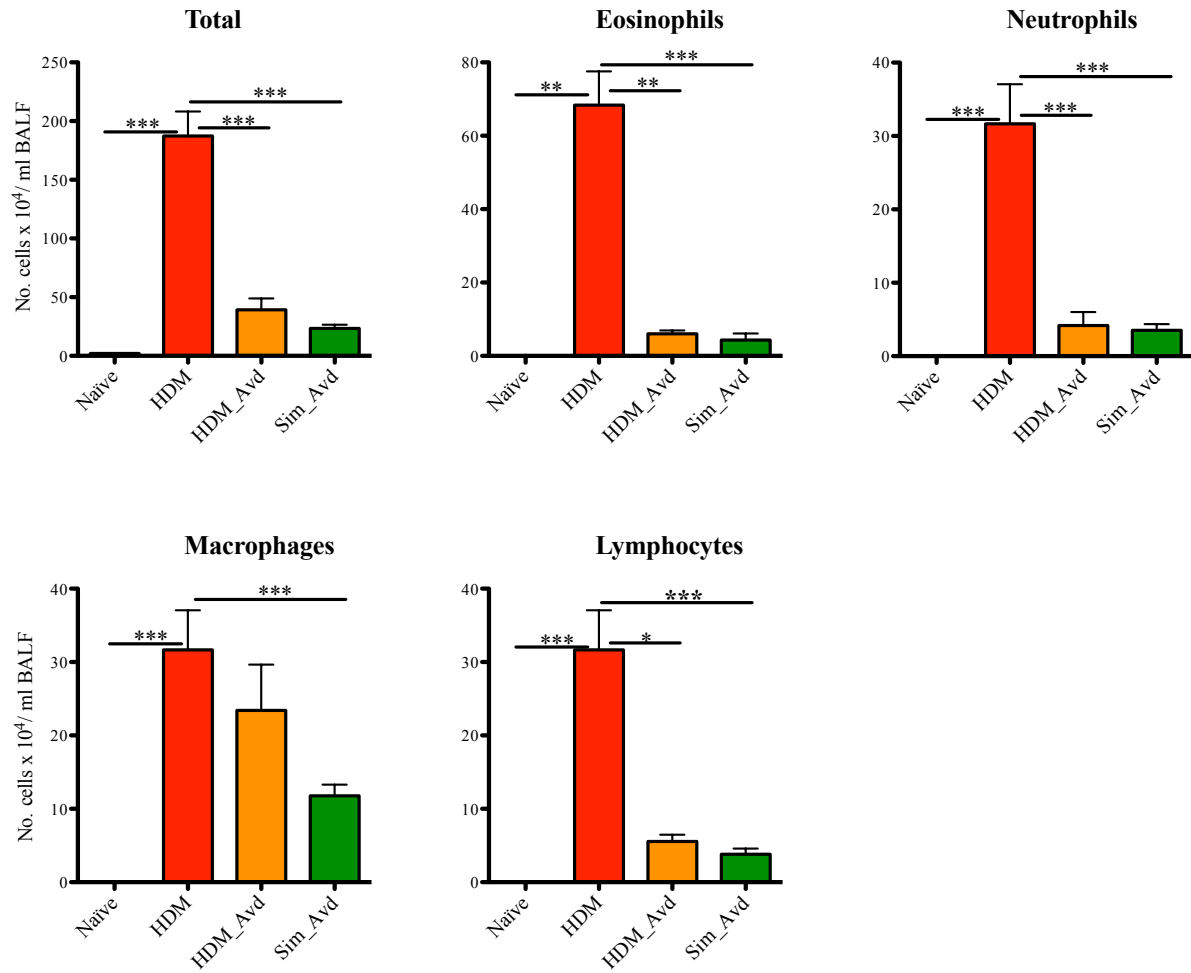


Figure 7.2: Impact of allergen avoidance and intranasal simvastatin on HDM- induced influx of total leukocyte number and differential in BALF.

Cell counts were determined 48 h after the end of one week of allergen avoidance, with (Sim_Avd) or without (HDM_Avd) simvastatin treatment. For all data, cell number is normalized to initial BALF volume and presented as mean $\pm$ SEM from 6 animals per group. Analysis was done using one-way ANOVA with Tukey multiple comparisons test. * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ (univariate analysis using SPSS20.0)

Figure 7.3

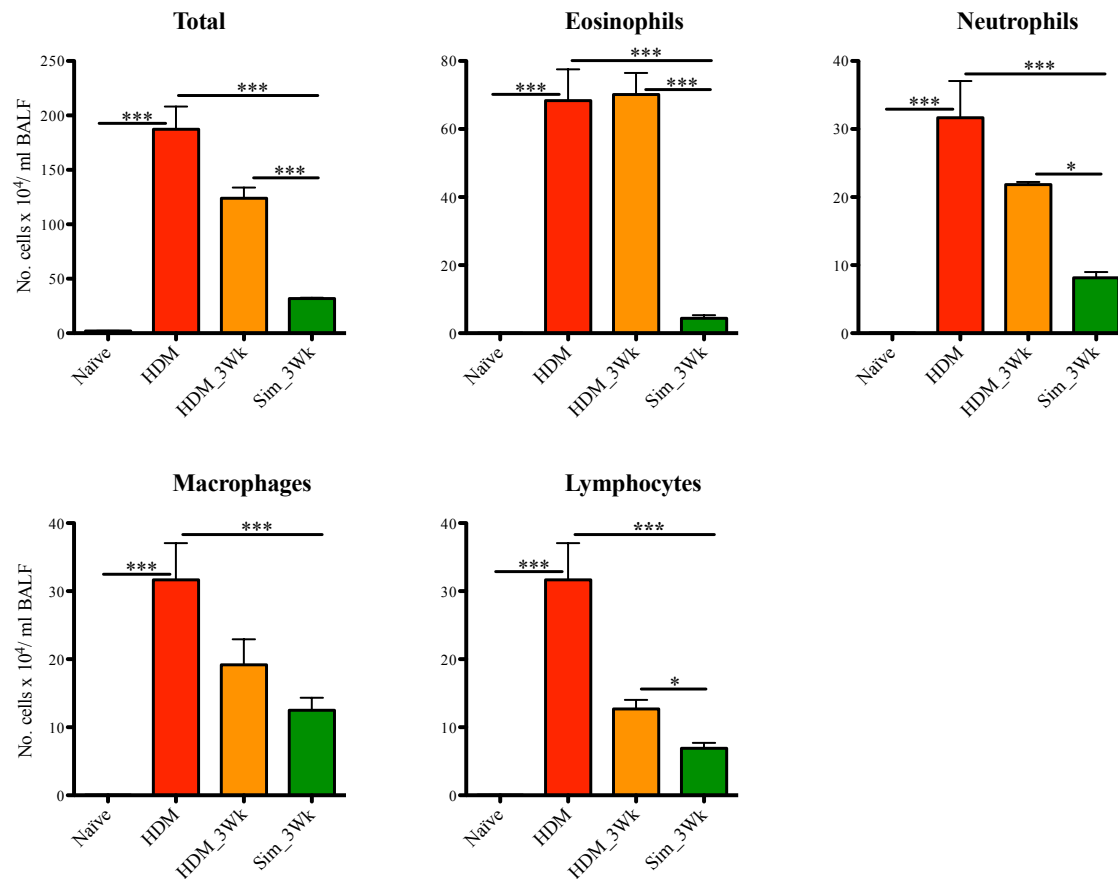


Figure 7.3: Reversal of inflammatory cell influx with 1-wk of i.n. simvastatin treatment.

Cell counts were determined 48 h after completing 3 weeks of HDM challenge with (Sim_Exp) or without (HDM_Exp) simvastatin treatment during the 3rd week. For all data, cell number is normalized to initial BALF volume and presented as mean $\pm$ SEM from 6 animals per group. Analysis was done using one-way ANOVA with Tukey multiple comparisons test: *: $p \leq 0.05$, ** ≤ 0.01 and *** ≤ 0.001 (univariate analysis using SPSS20.0)
Note: *: $p \leq 0.05$, ** ≤ 0.01 and *** ≤ 0.001 (univariate analysis using SPSS20.0)

Lung function:

Compared to naïve controls, acute HDM challenge induced a significant increase in R_N , G, and H ($p < 0.001$) (Figure 7.4). One week of allergen avoidance following acute HDM challenge did not alleviate the HDM-induced decline in lung function; i.e., R_N ,

G, and H remained elevated in response to the MCh challenge, similar to the level of acute HDM challenge only. When simvastatin (6.0 µg/kg, i.n.) was administered during the allergen avoidance in the third week, R_N was restored to the level of naïve controls, with statistical significance at 25 and 50mg/mL MCh (Figure 7.4A). Significant reversal of G was observed at 12 ($p<0.05$), 25 ($p<0.01$) and 50 ($p<0.01$) mg/mL MCh (Figure 7.4B), and H was reversed significantly at 25 and 50 mg/mL MCh (Figure 7.4C). Overall, lung function with simvastatin treatment was significantly different from both the acute HDM challenge group and the allergen avoidance group ($p<0.001$), and was similar to the allergen-naïve group for all parameters of lung function measured

Figure 7.4

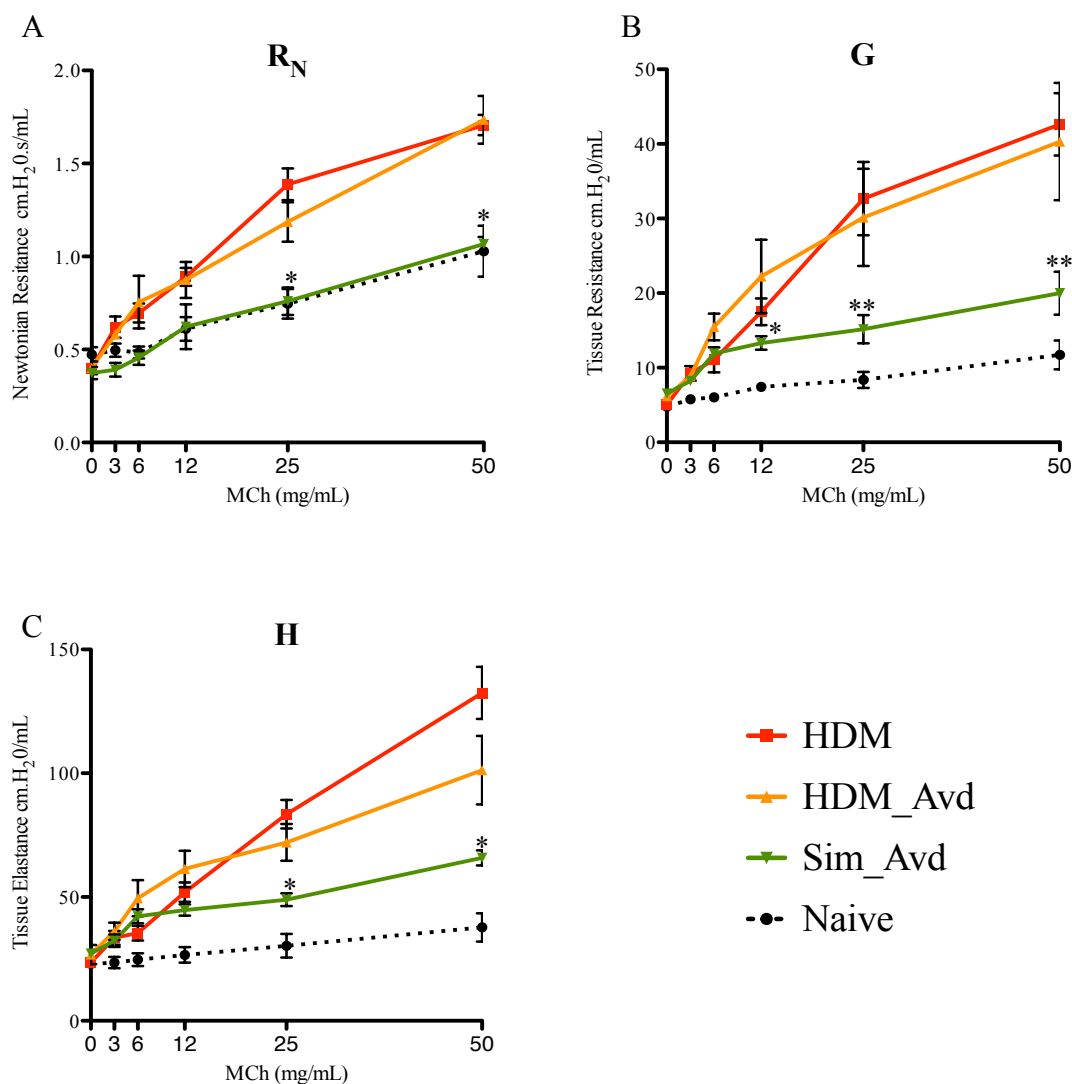


Figure 7.4: Impact of i.n. simvastatin therapy with allergen avoidance on respiratory mechanics.

Lung function was assessed using a flexiVent small animal ventilator 48 h after one week of allergen avoidance with (Sim_Avd) or without (HDM_Avd) simvastatin treatment. Control groups were challenged according to the acute protocol (2 weeks) with allergen (HDM) or saline (Naïve). A) Newtonian Resistance (RN), B) Tissue Resistance (G) and C) Tissue Elastance (H) were measured in response to sequential challenge with nebulized saline (0) or MCh (3-50mg/mL). Data are presented as mean $\pm$ SEM from 6 animals per group. Comparisons of naïve and each treatment were performed against acute 2-wks HDM challenge using Univariate analysis followed by Tukey multiple comparison test * $p \leq 0.05$, ** ≤ 0.01 and *** ≤ 0.001 (multivariate analysis using SPSS20.0) compared against HDM-avoidance group

In the allergen exposure arm, the allergen challenge was extended by an additional week, and lung function remained compromised to a level comparable to the acute (2 week) HDM challenge protocol (Figure 7.5). Although there was no statistical difference between the 3-wk and 2-wk of allergen challenge, however, the slope of the R_N (Figure 7.5A) curve increased (curve shifted leftward) by almost 28% in the HDM exposure arm. Treatment with simvastatin during the exposure arm resulted in a significant reversal in R_N at 25 ($p<0.05$) and 50mg/mL ($p<0.01$) MCh. G and H were also significantly reversed at the 25 and 50 mg/mL MCh dose with at least $p<0.05$ (Figure 7.5B&C).

Figure 7.5

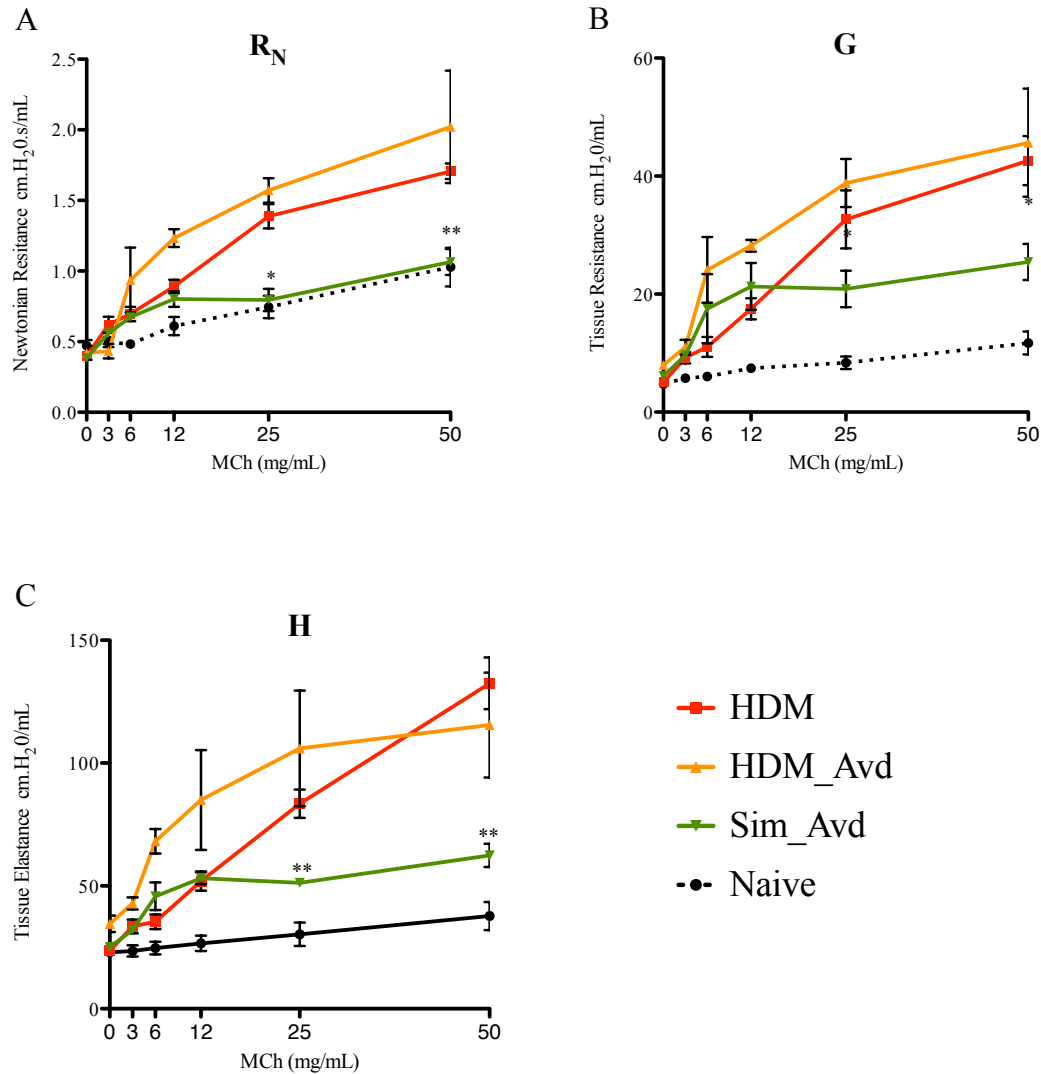


Figure 7.5: Impact of simvastatin treatment on respiratory mechanics during HDM exposure.

Lung function was assessed using a flexiVent small animal ventilator 48 h after completing 3 weeks total HDM challenge, or without simvastatin treatment only during the 3rd week. A) Newtonian Resistance (R_N), B) Tissue Resistance (G) and C) Tissue Elastance (H) were measured in response to sequential challenge with nebulized saline (0) or MCh (3-50 mg/mL). Data are presented as mean $\pm$ SEM from 6 animals per group. Comparisons of naïve and each treatment were performed against HDM challenge using Univariate analysis followed by Tukey multiple comparison test: * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ (multivariate analysis using SPSS20.0) compared against HDM-avoidance group.

Chronic Study: Allergen Exposure

Airway Inflammation:

As expected, five weeks of HDM challenge significantly increased the influx of total inflammatory cells $(123.6 \pm 11.3) \times 10^4$ in BALF, compared to the age-matched naïve controls $(2.9 \pm 0.4) \times 10^4$ ($p < 0.001$). Differential cell counts revealed that only macrophages were present in naïve controls. Chronic HDM challenge was associated with the significant influx of eosinophils $[(41.1 \pm 6.9) \times 10^4; p < 0.001]$, neutrophils $[(30 \pm 3.2) \times 10^4; p < 0.01]$, macrophages $[(43.3 \pm 1.3) \times 10^4; p < 0.001]$, and lymphocytes $[(14.6 \pm 3.3) \times 10^4; p < 0.01]$ (Figure 7.6). Three weeks of simvastatin treatment, beginning in the third week of allergen challenge, significantly reduced the influx of total inflammatory cells by 37.8% ($p < 0.01$). This included a significant reduction in eosinophils (68.7%), neutrophils (64.1%), macrophages (64.4%) and lymphocytes (67.3%).

Figure 7.6

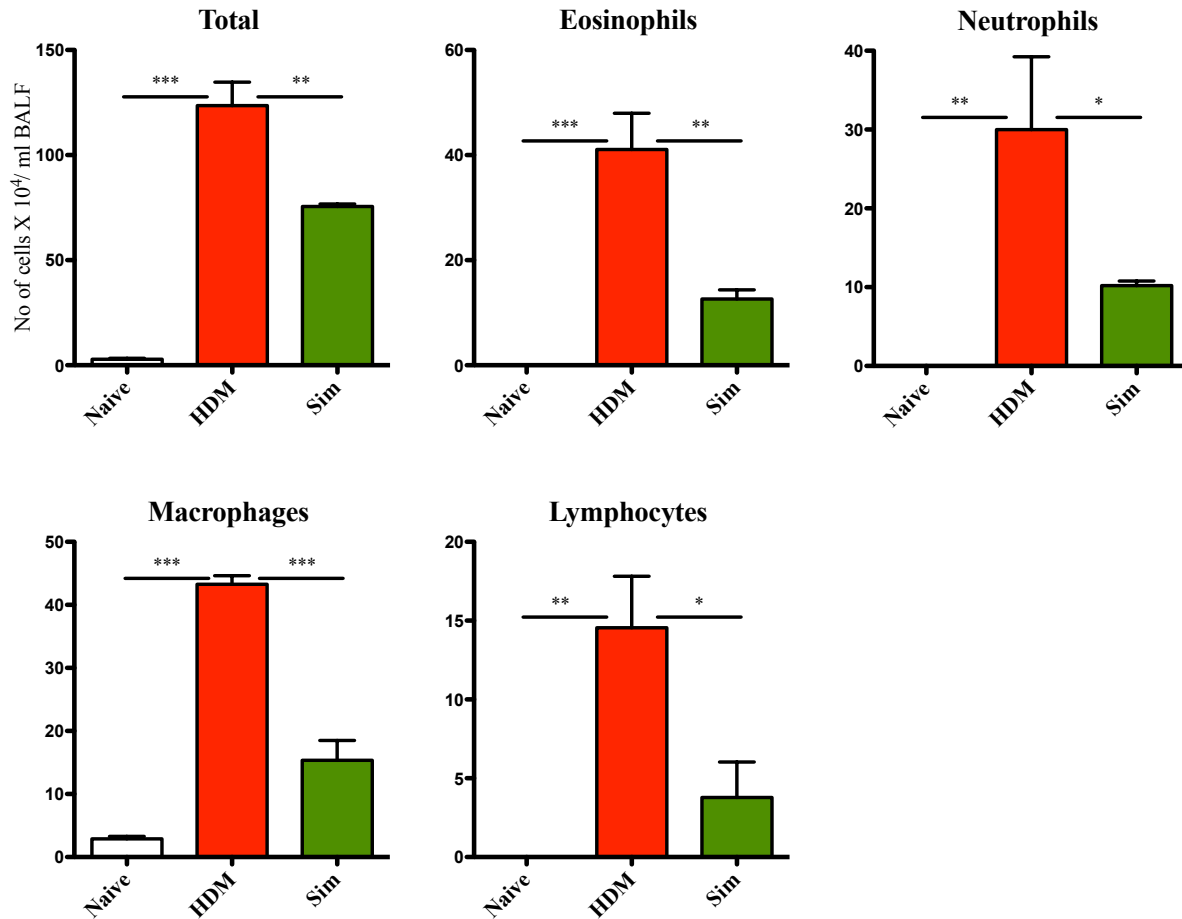


Figure 7.6: Reversal of inflammatory cell influx in BALF with intranasal simvastatin in a model of chronic HDM challenge.

Cell counts were determined 48 h after completing 5 weeks of HDM challenge, with and without simvastatin treatment beginning in the 3rd week. For all data, cell number was normalized to initial BALF volume and is presented as mean ± SEM from 6 animals per group. Analysis was carried out using one-way ANOVA with Tukey multiple comparisons test. *: p ≤ 0.05, ** ≤ 0.01 and *** ≤ 0.001 (univariate analysis using SPSS20.0)

To characterize the inflammation we used a mouse 31-plex cytokine assay (Eve Technologies) to the following cytokines in BALF: Eotaxin, G-CSF, GM-CSF, IFN γ , IL- 1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1 α , MIP-1 β , MIP-2, RANTES,

TNF- α , and VEGF. TGF β 1, 2, and 3 were also quantified using a TGF β array (Eve Technologies). Eighteen different cytokines were detected, grouped and shown as a heat map in Figure 7.7. It is evident from the heat map that most of the cytokines are increased with chronic HDM challenge; however, statistical significance between chronic HDM challenge and naïve controls was observed only for IL-4, IL-5, IL-7, TGF β 1, and TGF β 2. Statistical power was lacking to detect the significance for other cytokines. Similarly, we lacked the power to detect the impact of simvastatin on most cytokines except IL-5, which was significantly reversed by simvastatin treatment; however, the heat map (Figure 7.7) suggests that the simvastatin-treated animals were closer to the naïve group for most cytokines detected. Thus, we performed a supervised multivariate analysis to differentiate the treatment clusters.

Figure 7.7

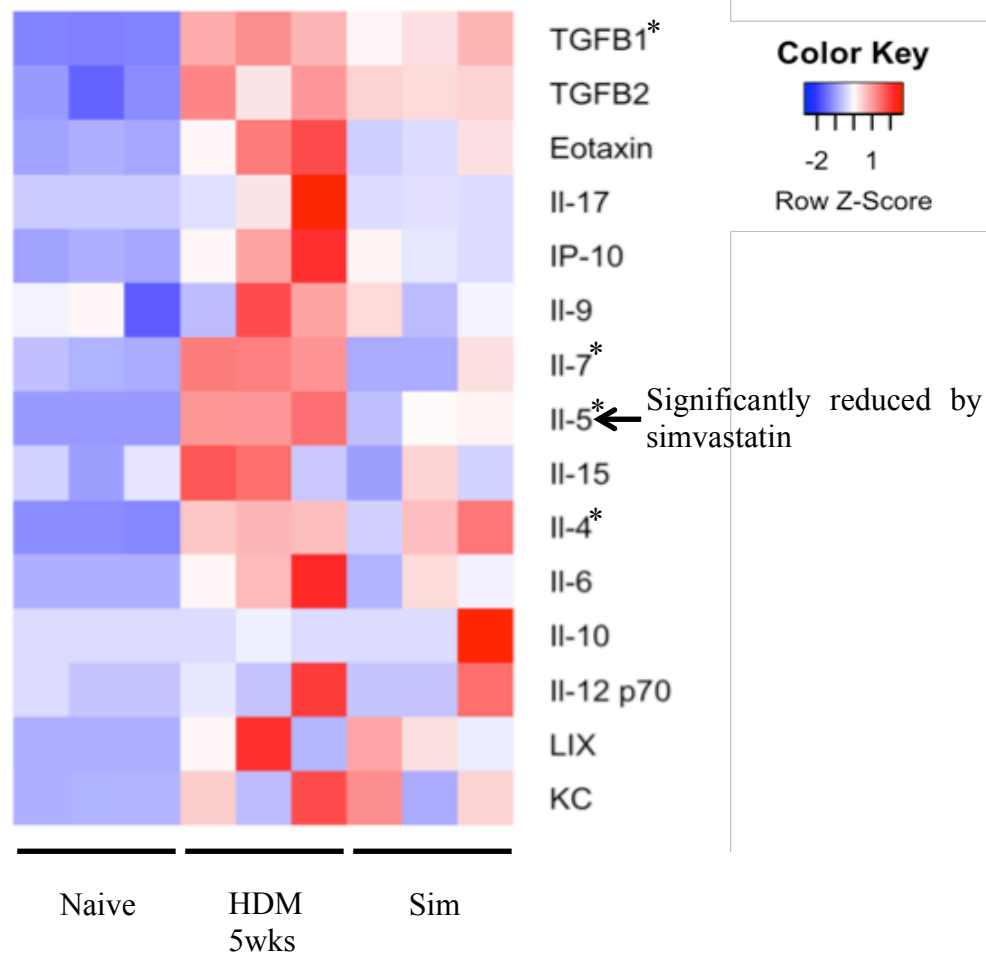


Figure 7.7: Reversal of HDM-induced accumulation of cytokines in BALF by i.n. simvastatin in a chronic model of HDM challenge.

Cytokine abundance determined by a 32-plex cytokine array (Eve Technology) 48 h after the final HDM challenge with and without simvastatin treatment. Y-axis contains 15 detected cytokines and X-axis represents the treatment groups (n= 3/group). The heatmap colour is based on the Row z score (color key). *(p<0.05), compared to HDM challenged group. Legend: Naïve = Age matched naïve control mice, HDM 5wks = 5-wks of chronic HDM challenge, Sim = 5wks of chronic HDM challenge with simvastatin treatment started at the end of 3rd-wks.

Lung Histology

The H&E staining of the lung sections from the left lobe shows marked influx of inflammatory cells in the tissue post chronic HDM challenge compared to the naïve animals. It also shows a change in the tissue structure post HDM challenge marked by the thickening of the epithelial layer along with epithelial shedding. Qualitatively, treatment with simvastatin reversed the epithelial thickening and influx of inflammatory cells into the tissues (Figure 7.8).

Figure 7.8

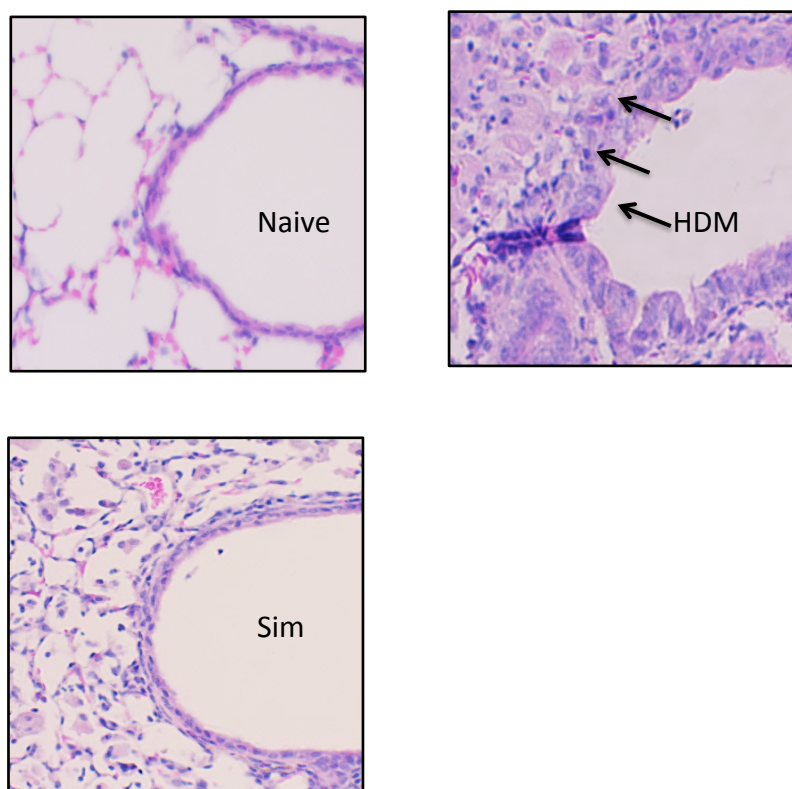


Figure 7.8: Reversal of HDM induced tissue inflammation by i.n. simvastatin following chronic HDM challenge.

Images of typical H&E staining of tissue inflammation 48h after completing the 5-wks protocol of chronic HDM challenge, with and without simvastatin treatment. Simvastatin treatment started from 3rd week of HDM challenge. Images were acquired using 200x magnification. Arrows indicate presence of inflammatory cells. Legend: Naïve = Age matched naïve control mice, HDM 5wks = 5-wks of chronic HDM challenge, Sim = 5wks of chronic HDM challenge with simvastatin treatment started at the end of 3rd-wks.

Similarly, PAS staining shows marked increase in the goblet cell number/ μm length of the basement membrane post chronic HDM challenge ($7.7 \pm 0.7/\mu\text{m}$ basement membrane, $p < 0.001$) in comparison to the naïve animals. Treatment with simvastatin reversed goblet cell hyperplasia to ($0.6 \pm 0.1/\mu\text{m}$ basement membrane, $p < 0.001$) (Figure 7.9A&B).

Figure 7.9

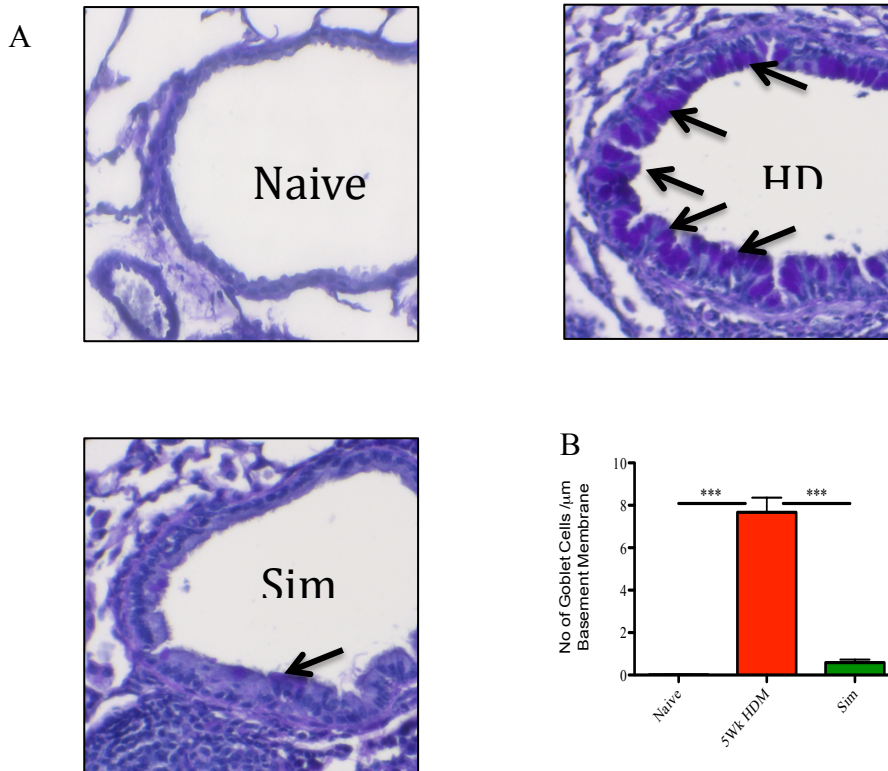


Figure 7.9: Reversal of HDM-induced goblet cell hyperplasia by i.n. simvastatin following chronic HDM challenge.

Images of typical PAS staining of goblet cells in bronchioles. A) Lung sections harvested 48 h after the final HDM challenge, with and without simvastatin treatment. Simvastatin treatment started in the beginning of the 3rd wk of the 5-week protocol. Images were acquired using 200x magnification. Arrows indicate purple mucous staining. B) Quantification of goblet cells using Image Pro software to determine goblet cell number in similar sized airways from each mouse (perimeter $161.1 \pm 28.5 \mu\text{m}$). Data represent mean $\pm$ SEM from 6 airways from each of 3 animals per group. Comparisons were done using one way ANOVA with Tukey multiple comparisons test: $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$ (univariate analysis using SPSS 20.0). Legend: Naïve = Age matched naïve control mice, HDM 5wks = 5-wks of chronic HDM challenge, Sim = 5wks of chronic HDM challenge with simvastatin treatment started at the end of 3rd-wks.

We also estimated the collagen deposition in the lung in response to the HDM challenge and evaluated the impact of simvastatin treatment by staining the lung sections with picrosirius red. Unfortunately, we observed a huge variability in the measurement of collagen fibres/unit basement membrane length. Thus, we could not interpret the results (Figure. 7.10).

Figure. 7.10

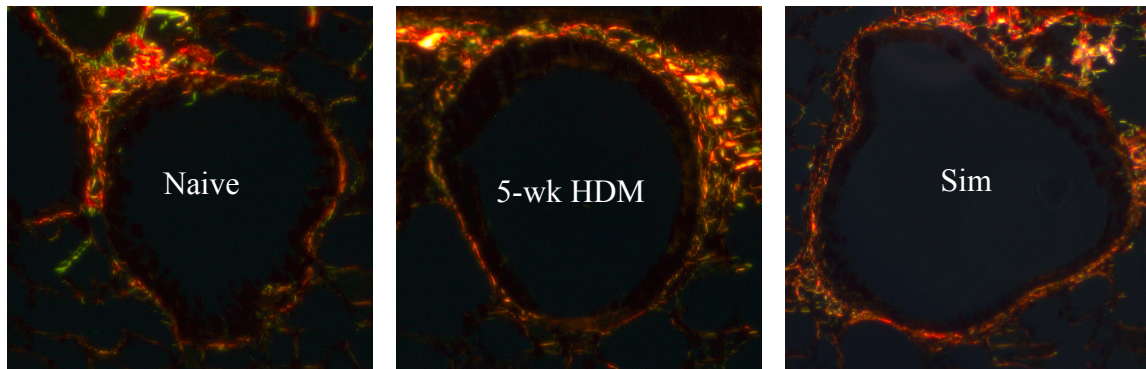


Figure 7.10: Collagen deposition after chronic HDM challenge, with and without intranasal simvastatin.

Polarized images of typical collagen fibers after picrosirius red staining, in lung sections, harvested 48 h after completing the 5-week chronic HDM challenge protocol, with and without simvastatin treatment beginning in the 3rd week of 5-wk HDM challenge protocol. Images were acquired using 200x magnification. Legend: Naïve = Age matched naïve control mice, HDM 5wks = 5-wks of chronic HDM challenge, Sim = 5wks of chronic HDM challenge with simvastatin treatment started at the end of 3rd-wks.

Lung function:

Chronic allergen challenge over 5 weeks induced a significant increase in the total airway resistance (Rrs) ($p < 0.001$), compared to naïve controls, at 25 and 50 mg/mL MCh (Fig 7.10A). Treatment with simvastatin reversed the HDM-induced increase in Rrs significantly at 50 mg/mL MCh ($p < 0.05$, Figure 7.10A). When the Rrs was broken down into R_N , G, and H, we observed that the HDM challenge significantly increased both G and H compared to age-matched naïve controls ($p < 0.001$) (Figure 7.10C&D). G was increased significantly through the entire range of MCh challenge (3-50 mg/mL, Figure 7.10C), whereas H differed from naïve controls beginning at 12mg/mL MCh ($p < 0.05$) and upward (Figure 7.10D). The observed power of 0.496 was insufficient to measure a statistical difference in R_N , in spite of a strong trend towards an increase in R_N after chronic HDM challenge (Figure 7.10B). Treatment with simvastatin significantly reversed the HDM-induced increase in G ($p < 0.05$; Figure 7.10C) but had no impact on the HDM-induced increase in H (Figure 7.10D). A positive impact of simvastatin on G compared to HDM challenged animals was observed at MCh dose of 50mg/mL ($p < 0.01$; Figure 7.10C). Simvastatin treatment also reversed the HDM-induced increase in R_N , but again, lack of power resulted in no statistical significance (Figure 7.10B).

We also performed a chronic reversal study, where allergen was withdrawn for two weeks with or without i.n. simvastatin treatment following five weeks of HDM challenge. However, we were unable to detect any statistically significant differences in lung function or lung inflammation between allergen avoidance group and the age-matched allergen naïve animals. Thus, it was impossible to decipher any impact of intranasal simvastatin in this model.

Figure 7.11

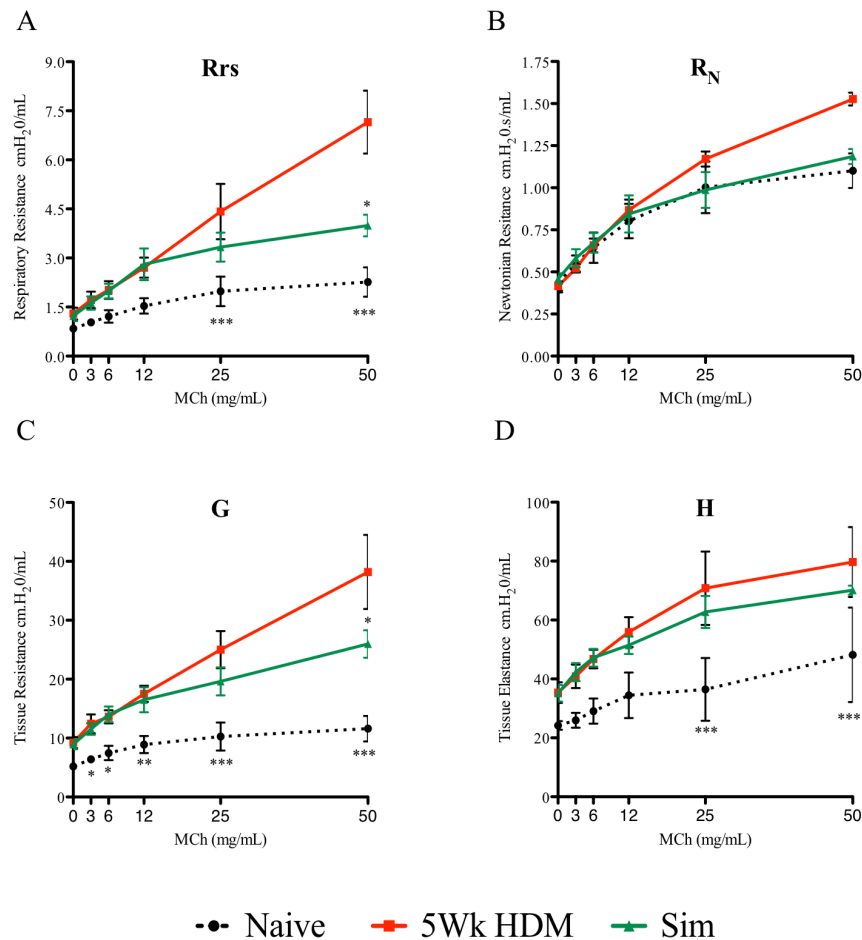


Figure 7.11: Effects of chronic HDM challenge and i.n simvastatin therapy on respiratory mechanics.

Lung function was assessed using a flexiVent small animal ventilator 48 h after the final HDM challenge, with and without simvastatin treatment beginning in the 3rd week of 5-wk HDM challenge protocol. A) Total Respiratory Resistance (R_{rs}), B) Newtonian Resistance (R_N), C) Tissue Resistance (G) and D) Tissue Elastance (H) were measured in response to sequential challenge with nebulized saline (0) or MCh (3-50mg/mL). Data are presented as mean ± SEM from 6 animals per group. Comparisons of Naïve and Simvastatin treatment groups were performed against 5 Wk HDM-challenged group by univariate analysis followed by Tukey multiple comparisons test. *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001 (univariate analysis SPSS 20.0). Legend: Naïve = Age matched naïve control mice, HDM 5wks = 5-wks of chronic HDM challenge, Sim = 5wks of chronic HDM challenge with simvastatin treatment started at the end of 3rd-wk.

Allergen Avoidance Study

Airway Inflammation:

Two weeks of allergen avoidance after 5 weeks of chronic HDM exposure negated the airway inflammation: there was no statistical difference in the total inflammatory cell count in BALF after allergen avoidance compared to naïve controls. Similarly, differential cell counts revealed no statistical differences for eosinophils, neutrophils, macrophages, or lymphocytes after allergen avoidance compared to naïve controls. Simvastatin treatment concurrent with allergen avoidance did not further reduce the airway inflammation (Figure. 7.12).

Figure 7.12

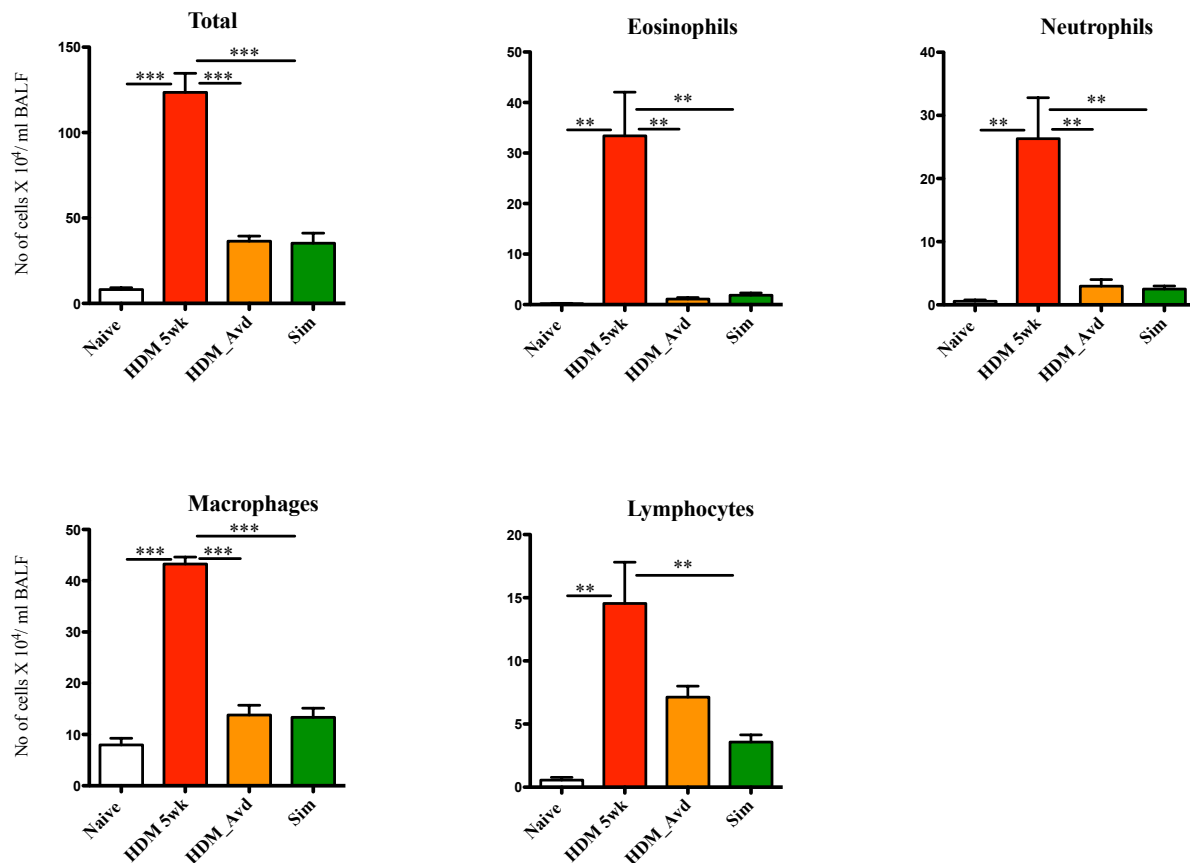


Figure 7.12: Total and differential cell counts in BALF with allergen avoidance after chronic HDM exposure.

Allergen avoidance lasted 2 weeks following 5 weeks of chronic HDM exposure. Cell counts determined 48 h after the end of the 7th week, with and without simvastatin treatment concurrent with allergen avoidance. For all data, the cell number was normalized to initial BALF volume and presented as mean $\pm$ SEM from 6 animals per group. Analysis was done using one-way ANOVA with Tukey multiple comparisons test. * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$ (univariate analysis using SPSS20.0). Legend: Naïve = Age matched naïve control mice, HDM 5wks = 5-wks of chronic HDM challenge, HDM_Avd = 5-wks of chronic HDM challenge followed by 2-wks of allergen abstinence. Sim = 5wks of chronic HDM challenge followed by 2-wks of allergen abstinence with simvastatin treatment.

Lung Function:

Two weeks of allergen avoidance significantly reversed the total respiratory resistance (R_{rs} ; Figure. 7.13A) and the tissue damping/resistance (G ; Figure. 7.13C) compared to 5 weeks of allergen challenge, at 50 mg/mL MCh. Statistical power was insufficient to observe a similar trend for Newtonian Resistance (R_N ; Figure. 7.13B) and Tissue Elastance (H ; Figure. 7.13D). Simvastatin treatment concurrent with allergen avoidance did not have any statistical effect compared to allergen avoidance alone.

Figure. 7.13

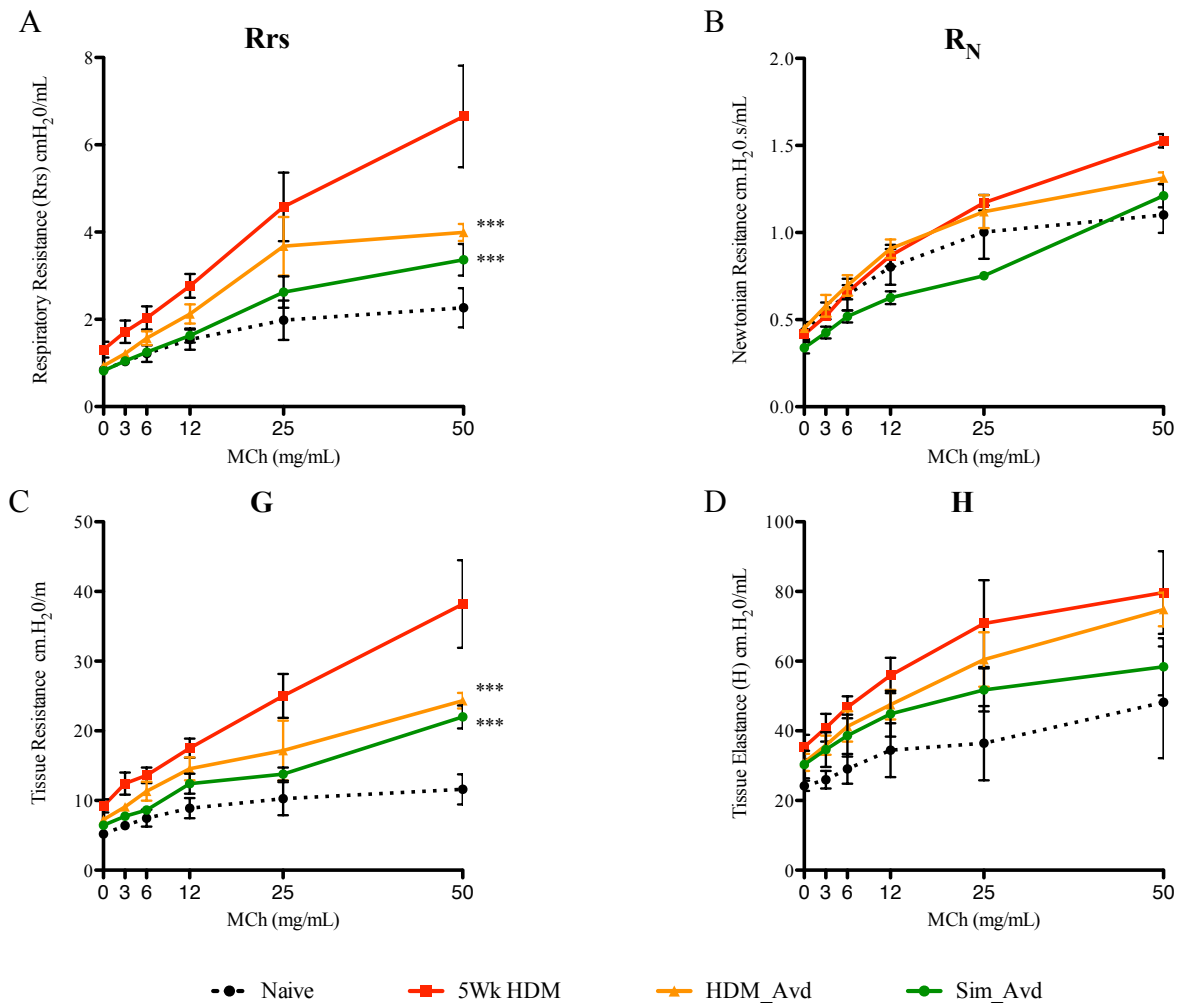


Figure. 7.13: Respiratory mechanics after allergen avoidance with or without i.n simvastatin following chronic HDM challenge.

Allergen avoidance lasted 2 weeks following 5 weeks of chronic HDM exposure. Lung function was assessed 48 h after the end of the 7th week, with and without simvastatin treatment concurrent with allergen avoidance. HDM (HDM 5wk) controls were administered HDM for 5 weeks, and naïve animals were age matched allergen naïve. A) Total Respiratory Resistance (Rrs), B) Newtonian Resistance (R_N), C) Tissue Resistance (G) and D) Tissue Elastance (H) were measured in response to sequential challenge with nebulized saline (0) or MCh (3-50 mg/mL). Data are presented as mean $\pm$ SEM from 6 animals per group. Comparisons of each treatment were performed against HDM 5wk controls using univariate analysis followed by Tukey multiple comparison test (* $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ (univariate analysis SPSS 20.0). Legend: Naïve = Age matched naïve control mice, HDM 5wks = 5-wks of chronic HDM challenge, HDM_Avd = 5-wks of chronic HDM challenge followed by 2-wks of allergen abstinence. Sim = 5wks of chronic HDM challenge followed by 2-wks of allergen abstinence with simvastatin treatment.

7.4 Discussion

Any animal model evaluating the efficacy of a drug for a disease should be designed to evaluate the potential to reverse the disease. Most studies with statins, however, including those in previous chapters here, use a prevention model, where treatment is given concomitantly to allergen challenge (173, 209, 222, 231, 236). This has its own validity in terms of exploring the potential of statins in preventing the allergen-induced changes in the lungs and providing proof of concept for efficacy; however, it does not mimic the clinical condition.

In previous chapters we showed that intranasal simvastatin could blunt allergen-induced airway inflammation, goblet cell hyperplasia and AHR (defined by increased respiratory resistance to the MCh challenge) at a dose 7000-fold less than reported in other preventative studies using systemic delivery. Here, for the first time, we used acute as well as a chronic reversal models, with allergen-avoidance and allergen-exposure arms, to evaluate the efficacy of intranasal simvastatin in reversing the established features of asthma in mice.

Since we had already established the development of robust airway inflammation, goblet cell hyperplasia and AHR after 2 weeks of allergen challenge, we began treatment 2 weeks after the initial HDM insult in the acute model. In the avoidance arm of the acute reversal study, we showed that one week of allergen avoidance significantly reduced inflammatory cell counts compared to age-matched naïve controls. However, lung function was unaffected, as all three parameters of respiratory resistance- R_N , G , and H were unchanged compared to acute HDM challenge alone.

In the allergen exposure arm, when mice were challenged with HDM for an additional week, the inflammatory cell counts remained elevated, especially eosinophils. Lung

function also remained compromised as all parameters of respiratory resistance remained significantly elevated. The additional week of allergen challenge showed a trend (not statistically significant) towards increased sensitivity of the airways to the MCh challenge.

Simvastatin treatment (6g/kg, i.n.) did not further reduce inflammatory cell counts when used in combination with allergen avoidance. However, all parameters of airway resistance were significantly reduced, compared to allergen avoidance alone. This is the first time in an animal model that simvastatin has demonstrated a reversal effect on HDM-induced lung function. Consistent with our acute prevention studies, as well as other preclinical studies (173, 209, 222, 231, 236), simvastatin treatment concurrent with continued allergen exposure for 3 weeks significantly reduced the accumulation of inflammatory cells in BALF, and also improved lung function evidenced by significantly decreasing respiratory resistance (R_N , G, and H).

The acute reversal study provided the rationale to move to a chronic murine model of allergic asthma, where mice were challenged with HDM for 5 weeks (described in Chapter 2). Using this model, we showed a significant increase in inflammation compared to age-matched naïve controls, 48 h after the final HDM challenge. These results are consistent with other chronic studies that used HDM as an allergen (168, 169). We also showed that there was a significant rise in proinflammatory cytokines IL-4, IL-5, IL-7, as well as $TGF\beta$ -1 and -2, out of 32 different cytokines probed. The HDM-induced increase in IL-4 and -5 is consistent with other studies and suggests a Th2 immune response (169, 274). Interestingly, HDM challenge also increased IL-7 levels, which has been implicated in steroid resistance and increased production of Th2 cytokines (291-293). Also, in our chronic model, we did not observe a rise in IL-10, consistent with human asthma where IL-10 levels are decreased, suggesting that chronic HDM exposure overwhelms the body's defense mechanism against inflammation, as observed in

human asthmatics (269). Interestingly, despite an observed increase in neutrophils, we did not observe a significant rise in the pro-neutrophilic cytokine KC/Gro. However, HDM challenge induced a rise in TGF β , which is associated with allergic inflammation (294). Overall, the results suggest that the observed inflammation is predominantly Th2 oriented and eosinophilic. We did not observe any significant changes in other cytokines. It is possible that 48 h following the final HDM challenge may not be an appropriate time point for cytokine assessment, as most cytokines would peak earlier, to elicit their effects on physiological outcomes observed later.

In our model, we also showed that HDM challenge induced goblet cell hyperplasia; unfortunately, this was the only evidence of airway remodelling, as collagen staining was inconclusive. However, chronic HDM challenge significantly compromised lung function: There was a significant rise in the total respiratory resistance (Rrs), which included a significant rise in the tissue resistance (G) and the tissue elastance (H), in line with other studies(168, 169). However, in our model, the HDM induced change in the Newtonian resistance (R_N) to the MCh challenge was statistically not different from the naïve controls, but most studies to our knowledge only report the Total Respiratory Resistance (Rrs) and not the individual components (169, 269, 295).

In the allergen avoidance arm, airway inflammation was completely reversed, according to total and differential cells counts. Rrs and G were also significantly reversed compared to naïve controls, and R_N and H showed a similar trend albeit in the absence of statistical significance. In literature, studies that employed a chronic model, using alternate day HDM challenge, failed to reproduce airway remodelling and sometimes AHR as well (295). Such outcomes suggest that using an alternate day HDM challenge results in some degree of

resistance, which is not observed in models that use HDM challenge 5days/wk for 5-7 weeks (169).

All this being said, the aim of the current study was to evaluate the efficacy of simvastatin in a chronic model of allergic asthma. As a reversal study, the treatment with simvastatin started only after the 2 weeks of allergen challenge. In our chronic study, we reduced the dose of simvastatin (3 $\mu\text{g/kg}$) compared to the dose used in the acute study (6 $\mu\text{g/kg}$) as we were increasing the duration of treatment to 3 weeks.

Consistent with our previous findings using 6 $\mu\text{g/kg}$ simvastatin, we showed that 3 $\mu\text{g/kg}$ simvastatin also reversed the HDM induced airway inflammation in BALF as well as in lung tissue supporting its anti-inflammatory potential in a chronic model of asthma. Simvastatin treatment also reversed established goblet cell hyperplasia in mice challenged with HDM, which is a promising sign for its potential to reverse established remodelling in the mice, and consistent with a prevention study that showed statins could blunt goblet cell hyperplasia (233). However, to prove this, other aspects of lung remodelling need to be assessed through diligent lung morphometric analysis.

Most importantly, simvastatin reversed the established AHR to MCh challenge, as evidenced by the recovery of R_{rs} and G , although without significant impact on H . Simvastatin treatment had a very strong impact on R_N ; however, a lack of statistical power meant we could not statistically differentiate the impact of simvastatin on R_N compared to HDM-challenged animals, even though R_N was restored to the level of naïve controls. The significant impact of simvastatin on G means there is less tissue drag on the air while breathing, consistent with the impact on airway inflammation observed in both BALF cell count and tissue histology. By reversing the influx of inflammatory cells (and possibly the accompanying fluid) in the lung

tissue, simvastatin improved the ability of the tissue to expand, thereby improving compliance of the lungs. The inability of simvastatin to impact H implies that in this model, simvastatin did not improve the impaired elasticity of the lung resulting from HDM challenge, even though the results from our acute prevention and reversal studies showed that simvastatin significantly improves this parameter (217, 296).

Overall, this is the first report of intranasal simvastatin reversing HDM-induced lung inflammation, AHR, and possibly remodelling in both acute as well as chronic studies. The dose used in these studies can safely translate to clinical trials not only in adults but also in children, as previously in Chapter 5 we have shown that i.n. simvastatin does not enter the systemic circulation even at twice the dose used in the current studies. However, more work is needed to evaluate local toxicity before clinical trials become a possibility.

Chapter 8: Conclusion

Asthma has reached almost epidemic proportions worldwide (2, 297). It is apparent that asthma is as much a syndrome as a disease (134, 135), highlighting the complexity of pathophysiology and biological mechanisms that create a significant hurdle for development of effective therapies. However, inflammation, airway remodelling, and hyperresponsiveness are the hallmark features of asthma, and although oxidative stress is also a known pathogenic feature, it is less well developed as a target for therapy, directly or indirectly. Oxidative stress is the result of a combination of environmental factors and possibly impaired antioxidant mechanisms in asthmatics in combination with chronic inflammation (20). Oxidative stress produces free radicals, which attack the unsaturated fatty acid chains in phospholipids, especially PCs (the most abundant phospholipids in biological membranes and lung surfactant. The lipid tails are oxidized to produce bioactive species that can influence local inflammation and tissue remodelling (24, 37).

Currently, symptoms of asthma are treated with rescue/reliever medication and inhaled corticosteroids to control inflammation (the standard controller therapy) (11). However, there is no treatment targeting oxidative stress and tissue remodelling in asthmatics, and this may contribute to intrinsic refractoriness or progressive desensitization to therapy observed in many patients (20, 39). These patients remain symptomatic, resulting in an impaired quality of life for asthmatics and their family members, and an economic burden on society (3-7).

Statins were originally developed to reduce circulating cholesterol (48). However, due to their pleiotropic anti-inflammatory and antioxidant effects, they are increasingly used in the clinic to reduce inflammation and oxidative stress in patients with cardiovascular mortality and morbidity (195, 197, 198, 202). Since inflammation and oxidative stress are also pathogenic features of asthma, this provides the rationale for exploring statins as therapy in asthma.

However, clinical and preclinical results are in conflict with respect to the efficacy of statins in asthma: randomized clinical trials have yet to support any real benefit, in contrast to results from preclinical studies of allergic asthma (173, 298). Retrospective epidemiological studies strongly infer that statin use for cardiovascular indication has beneficial effects on asthma control, measured by lung function and quality of life indices (207). Thus, a knowledge gap exists in the translation of the benefits of statins from the laboratory to the clinic. Preclinical studies have focused on assessing acute efficacy without considering the feasibility of dosage and relevance of animal models to the human disease condition (173, 194, 209). Consequently, clinical trials were designed to assess a relatively acute impact on asthma symptoms, and likely underestimated the pharmacokinetics of statins and the need for long-term therapy. Another limitation of these trials is that their cohorts included mild to moderate asthmatics with already well-controlled symptoms (210, 211, 213). Thus, it is not completely surprising that these trials did not reveal any improvement in asthma with statin use.

The projects in this thesis were designed to (i) evaluate the effects of simvastatin therapy in a clinically relevant murine model of allergic asthma, (ii) find the optimum route of delivery to reduce the dose required to produce maximal benefits, and (iii) unravel the mechanism of action. These objectives represent an attempt to bridge the existing knowledge gap to bring statins closer to clinical use for the benefit of the people with asthma.

In addressing the first objective, we showed that systemically delivered simvastatin prevents inflammation and goblet cell hyperplasia induced by HDM challenge, but does not improve lung function. Even prophylactic use of systemically delivered simvastatin was insufficient to improve lung function, despite more effective inhibition of allergen-induced airway inflammation and goblet cell hyperplasia. This observation prompted us to measure

simvastatin and its active form (simvastatin hydroxy acid) in lung tissue as well as in the circulation. Interestingly, we detected the active form of simvastatin in serum but not in the lungs, suggesting one possible barrier in translating the benefits of statins on systemic cardiovascular pathophysiology to the lung tissue itself.

Thus, we proceeded to target lungs directly with local, intranasal (i.n.) delivery of simvastatin. The dose was calculated based on the effective circulatory levels of simvastatin determined from our systemic delivery studies. Using a combination of ethanol and water (0.002:1; v/v), we solved the problem of simvastatin solubility in water. At this dose, simvastatin prevented the HDM-induced decline in lung function in mice, in conjunction with other benefits on airway inflammation and remodelling. Interestingly, this dose was 1/7000th of that used systemically. At this low, locally delivered dose, the impact of simvastatin was equivalent to the highest dose of fluticasone, a gold standard inhaled corticosteroid for asthma therapy. Since these were all preventive studies, we also aimed to test simvastatin in acute as well as chronic reversal studies. Despite some model-related setbacks, we were able to show that intranasal simvastatin was able to reverse established lung inflammation, goblet cell hyperplasia (a feature of lung remodelling), and AHR. Thus, this work has provided evidence to lessen the knowledge gap between preclinical and clinical studies regarding the efficacy of statins in asthma, and points to the need for direct targeting of the lungs using local delivery of simvastatin. Although the results using intranasal simvastatin are impressive in this murine model of asthma, the battle is far from over, as the next challenge will be to translate these findings into humans. This is itself is a hurdle, considering the abysmally slow rate of knowledge translation from preclinical studies to clinical practice. A significant barrier is the current lack of a local (inhaled) simvastatin formulation that can be used with an inhaler device for human asthmatics. Attempts

have been made to formulate inhalable simvastatin in the form of metered dose inhalers (MDI) or micronized powder, however it further needs refinement before it could be used in humans (299, 300). Our current understanding about the optimal doses of simvastatin could help further refining of the inhalable formulation of simvastatin. Moreover, the local toxicity of inhaled simvastatin would need to be tested in a phase I trial; even though a relatively safe and approved human drug is being re-purposed, changing the route of delivery of the drug warrants such testing.

The mechanism of action of intranasal simvastatin remains unknown. The anti-inflammatory potential of statins has been linked to inhibition of the mevalonate pathway, essential for prenylation of small G-proteins. Prenylation helps these proteins to anchor to the plasma membrane and become activated, driving the downstream cascade responsible for inflammation, cell migration and proliferation (71, 193, 200, 222, 233). Activation of Rho-associated kinase (ROCK) via prenylation of RhoA is an example of this process. However, statins seem to affect lung function independent of the mevalonate cascade, as mevalonate supplementation does not reverse the impact of statins on lung function (222). Also, oxidative stress is a major pathologic feature of asthma, driving inflammation, remodelling, and AHR, and is induced by the environmental factors (such as pollution and allergen exposure) as well as ongoing inflammation in asthma (20). We already know that oxidative stress damages the lipids and converts them to highly bioactive molecules (OxPCs) involved in pathologies of various diseases (22, 283). Since the lung is a lipid-rich organ with PC being the chief component of cell membranes and surfactant (21, 27), our goal was to understand if OxPCs are implicated in asthma pathobiology *in vivo* in animals and humans. Also, OxPCs induced various processes responsible for inflammatory cell tethering and migration (e.g., activation of VCAMs and E-

selectin), along with ROCK activation (22, 34, 74). Interestingly, these processes are negatively affected by statins (173, 222) and thus it was of interest to evaluate the impact of statins on lung OxPCs following allergen challenge.

We used a cutting-edge lipidomics platform to show that HDM challenge induces significant accumulation of OxPCs in the lungs. We determined that OxPCs in the lungs of human asthmatics share >90% homology with OxPCs identified in HDM-challenged mice. Also, OxPCs that define HDM-challenged clustering in our animal model are also important in defining clusters of human asthmatics allergic to and challenged with HDM. Lipidomics analysis also showed that the severity of the late asthma response after allergen challenge has a distinct signature of OxPCs. Currently, we are working to evaluate if intranasal administration of OxPCs in mice can induce a similar response to that of HDM, specifically with respect to lung function. My preliminary studies indicate that work in this area will be challenging, and will require a suite of studies with *in vivo*, *in vitro* and *ex vivo* models using selective inhibitors as well as genetically engineered animal models to decipher the exact pathobiology, signalling pathways and critical regulators associated with OxPC activities. However, the preliminary data reported here can help direct research focus in this regard: intranasal simvastatin significantly blunted the accumulation of OxPCs in the lungs, but with an OxPC signature distinct from that of low dose fluticasone, yet both inhibited lung inflammatory cell infiltration. Conversely, only intranasal simvastatin was sufficient to prevent and reverse AHR. Supervised and unsupervised multivariate analysis showed that HDM-challenged animals that also received treatment with simvastatin were closer to age-matched allergen-naïve animals. This suggests a central role for OxPCs in the observed difference in therapeutic effects of simvastatin and fluticasone. Current work within our group supports the hypothesis that simvastatin is enhancing the clearance of

OxPCs through HDL. As HDL plays an important role in clearing the oxidized lipids (47), unpublished data from our group suggest that simvastatin reduces the allergen-induced proinflammatory proteins in HDL. Simvastatin is also known to enhance the essential structural component (ApoA1) in HDL that is required the activity of various HDL associated enzymes responsible for the clearance of oxidized lipids (47). It is also important to note here that clinical trials have established that simvastatin increases the production of HDL (48). Thus simvastatin not only helps in preserving the functionality of HDL but also increases its production, and thereby prevents the accumulation of OxPCs in the allergen-challenged lungs in mice. In addition, it also prevents mevalonate mediated ROS generation and activates HO-1 gene (199, 201, 202). Together these effects highlight the antioxidant potential of i.n. simvastatin.

Overall, this work supports a role for OxPCs in asthma pathogenesis, with the potential to define asthma sub-phenotypes. Since the impact of simvastatin treatment was strongly correlated with improved lung health, OxPCs can act as a marker for disease control. Also, the ability of simvastatin to blunt the accumulation of HDM-induced OxPCs supports a role for simvastatin in countering oxidative stress-induced pathologies. However, it is not clear if simvastatin is preventing OxPC accumulation via antioxidant mechanisms, or if it is somehow mitigating the downstream effects of oxidative stress. The collection of studies in this thesis has opened many exciting avenues for future research to evaluate the potential of OxPCs (i) in asthma pathobiology, (ii) in defining phenotypes of asthma, (iii) as markers for disease severity and control, and (iv) as a target for future therapeutics. The main findings from this thesis and the future directions have been summarized in Figure 8.1.

Figure. 8.1

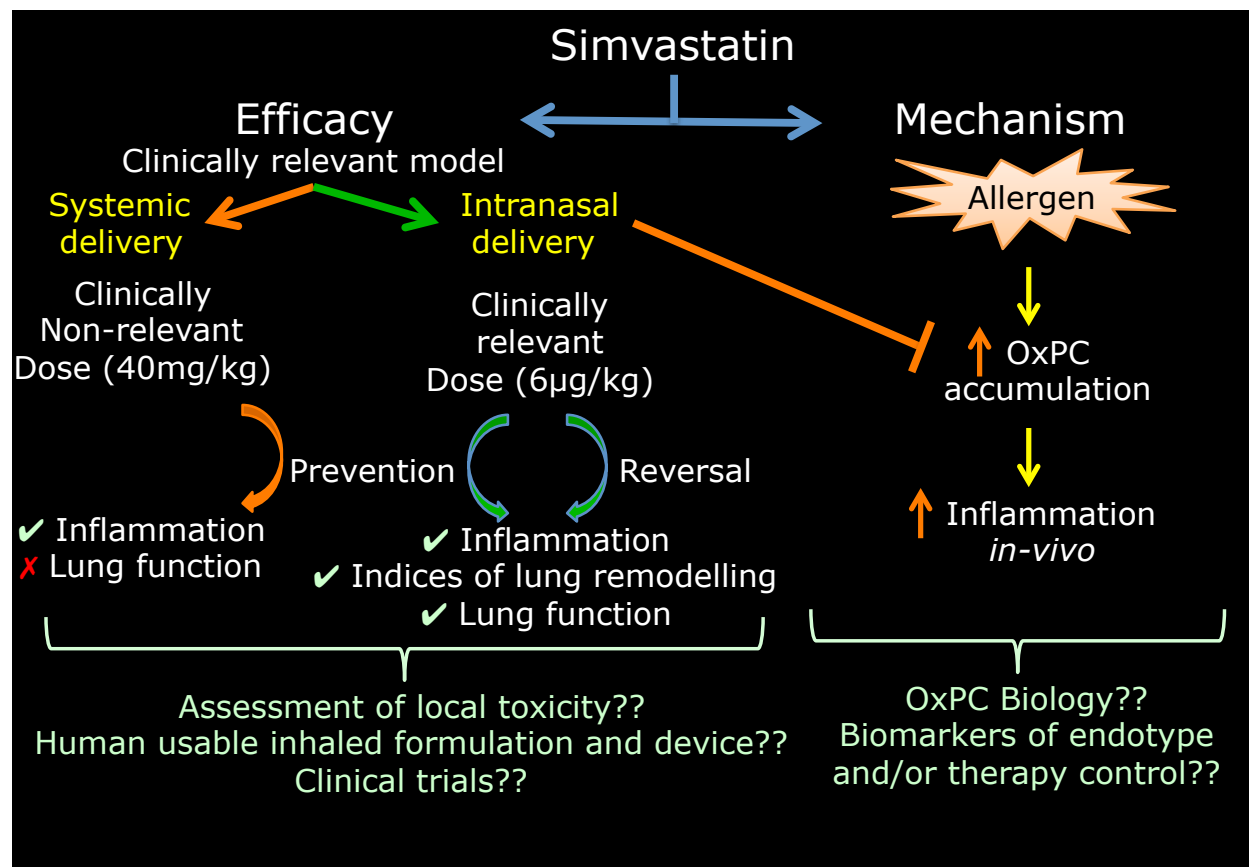


Figure 8.1: Summary of the benefits of simvastatin on allergen compromised lung health OxPC accumulation, a novel disease pathology and future directions.

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